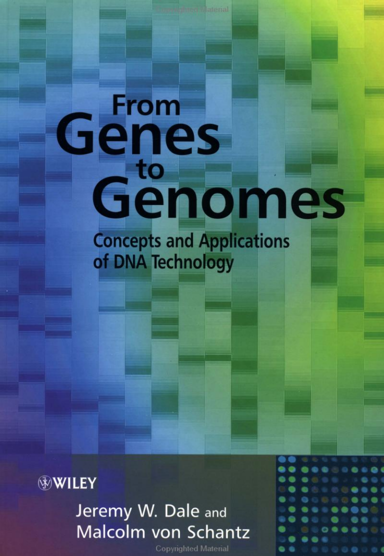

From Genes to Genomes

Concepts and Applications of DNA Technology

WILEY

Jeremy W. Dale and
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Preface

Over the last 30 years, a revolution has taken place that has put molecular biology at the heart of all the biological sciences, and has had extensive implications in many fields, including the political arena. A major impetus behind this revolution was the development of techniques that allowed the isolation of specific DNA fragments and their replication in bacterial cells (*gene cloning*). These techniques also included the ability to engineer bacteria (and subsequently other organisms including plants and animals) to have novel properties, and the production of pharmaceutical products. This has been referred to as *genetic engineering*, *genetic manipulation*, and *genetic modification* – all meaning essentially the same thing. However, many of the applications extend further than that, and do not involve cloning of genes or genetic modification of organisms, although they draw on the knowledge derived in those ways. This includes techniques such as *nucleic acid hybridization* and the *polymerase chain reaction* (*PCR*), which can be applied in a wide variety of ways ranging from the analysis of differentiation of tissues to forensic applications of DNA fingerprinting and the diagnosis of human genetic disorders. In an attempt to cover this range of techniques and applications, we have used the term *DNA technology* in the subtitle.

The main title of the book, *From Genes to Genomes*, is derived from the progress of this revolution. It signifies the move from the early focus on the isolation and identification of specific genes to the exciting advances that have been made possible by the sequencing of complete genomes. This has in turn spawned a whole new range of technologies (*post-genomics*) that are designed for genome-wide analysis of gene structure and expression, including computer-based analyses of such large data sets (*bioinformatics*).

The purpose of this book is to provide an introduction to the concepts and applications of this rapidly-moving and fascinating field. In writing this book, we had in mind its usefulness for undergraduate students in the biological and biomedical sciences (who we assume will have a basic grounding in molecular biology). However, it will also be relevant for many others, ranging from research workers who want to update their knowledge of related areas to anyone who would like to understand rather more of the background to current controversies about the applications of some of these techniques.

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Malcolm von Schantz

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1 Introduction

This book is about the study and manipulation of nucleic acids, and how this can be used to answer biological questions. Although we hear a lot about the commercial applications, in particular (at the moment) the genetic modification of plants, the real revolution lies in the incredible advances in our understanding of how cells work. Until about 30 years ago, genetics was a patient and laborious process of selecting variants (whether of viruses, bacteria, plants or animals), and designing breeding experiments that would provide data on how the genes concerned were inherited. The study of human genetics proceeded even more slowly, because of course you could only study the consequences of what happened naturally. Then, in the 1970s, techniques were discovered that enabled us to cut DNA precisely into specific fragments, and join them together again in different combinations. For the first time it was possible to isolate and study specific genes. Since this applied equally to human genes, the impact on human genetics was particularly marked. In parallel with this, *hybridization* techniques were developed that enabled the identification of specific DNA sequences, and (somewhat later) methods were introduced for determining the sequence of these bits of DNA. Combining those advances with automated techniques and the concurrent advance in computer power has led to the determination of the full sequence of the human genome.

This revolution does not end with understanding how genes work and how the information is inherited. Genetics, and especially modern molecular genetics, underpins all the biological sciences. By studying, and manipulating, specific genes, we develop our understanding of the way in which the products of those genes interact to give rise to the properties of the organism itself. This could range from, for example, the mechanism of motility in bacteria to the causes of human genetic diseases and the processes that cause a cell to grow uncontrollably giving rise to a tumour. In many cases, we can identify precisely the cause of a specific property. We can say that a change in one single base in the genome of a bacterium will make it resistant to a certain antibiotic, or that a change in one base in human DNA could cause debilitating disease. This only scratches the surface of the power of these techniques, and indeed this book can only provide an introduction to them. Nevertheless, we hope that by the time you have studied it, you will have some appreciation of what can be (and indeed has been) achieved.

Genetic manipulation is traditionally divided into *in vitro* and *in vivo* work. Traditionally, investigators will first work *in vitro*, using enzymes derived from various organisms to create a *recombinant DNA molecule* in which the DNA they want to study is joined to a *vector*. This recombinant vector molecule is then processed *in vivo* inside a *host* organism, more often than not a strain of the *Escherichia coli* (*E. coli*) bacterium. A *clone* of the host carrying the foreign DNA is grown, producing a great many identical copies of the DNA, and sometimes its products as well. Today, in many cases the *in vivo* stage is bypassed altogether by the use of PCR (polymerase chain reaction), a method which allows us to produce many copies of our DNA *in vitro* without the help of a host organism.

In the early days, *E. coli* strains carrying recombinant DNA molecules were treated with extreme caution. *E. coli* is a bacterium which lives in its billions within our digestive system, and those of other mammals, and which will survive quite easily in our environment, for instance in our food and on our beaches. So there was a lot of concern that the introduction of foreign DNA into *E. coli* would generate bacteria with dangerous properties. Fortunately, this is one fear that has been shown to be unfounded. Some natural *E. coli* strains *are* pathogenic – in particular the O157:H7 strain which can cause severe disease or death. By contrast, the strains used for genetic manipulation are harmless disabled laboratory strains that will not even survive in the gut. Working with genetically modified *E. coli* can therefore be done very safely (although work with *any* bacterium has to follow some basic safety rules). However, the most commonly used type of vector, plasmids, are shared readily between bacteria; the transmission of plasmids between bacteria is behind much of the natural spread of antibiotic resistance. What if our recombinant plasmids were transmitted to other bacterial strains that *do* survive on their own? This, too, has turned out not to be a worry in the majority of cases. The plasmids themselves have been manipulated so that they cannot be readily transferred to other bacteria. Furthermore, carrying a gene such as that coding for, say, dogfish insulin, or an artificial chromosome carrying 100 000 bases of human genomic DNA is a great burden to an *E. coli* cell, and carries no reward whatsoever. In fact, in order to make them accept it, we have to create conditions that will kill all bacterial cells *not* carrying the foreign gene. If you fail to do so when you start your culture in the evening, you can be sure that your bacteria will have dropped the foreign gene the next morning. Evolution in progress!

Whilst nobody today worries about genetically modified *E. coli*, and indeed diabetics have been injecting genetically modified insulin produced by *E. coli* for decades, the issue of genetic engineering is back on the public agenda, this time pertaining to higher organisms. It is important to distinguish the *genetic* *modification* of plants and animals from *cloning* plants and animals. The latter simply involves the production of genetically identical individuals; it does not involve any genetic modification whatsoever. (The two technologies can be used in tandem, but that is another matter.) So, we will ignore the cloning of higher organisms here. Although it is conceptually very similar to producing a clone of a genetically modified *E. coli*, it is really a matter of reproductive cell biology, and frankly relatively uninteresting from the molecular point of view. By contrast, the genetic modification of higher organisms is both conceptually similar to the genetic modification of bacteria, and also very pertinent as it is a potential and, in principle, fairly easy application following the isolation and analysis of a gene.

At the time of writing, the ethical and environmental consequences of this application are still a matter of vivid debate and media attention, and it would be very surprising if this is not still continuing by the time you read this. Just as in the laboratory, the genetic modification as such is not necessarily the biggest risk here. Thus, if a food crop carries a gene that makes it tolerant of herbicides (weedkillers), it would seem reasonable to worry more about increased levels of herbicides in our food than about the genetic modification itself. Equally, the worry about such an organism escaping into the wild may turn out to be exaggerated. Just as, without an evolutionary pressure to keep the genetic modification, our *E. coli* in the example above died out overnight, it appears quite unlikely that a plant that wastes valuable resources on producing a protein that protects it against herbicides will survive long in the wild in the absence of herbicide use.

Nonetheless, this issue is by no means as clear-cut as that of genetically modified bacteria. We cannot test these organisms in a contained laboratory. They take months or a year to produce each generation, not 20 minutes as *E. coli* does. And even if they should be harmless in themselves, there are other issues as well, such as the one exemplified above. Thus, this is an important and complicated issue, and to understand it fully you need to know about evolution, ecology, food chemistry, nutrition, and molecular biology. We hope that reading this book will be of some help for the last of these. We also hope that it will convey some of the wonder, excitement, and intellectual stimulation that this science brings to its practitioners. What better way to reverse the boredom of a long journey than to indulge in the immense satisfaction of constructing a clever new screening algorithm? Who needs jigsaw and crossword puzzles when you can figure out a clever way of joining two DNA fragments together? And how can you ever lose the fascination you feel about the fact that the drop of enzyme that you're adding to your test tube is about to manipulate the DNA molecules in it with surgical precision?

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2 Basic Molecular Biology

In this book, we assume you already have a working knowledge of the basic concepts of molecular biology. This chapter serves as a reminder of the key aspects of molecular biology that are especially relevant to this book.

2.1 Nucleic Acid Structure

2.1.1 The DNA backbone

Manipulation of nucleic acids in the laboratory is based on their physical and chemical properties, which in turn are reflected in their biological function. Intrinsically, DNA is a very stable molecule. Scientists routinely send DNA samples in the post without worrying about refrigeration. Indeed, DNA of high enough quality to be cloned has been recovered from frozen mammoths and mummified Pharaohs thousands of years old. This stability is provided by the robust repetitive phosphate–sugar backbone in each DNA strand, in which the phosphate links the $5'$ position of one sugar to the $3'$ position of the next (Figure 2.1). The bonds between these phosphorus, oxygen, and carbon atoms are all *covalent bonds*. Controlled degradation of DNA requires enzymes (nucleases) that break these covalent bonds. These are divided into *endonucleases*, which attack internal sites in a DNA strand, and *exonucleases*, which nibble away at the ends. We can for the moment ignore other enzymes that attack for example the bonds linking the bases to the sugar residues. Some of these enzymes are non-specific, and lead to a generalized destruction of DNA. It was the discovery of *restriction endonucleases* (or *restriction enzymes*), which cut DNA strands at specific positions, that opened up the possibility of *recombinant DNA technology* ('*genetic engineering*'), coupled with *DNA ligases*, which can join two double-stranded DNA molecules together.

RNA molecules, which contain the sugar ribose (Figure 2.2), rather than the deoxyribose found in DNA, are less stable than DNA. This is partly due to their greater susceptibility to attack by nucleases (*ribonucleases*), but they are also more susceptible to chemical degradation, especially by alkaline conditions.

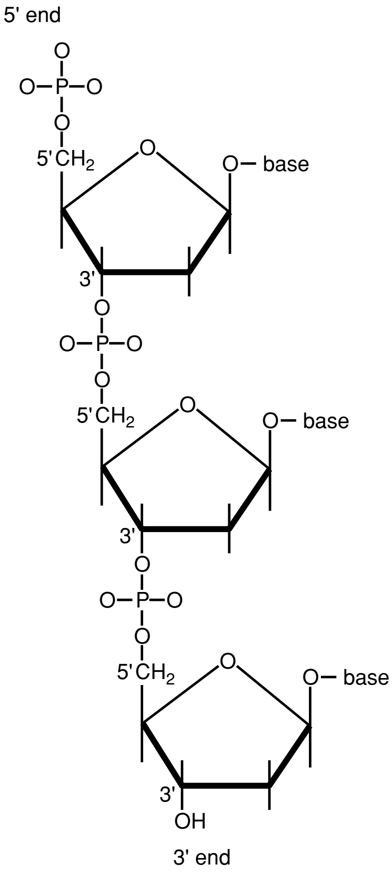

Figure 2.1 DNA backbone

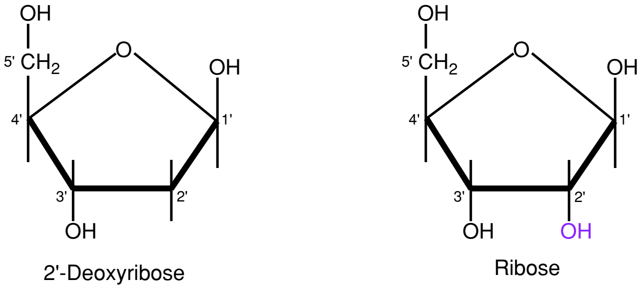

Figure 2.2 Nucleic acid sugars

2.1.2 The base pairs

In addition to the sugar (2′deoxyribose) and phosphate, DNA molecules contain four nitrogen-containing bases (Figure 2.3): two pyrimidines, thymine (T) and cytosine (C), and two purines, guanine (G) and adenine (A). (Other bases can be incorporated into synthetic DNA in the laboratory, and sometimes other bases occur naturally.) Since the purines are bigger than the pyrimidines, a regular double helix requires a purine in one strand to be matched by a pyrimidine in the other. Furthermore, the regularity of the double helix requires specific hydrogen bonding between the bases so that they fit together, with an A opposite a T, and a G opposite a C (Figure 2.4). We refer to these pairs of bases as *complementary*, and hence to one strand as the *complement* of the other. Note that the two DNA strands run in opposite directions. In a conventional representation of a double-stranded sequence the ‘top’ strand has a 5′ hydroxyl group at the left-hand end (and is said to be written in the 5′ to 3′ direction), while the ‘bottom’ strand has its 5′ end at the right-hand end. Since the two strands are complementary, there is no information in the second strand that cannot be deduced from the first one. Therefore, to save space, it is common to represent a double-stranded DNA sequence by showing the sequence of only one strand. When only one strand is

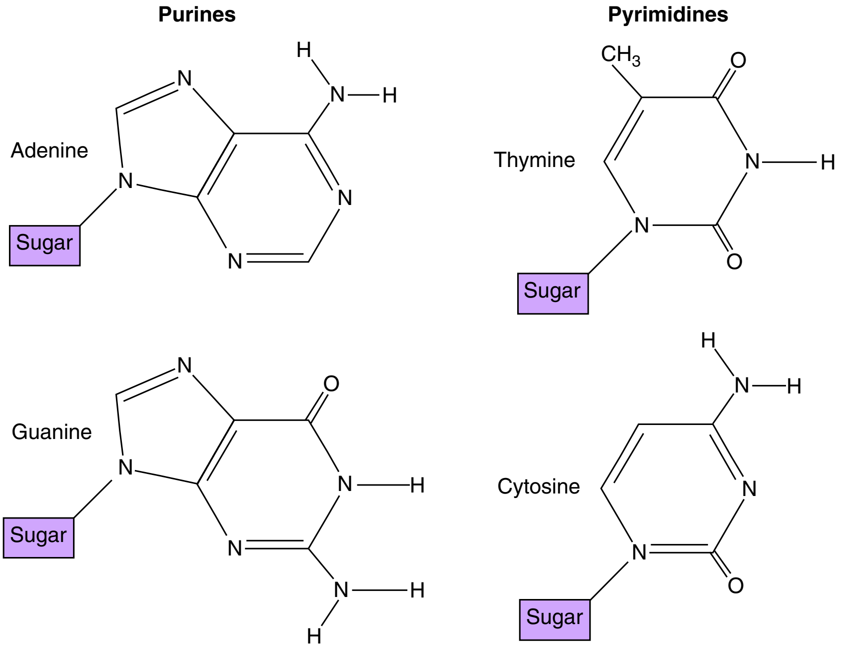

Figure 2.3 Nucleic acid bases

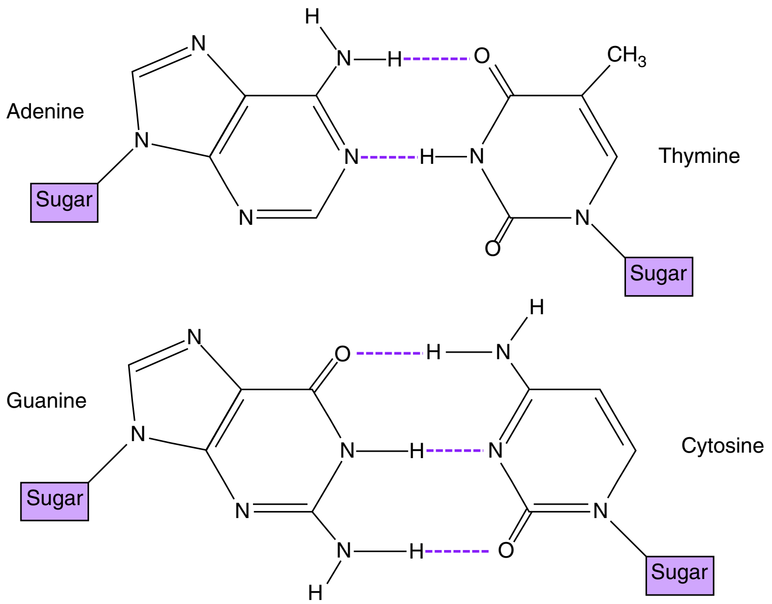

Figure 2.4 Base-pairing in DNA

Box 2.1 Complementary sequences

DNA sequences are often represented as the sequence of just one of the two strands, in the 5′ to 3′ direction, reading from left to right. Thus the double-stranded DNA sequence

5′-AGGCTG-3′
3′-TCCGAC-5′

would be shown as AGGCTG, with the orientation (i.e., the position of the 5′ and 3′ ends) being inferred.

To get the sequence of the other (complementary) strand, you must not only change the A and G residues to T and C (and vice versa), but you must also reverse the order.

So in this example, the complement of AGGCTG is CAGCCT, reading the lower strand from right to left (again in the 5′ to 3′ direction).

shown, we use the 5′ to 3′ direction; the sequence of the second strand is inferred from that, and you have to remember that the second strand runs in the opposite direction. Thus a single strand sequence written as AGGCTG (or more fully 5′AGGCTG3′) would have as its complement CAGCCT (5′CAGCCT3′) (see Box 2.1).

Thanks to this base-pairing arrangement, the two strands can be safely separated – both in the cell and in the test tube – under conditions which disrupt the hydrogen bonds between the bases but are much too mild to pose any threat to the covalent bonds in the backbone. This is referred to as *denaturation* of DNA and, unlike the denaturation of many proteins, it is reversible. Because of the complementarity of the base pairs, the strands will easily join together again and *renature*. In the test tube, DNA is readily denatured by heating, and the denaturation process is therefore often referred to as *melting* even when it is accomplished by means other than heat (e.g. by NaOH). Denaturation of a double-stranded DNA molecule occurs over a short temperature range, and the midpoint of that range is defined as the *melting temperature* (T_m). This is influenced by the base composition of the DNA. Since guanine:cytosine (GC) base pairs have three hydrogen bonds, they are stronger (i.e. melt less easily) than adenine:thymine (AT) pairs, which have only two hydrogen bonds. It is therefore possible to estimate the melting temperature of a DNA fragment if you know the sequence (or the base composition and length). These considerations are important in understanding the technique known as *hybridization*, in which *gene probes* are used to detect specific nucleic acid sequences. We will look at hybridization in more detail in Chapter 8.

Although the normal base pairs (A–T and G–C) are the only forms that are fully compatible with the Watson–Crick double helix, pairing of other bases can occur, especially in situations where a regular double helix is less important (such as the folding of single-stranded nucleic acids into secondary structures – see below).

In addition to the hydrogen bonds, the double stranded DNA structure is maintained by *hydrophobic interactions* between the bases. The hydrophobic nature of the bases means that a single-stranded structure, in which the bases are exposed to the aqueous environment, is unstable. Pairing of the bases enables them to be removed from interaction with the surrounding water. In contrast to the hydrogen bonding, hydrophobic interactions are relatively non-specific. Thus, nucleic acid strands will tend to stick together even in the absence of specific base-pairing, although the specific interactions make the association stronger. The specificity of the interaction can therefore be increased by the use of chemicals (such as formamide) that reduce the hydrophobic interactions.

What happens if there is only a single nucleic acid strand? This is normally the case with RNA, but single-stranded forms of DNA also exist. For example, in some viruses the genetic material is single-stranded DNA. A single-stranded nucleic acid molecule will tend to fold up on itself to form localized double-stranded regions, including structures referred to as hairpins or stem-loop structures. This has the effect of removing the bases from the surrounding water. At room temperature, in the absence of denaturing agents, a single-stranded nucleic acid will normally consist of a complex set of such localized secondary structure elements, which is especially evident with RNA molecules such as transfer RNA (tRNA) and ribosomal RNA (rRNA). This can also happen to a limited extent with double stranded DNA, where short sequences can tend to loop out of the regular double helix. Since this makes it easier for enzymes to unwind the DNA, and to separate the strands, these sequences can play a role in the regulation of gene expression, and in the initiation of DNA replication.

A further factor to be taken into account is the negative charge on the phosphate groups in the nucleic acid backbone. This works in the opposite direction to the hydrogen bonds and hydrophobic interactions; the strong negative charge on the DNA strands causes electrostatic repulsion that tends to repel the two strands. In the presence of salt, this effect is counteracted by the presence of a cloud of counterions surrounding the molecule, neutralizing the negative charge on the phosphate groups. However, if you reduce the salt concentration, any weak interactions between the strands will be disrupted by electrostatic repulsion – and therefore we can use low salt conditions to increase the specificity of hybridization (see Chapter 8).

2.1.3 RNA structure

Chemically, RNA is very similar to DNA. The fundamental chemical difference is that the RNA backbone contains ribose rather than the 2′-deoxyribose (i.e. ribose without the hydroxyl group at the 2′ position) present in DNA (Figure 2.5). However, this slight difference has a powerful effect on some properties of the nucleic acid, especially on its stability. Thus, RNA is readily destroyed byexposure to high pH. Under these conditions, DNA is stable: although the strands will separate, they will remain intact and capable of renaturation when the pH is lowered again. A further difference between RNA and DNA is that the former contains uracil rather than thymine (Figure 2.5).

Generally, while most of the DNA we use is double stranded, most of the RNA we encounter consists of a single polynucleotide strand – although we must remember the comments above regarding the folding of single-stranded nucleic acids. However, this distinction between RNA and DNA is not an inherent property of the nucleic acids themselves, but is a reflection of the natural roles of RNA and DNA in the cell, and of the method of production. In all *cellular* organisms (i.e. excluding viruses), DNA is the inherited material responsible for the genetic composition of the cell, and the replication process that has evolved is based on a double-stranded molecule; the roles of RNA in the cell do not require a second strand, and indeed the presence of a second, complementary, strand would preclude its role in protein synthesis. However, there are some viruses that have double-stranded RNA as their genetic material,

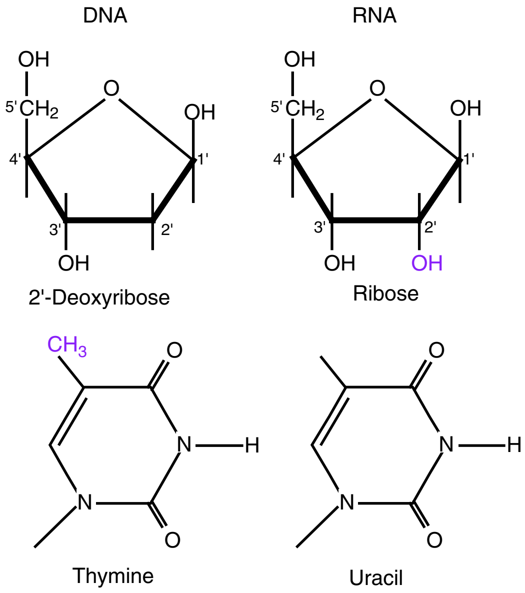

Figure 2.5 Differences between DNA and RNA

as well as some with single-stranded RNA, and some viruses (as well as some plasmids) replicate via single-stranded DNA forms.

2.1.4 Nucleic acid synthesis

We do not need to consider all the details of how nucleic acids are synthesized. The basic features that we need to remember are summarized in Figure 2.6, which shows the addition of a nucleotide to the growing end (3′-OH) of a DNA strand. The substrate for this reaction is the relevant deoxynucleotide triphosphate (dNTP), i.e. the one that makes the correct base-pair with the corresponding residue on the template strand. The DNA strand is always extended at the 3′-OH end. For this reaction to occur it is essential that the residue at the 3′-OH end, to which the new nucleotide is to be added, is accurately base-paired with its partner on the other strand.

RNA synthesis occurs in much the same way, as far as this description goes, except that of course the substrates are nucleotide triphosphates (NTPs) rather than the deoxynucleotide triphosphates (dNTPs). There is one very important difference though. DNA synthesis only occurs by extension of an existing strand – it always needs a *primer* to get it started. RNA polymerases on the other hand are capable of starting a new RNA strand from scratch, given the appropriate signals.

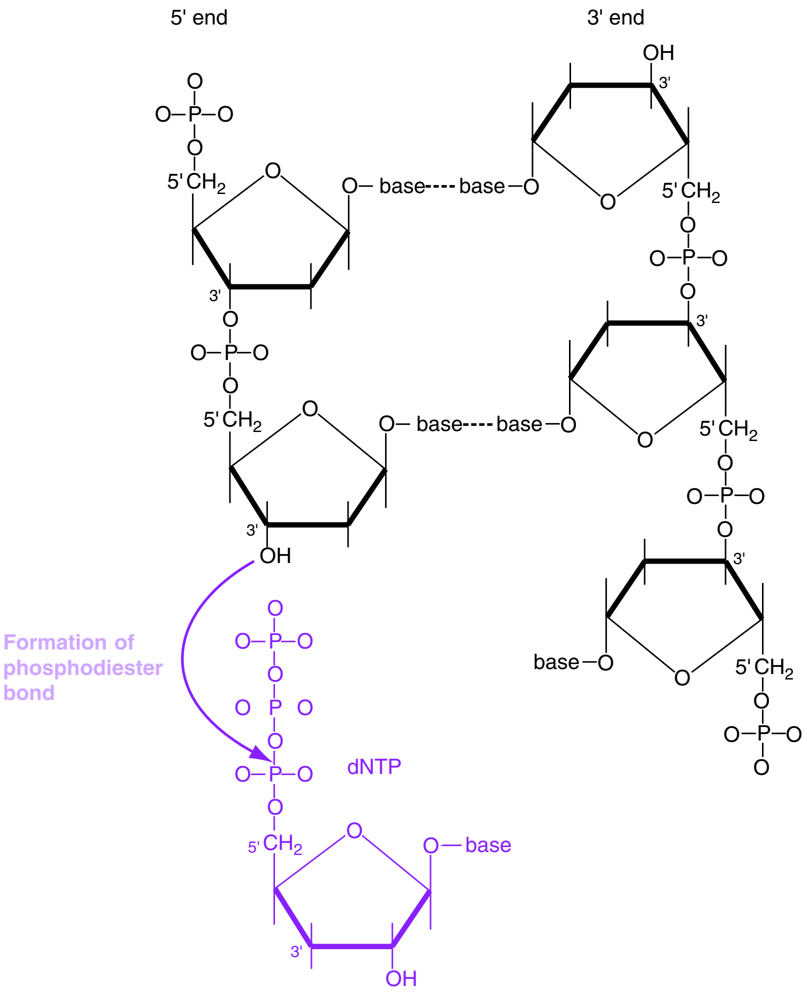

Figure 2.6 DNA synthesis

2.1.5 Coiling and supercoiling

DNA can be denatured and renatured, deformed and reformed, and still retain unaltered function. This is a necessary feature, because as large a molecule as DNA will need to be packaged if it is to fit within the cell that it controls. The DNA of a human chromosome, if it were stretched out into an unpackaged double helix, would be several centimetres long. Thus, cells are dependent on the packaging of DNA into modified configurations for their very existence.

Double-stranded DNA, in its relaxed state, normally exists as a right-handed double helix with one complete turn per 10 base pairs; this is known as the *B* *form* of DNA. Hydrophobic interactions between consecutive bases on the same strand contribute to this winding of the helix, as the bases are brought closer together enabling a more effective exclusion of water from interaction with the hydrophobic bases.

There are other forms of double helix that can exist, notably the *A form* (also right-handed but more compact, with 11 bases per turn) and *Z-DNA* which is a left-handed double helix with a more irregular appearance (a zigzag structure, hence its designation). The latter is of especial interest as certain regions of DNA sequence can trigger a localized switch between the right-handed B form and the left-handed Z form. However, natural DNA resembles most closely the B form, for most of its length.

However, that is not the complete story. There are higher orders of conformation. The double helix is in turn coiled on itself – an effect known as *supercoiling*. There is an interaction between the coiling of the helix and the degree of supercoiling. As long as the ends are fixed, changing the degree of coiling will alter the amount of supercoiling, and vice versa. The effect is easily demonstrated (and probably already familiar to you) with a telephone cord. If you rotate the receiver so as to coil up the cord more tightly and then move the receiver towards the phone you will not only see the supercoiling of the cord but also, if you look more closely, you will see that the tightness of the winding of the cord reduces as it becomes supercoiled.

DNA *in vivo* is constrained; the ends are not free to rotate. This is most obviously true of circular DNA structures such as (most) bacterial plasmids. The net effect of coiling and supercoiling (a property known as the *linking number*) is therefore fixed, and cannot be changed without breaking one of the strands. In nature, there are enzymes known as *topoisomerases* (including DNA gyrase) that do just that: they break the DNA strands, and then in effect rotate the ends and reseal them. This alters the degree of winding of the helix and thus affects the supercoiling of the DNA. Topoisomerases also have an ingenious use in the laboratory, which we will consider in Chapter 5.

So the plasmids that we will be referring to frequently in later pages are naturally supercoiled when they are isolated from the cell. However, if one of the strands is broken at any point, the DNA is then free to rotate at that point and can therefore relax into a non-supercoiled form, with the characteristic B form of the helix. This is known as an *open circular* form (in contrast to the *covalently closed circular* form of the native plasmid). The plasmid will also be in a relaxed form after insertion of a foreign DNA fragment, or other manipulations. Although we have resealed all the nicks in the DNA, we have not altered the supercoiling of the molecule; that will not happen until it has been reinserted into a bacterial cell. Some of the properties of the manipulated plasmid, such as its transforming ability and its mobility on an agarose gel, are therefore not the same as those of the native plasmid isolated from a bacterial cell.

2.2 Gene Structure and Organization

The definition of a 'gene' is rather imprecise. Its origins go back to the early days of genetics, when it could be used to described the unit of inheritance of an observable characteristic (a *phenotype*). As the study of genetics progressed, it became possible to use the term gene as meaning a DNA sequence coding for a specific polypeptide, although this ignores those 'genes' that code for RNA molecules such as ribosomal RNA and transfer RNA, which are not translated into proteins. It also ignores regulatory regions which are necessary for proper expression of a gene although not themselves transcribed or translated.

We often use the term 'gene' as being synonymous with 'open reading frame' (ORF), i.e. the region between the start and stop codons (although even that definition is still vague as to whether we should or should not include the stop codon itself). In bacteria, this takes place in an uninterrupted sequence. In eukaryotes, the presence of introns (see below) makes this definition more difficult; the region of the chromosome that contains the information for a specific polypeptide may be many times longer than the actual coding sequence. Basically, it is not possible to produce an entirely satisfactory definition. However, this is rarely a serious problem. We just have to be careful as to how we use the word depending on whether we are discussing only the coding region (ORF), the length of sequence that is transcribed into mRNA (including untranslated regions), or the whole unit in the widest sense (including regulatory elements that are beyond the translation start site).

In this section we want to highlight some of the key differences in 'gene' organization between eukaryotes and prokaryotes (bacteria), as these differences play a major role in the discussion of the application of molecular biology techniques and their use in different systems.

2.2.1 Operons

In bacteria, it is quite common for a group of genes to be transcribed from a single promoter into one long RNA molecule; this group of genes is known as an *operon* (Figure 2.7). If we are considering protein-coding genes, the transcription product, messenger RNA (mRNA), is then translated into a number of separate polypeptides. This can occur by the ribosomes reaching the stop codon at the end of one polypeptide-coding sequence, terminating translation and releasing the product before re-initiating (without dissociation from the mRNA). Alternatively, the ribosomes may attach independently to internal ribosome binding sites within the mRNA sequence. Generally, the genes involved are responsible for different steps in the same pathway, and this

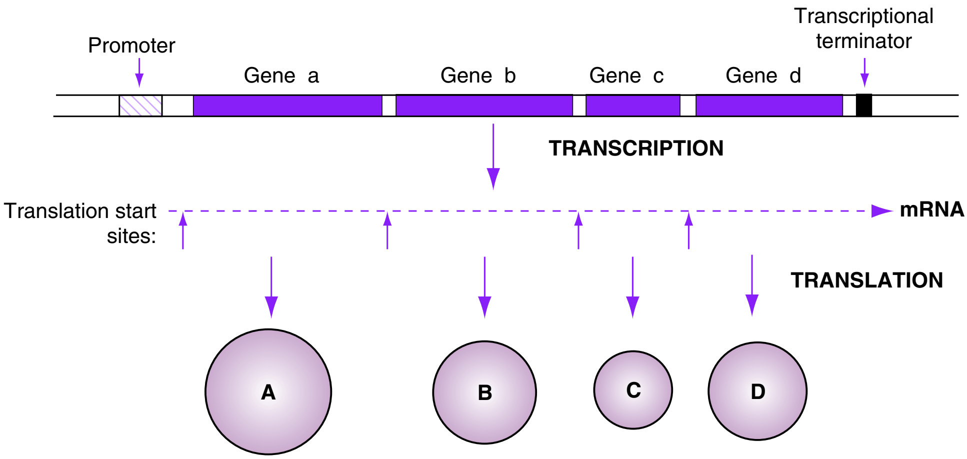

Figure 2.7 Structure of an operon

arrangement facilitates the co-ordinate regulation of those genes, i.e. expression goes up or down together in response to changing conditions.

In eukaryotes, by contrast, the way in which ribosomes initiate translation is different, which means that they cannot produce separate proteins from a single mRNA in this way. There are ways in which a single mRNA can give rise to different proteins, but these work in different ways, such as different processing of the mRNA (see below) or by producing one long polyprotein or precursor which is then cleaved into different proteins (as occurs in some viruses). A few viruses do actually have internal ribosome entry sites.

2.2.2 Exons and introns

In bacteria there is generally a simple one-for-one relationship between the coding sequence of the DNA, the mRNA and the protein. This is usually not true for eukaryotic cells, where the initial transcription product is many times longer than that needed for translation into the final protein. It contains blocks of sequence (*introns*) which are removed by processing to generate the final mRNA for translation (Figure 2.8).

Introns do occur in bacteria, but quite infrequently. This is partly due to the need for economy in a bacterial cell; the smaller genome and generally more rapid growth provides an evolutionary pressure to remove unnecessary material from the genome. A further factor arises from the nature of transcription and translation in a bacterial cell. As the ribosomes are translating the mRNA while it is being made, there is usually no opportunity for sections of the RNA to be removed before translation.

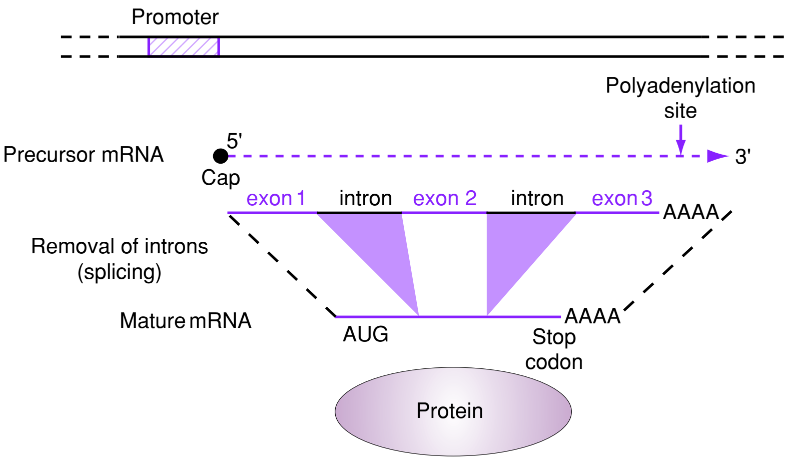

Figure 2.8 Exons and introns

2.3 Information Flow: Gene Expression

The way in which genes are expressed is sufficiently central to so much of the subsequent material in this book that it is worth reviewing briefly the salient features. The basic dogma (Figure 2.9) is that while DNA is the basic genetic material that carries information from one generation to the next, its effect on the characteristics of the cell requires firstly its copying into RNA (transcription), and then the translation of the mRNA into a polypeptide by ribosomes. Further processes are required before its proper activity can be manifested: these include the folding of the polypeptide, possibly in association with other subunits to form a multi-subunit protein, and in some cases modification, e.g. by glycosylation or phosphorylation. It should be noted that in some cases, RNA rather than protein is the final product of a gene (ribosomal and transfer RNA molecules for example).

2.3.1 *Transcription*

Transcription is carried out by RNA polymerase. RNA polymerase recognizes and binds to a specific sequence (the *promoter*), and initiates the synthesis of mRNA from an adjacent position.

A typical bacterial promoter carries two *consensus* sequences (i.e. sequences that are closely related in all genes): TTGACA centred at position −35 (i.e. 35 bases before the transcription start site), and TATAAT at −10 (Figure 2.10). It is important to understand the nature of a consensus: few bacterial promoters

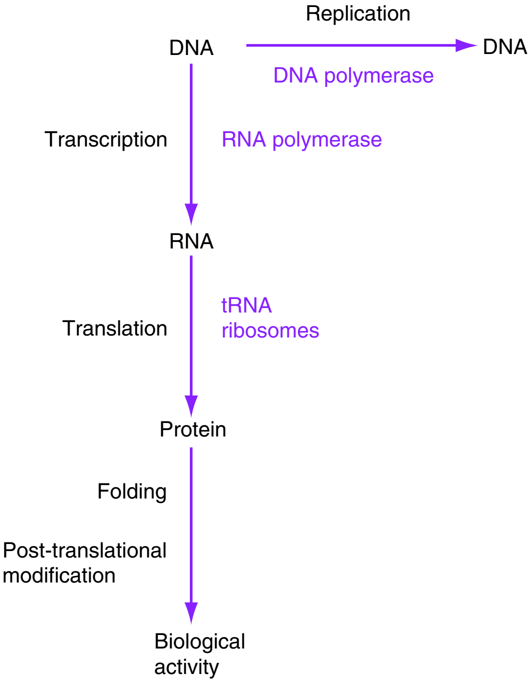

Figure 2.9 Information flow

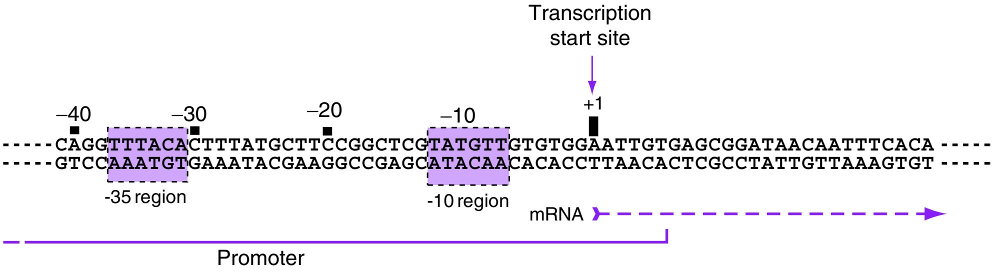

Figure 2.10 Structure of the promoter region of the *lac* operon; note that the −35 and −10 regions of the *lac* promoter do not correspond exactly with the consensus sequences TTGACA and TATAAT respectively

have exactly the sequences shown but if you line up a large number of promoters you will see that at any one position a large number of them have the same base (see Box 2.2). The RNA polymerase has higher affinity for some promoters than others – depending not only on the exact nature of the two consensus sequences but to a lesser extent on the sequence of a longer region. The nature and regulation of bacterial promoters, including the existence of alternative types of promoters, is considered further in Chapter 13.

Box 2.2 Examples of E. coli promoters

```
          –35                      –10       1
TGGCGGTGTTGACATAAATA CCACTGGCGGTGATACTGAGCA CA  Lambda PL
CGTGCGTGTTGACTATTTTA CCTCTGGCGGTGATAATGGTTG CA  Lambda PR
TGCCGAAGTTGAGTATTTTTTGCTGTATTTGTCATAATGACTCCTG  Lambda PO
ATGAGCTGTTGACAATTAAT CATCGAACTAGTTAACTAGTACGCA  trp
CATCGAATGGCGCAAAACCTTTCGCGGTATGGCATGATAGCGCCCG  lacI
CCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGG A  lacZ
CGTAACACTTTACAGCGGCG  CGTCATTTGATATGATGCGCC CG  tyr tRNA
        TTGACA                  TATAAT          consensus
```

Bases matching the – 10 and 35 consensus sequences are boxed. Spaces are inserted to optimize the alignment. Note that the consensus is derived from a much larger collection of characterized promoters.

Position 1 is the transcription start site.

In eukaryotes, by contrast, the promoter is a considerably larger area around the transcription start site, where a number of *trans*-acting transcription factors (i.e. DNA-binding proteins encoded by genes in other parts of the genome) bind to a number of *cis*-acting promoter elements (i.e. elements that affect the expression of the gene next to them) in a considerably more complex scenario. The need for this added complexity can easily be imagined; if cells carrying the same genome are differentiated into a multitude of cell types fulfilling very different functions, a very sophisticated control system is needed to provide each cell type with its specific repertoire of genes, and to fine-tune the degree of expression for each one of them. Nonetheless, the promoter region, however simple or complex, gives rise to different levels of transcription of various genes.

In eukaryotes, the primary transcript, *heteronuclear RNA* (hnRNA), is very short-lived as such, as it is processed in a number of steps. A specialized nucleotide *cap* is added to the 5′ end; this is the site recognized by the ribosomes in protein synthesis (see below). The precursor mRNA is cleaved at a specific site towards the 3′ end and a *poly-A tail*, consisting of a long sequence of adenosine residues, is added to the cut end.. This is a specific process, governed by polyadenylation recognition sequences in the 3′ untranslated region. Nature's 'tagging' of mRNA molecules comes in very useful in the laboratory for the isolation of eukaryotic mRNA (see Chapter 7). Finally, in the process of *splicing*, the introns are spliced out and the exons are joined together.

In bacteria, the processes of transcription and translation take place in the same compartment and simultaneously. In other words, the ribosomes translating the mRNA follow closely behind the RNA polymerase, and polypeptide production is well under way long before the mRNA is complete. In eukaryotes, by contrast, the mature mRNA molecule is transported out of the nucleus to the cytoplasm where translation takes place.

The resulting level of protein production is dependent on the amount of the specific mRNA available, rather than just the rate of production. The level of an mRNA species will be affected by its rate of degradation as well as by its rate of synthesis. In bacteria, most mRNA molecules are degraded quite quickly (with a half life of only a few minutes), although some are much more stable. The instability of the majority of bacterial mRNA molecules means that bacteria can rapidly alter their profile of gene expression by changing the transcription of specific genes. The lifespans of most eukaryotic mRNA molecules are measured in hours rather than minutes. Again, this is a reflection of the fact that an organism that is able to control its own environment to a varying extent is subjected to less radical environmental changes. Consequently, mRNA molecules tend to be more stable in multicellular organisms than in, for example, yeast. Nonetheless, the principle remains: the level of an mRNA is a function of its production and degradation rates. We will discuss how to study and disentangle these parameters in Chapter 13.

2.3.2 Translation

In bacteria, translation starts when ribosomes bind to a specific site (the *ribosome binding site, RBS*) which is adjacent to the start codon. The sequence of the ribosome binding site (also known as the *Shine–Dalgarno sequence*) has been recognized as being complementary to the 3′ end of the 16S rRNA (Figure 2.11). The precise sequence of this site, and its distance from the start codon does affect the efficiency of translation, although in nature this is less important than transcriptional efficiency in determining the level of gene expression. Translation efficiency will also depend on the codon usage, i.e. the match between synonymous codons and the availability of tRNA that will recognize each codon. This concept is explored more fully in Chapter 15.

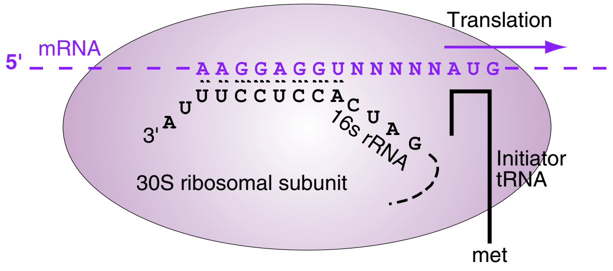

Figure 2.11 Bacterial ribosome binding site

In bacterial systems, where transcription and translation occur in the same compartment of the cell, ribosomes will bind to the mRNA as soon as the RBS has been synthesized. Thus there will be a procession of ribosomes following close behind the RNA polymerase, translating the mRNA as it is being produced. So, although the mRNA may be very short-lived, the bacteria are capable of producing substantial amounts of the corresponding polypeptide.

In eukaryotes, the mechanism (as usual) is much more complicated. Instead of binding just upstream of the initiation codon, the ribosome binds at the very 5′ end of the mRNA to the cap, and reads along the 5′ untranslated region (UTR) until it reaches an initiation codon. The sequence AUG may be encountered on the way without initiation; the surrounding sequence is also important to define the start of protein synthesis. The fact that the 5′ UTR is scanned in its full length by the ribosome makes it an important region for specifying translation efficiency, and different secondary structures can have either a positive or a negative effect on the amount of protein that is produced.

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3 How to Clone a Gene

3.1 What is Cloning?

Cloning means using asexual reproduction to obtain organisms that are genetically identical to one another, and to the ‘parent’. Of course, this contrasts with sexual reproduction, where the offspring are not usually identical. It is worth stressing that clones are only identical *genetically*; the actual appearance and behaviour of the clones will be influenced by other factors such as their environment. This applies equally to all organisms, from bacteria to humans.

Despite the emotive language that increasingly surrounds the use of the word ‘cloning’, this is a concept that will be surprisingly familiar to many people. In particular, anyone with an interest in gardening will know that it is possible to propagate plants by taking cuttings, and that in this way you will produce a number of plants that are identical to the parent. These are clones. Similarly, the routine bacteriological procedure of purifying a bacterial strain by picking a single colony for inoculating a series of fresh cultures is also a form of cloning.

The term *cloning* is also applied to genes, as an extension of the concept. If you introduce a foreign gene into a bacterium, or any other type of cell, in such a way that it will be copied when the cell replicates, then you will produce a large number of cells all with identical copies of that piece of DNA – you have *cloned* the gene (Figure 3.1). By producing a large number of copies in this way, you can sequence it or label it as a probe to study its expression in the organism it came from. You can express its protein product in bacterial or eukaryotic cells. You can mutate it and study what difference that mutation makes to the properties of the gene, its protein product, or the cell that carries it. You can even purify the gene from the bacterial clone and inject it into a mouse egg, and produce a line of transgenic mice that express it. Behind all of these applications lies a cloning process with the same basic steps.

In subsequent chapters, we will consider how this process is achieved, initially with bacterial cells (mainly *E. coli*) as the host and later extending the discussion to alternative host cells. The purpose of this chapter is to present an overview of the process, with the details of the various steps being considered further in the subsequent chapters.

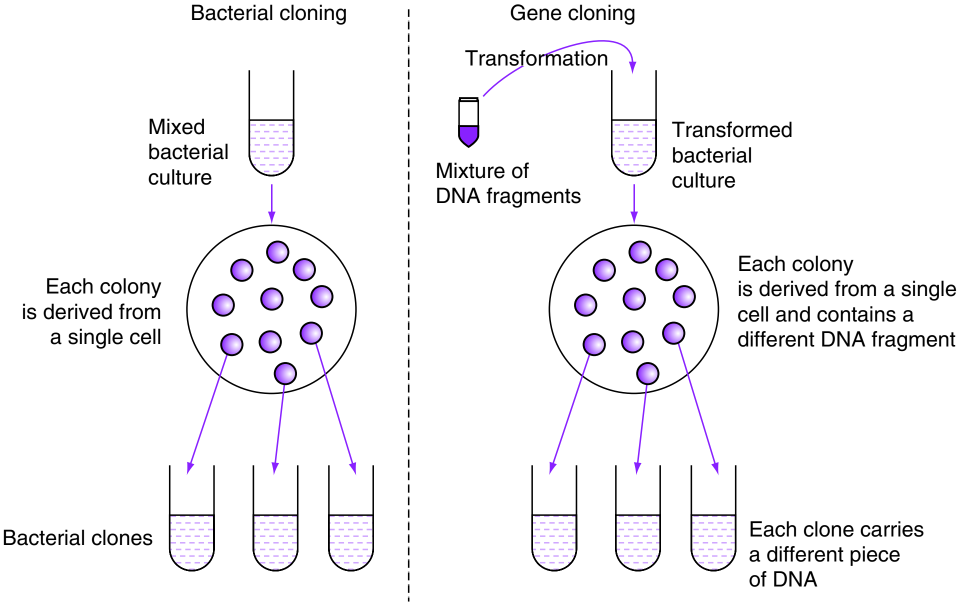

Figure 3.1 Comparison of bacterial cloning and gene cloning

3.2 Overview of the Procedures

Some bacterial species will naturally take up DNA by a process known as *transformation*. However, most species have to be subjected to chemical or physical treatments before DNA will enter the cells. In all cases, the DNA will not be replicated by the host cell unless it either recombines with (i.e. is inserted into) the host chromosome or alternatively is incorporated into a molecule that is recognized by enzymes within the host cell as a substrate for replication. For most purposes the latter process is the relevant one. We use *vectors* to carry the DNA and allow it to be replicated. There are many types of vectors for use with bacteria. Some of these vectors are *plasmids*, which are naturally occurring pieces of DNA that are replicated independently of the chromosome, and are inherited by the two daughter cells when the cell divides. (In Chapter 6 we will encounter other types of vectors, including viruses that infect bacteria; these are known as *bacteriophages*, or phages for short.)

The DNA that we want to clone is inserted into a suitable vector, producing a *recombinant molecule* consisting of vector plus insert (Figure 3.2). This recombinant molecule will be replicated by the bacterial cell, so that all the cells descended from that initial transformant will contain a copy of this piece of recombinant DNA. A bacterium like *E. coli* can replicate very rapidly under laboratory conditions, doubling every 20 minutes or so. This exponential

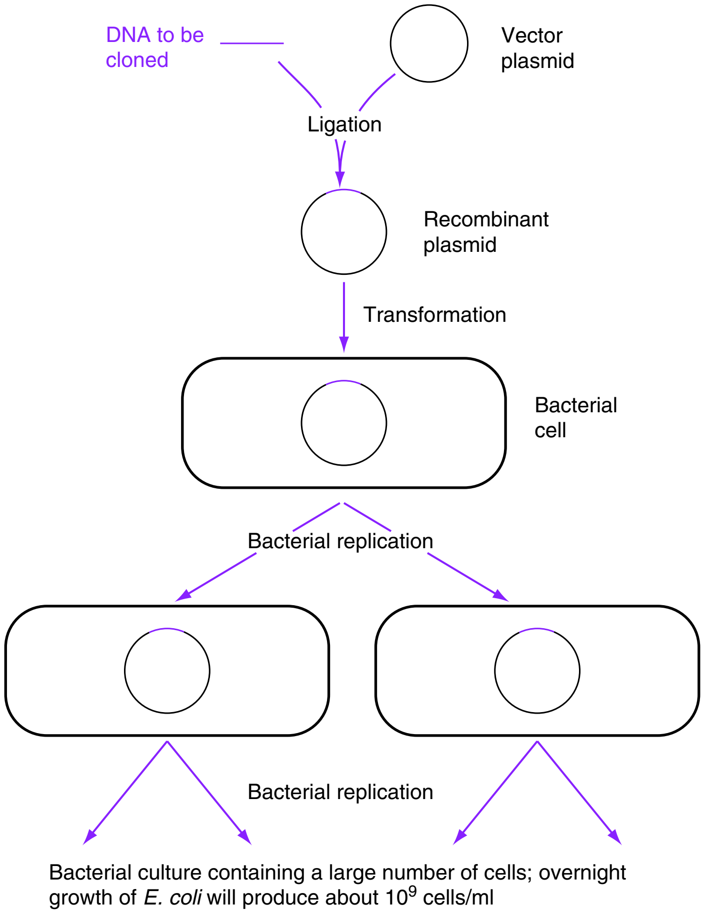

Figure 3.2 Basic outline of gene cloning

growth gives rise to very large numbers of cells; after 30 generations (10 hours), there will be 1×10^9 (one thousand million, or 1 000 000 000) descendants of the initial transformant. Each one of these cells carries a copy of the recombinant DNA molecule, so we will have produced a very large number of copies of the cloned DNA.

Of course exponential growth does not continue indefinitely; after a while, the bacteria start to run out of nutrients, and stop multiplying. (The reasons for growth stopping are actually rather more complex than that, but depletion of nutrients, including diffusion of oxygen, is the main factor.) With *E. coli*, this commonly occurs with about 1×10^9 bacteria per ml of culture. However, if we take a small sample and add it to fresh medium, exponential growth will resume. The clone can thus be propagated, and in this way we can effectively produce unlimited quantities of the cloned DNA. If we can get the bacteria to express the cloned gene, we can also get very large amounts of the product of that gene.

In order to carry out this procedure, we require a method for joining pieces of DNA to such a vector, as well as a way of cutting the vector to provide an opportunity for this joining to take place. The key to the development of gene cloning technology was the discovery of enzymes that would carry out these reactions in a very precise way. The main enzymes needed are *restriction endonucleases* which break the sugar–phosphate backbone of DNA molecules at precise sites, and *DNA ligases* which are able to join together the fragments of DNA that are generated in this way (Figure 3.3). These enzymes, and the ways in which they are used, are described in more detail in Chapter 5.

Once a piece of DNA has been inserted into a plasmid (forming a recombinant plasmid) it then has to be introduced into the bacterial host by a transformation process. Generally this process is not very efficient so only a small proportion of bacterial cells actually take up the plasmid. However, by using a plasmid vector that carries a gene coding for resistance to a specific antibiotic, we can simply plate out the transformed bacterial culture onto agar plates

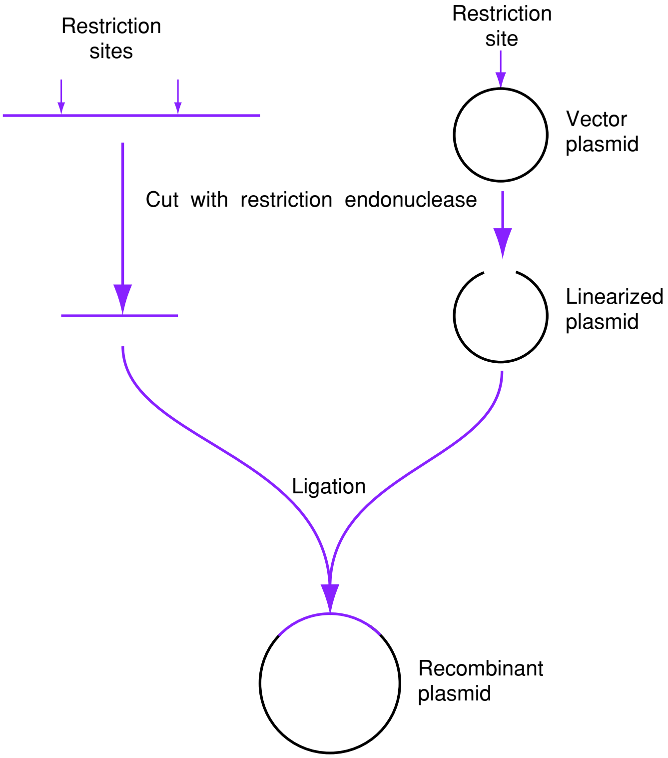

Figure 3.3 Cutting and joining DNA

containing that antibiotic, and only the cells which have received the plasmid will be able to grow and form colonies.

This description does not consider how we get hold of a piece of DNA carrying the specific gene that we want to clone. Even a small and relatively simple organism like a bacterium contains thousands of genes, and they are not arranged as discrete packets but are regions of a continuous DNA molecule. We have to break this molecule into smaller fragments, which we can do specifically (using restriction endonucleases) or non-specifically (by mechanical shearing). But however we do it, we will obtain a very large number of different fragments of DNA with no easy way of reliably purifying a specific fragment, let alone isolating the specific fragment that carries the required gene. The only way of separating the fragments is by size, but there are so many fragments that there will be a lot of different pieces of DNA that are so similar in size that they cannot be separated.

3.3 Gene Libraries

Fortunately it is not necessary to try to purify specific DNA fragments. One of the strengths of gene cloning is that it provides another, more powerful, way of finding a specific piece of DNA. Rather than attempting to separate the DNA fragments, we take the complete mixture and use DNA ligase to insert the fragments into the prepared vector. Under the right conditions, only one fragment will be inserted into each vector molecule. In this way, we produce a mixture of a large number of different recombinant vector molecules, which is known as a *gene library* (or more specifically a *genomic library*, to contrast it with other forms of gene library that will be described in Chapter 7). When we transform a bacterial culture with this library, each cell will only take up one molecule. When we then plate the transformed culture, each colony, which arises from a single transformed cell, will contain a large number of bacteria all of which carry the same recombinant plasmid, with a copy of the same piece of DNA from our starting mixture. So instead of a mixture of thousands (or millions, or tens of millions) of different DNA fragments, we have a large number of bacterial colonies each of which carries one fragment only (Figure 3.4). The production and screening of gene libraries is considered in Chapters 7 and 8, where we will see that a variety of different vectors, other than simple plasmids, are generally used for constructing genomic libraries.

We still have a very complex mixture, but whereas purifying an individual DNA fragment is extremely difficult, it is simple to isolate individual bacterial colonies from this mixture – we just pick them from a plate. Each individual bacterial colony will carry a different piece of DNA from our original complex mixture, so if we can identify which bacterial colony carries the gene that we are interested in, purifying it becomes a simple matter. We just have to pick the

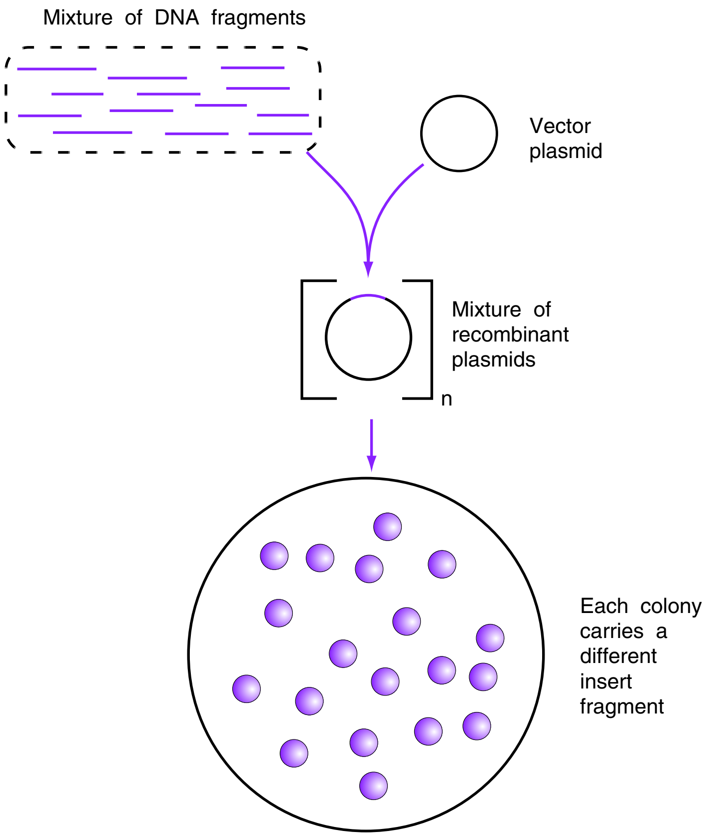

Figure 3.4 Making a genomic library

right colony and inoculate it into fresh medium. However, we still have the problem of knowing which of these thousands/millions of bacterial colonies does actually carry the gene that we want. This is considered more fully in Chapter 8, but one commonly used and very powerful method can be introduced here as an example. This depends on the phenomenon of *hybridization*.

3.4 Hybridization

If a double-stranded DNA fragment is heated, the non-covalent bonds holding the two strands together will be disrupted, and the two strands will separate. This is known as *denaturation*, or less formally (and less accurately) as ‘*melting*’. When the solution is allowed to cool again, these bonds will reform and the original double-stranded fragment will be re-formed (the two strands are said to *anneal*).

We can utilize this phenomenon to identify a specific piece of DNA in a complex mixture by labelling a specific DNA sequence (the *probe*), and mixing the labelled probe with the denatured mixture of fragments. When the mixture is cooled down, the probe will tend to hybridize to any related DNA fragments (Figure 3.5), which enables us to identify the specific DNA fragments that we want.

For screening a gene library, the labelled probe will hybridize to DNA from any colony that carries the corresponding gene or part of it; we can then recover that colony and grow up a culture from it, thus producing an unlimited amount of our cloned gene.

Of course it is not quite as simple as that – we cannot hybridize the probe to the colonies on an agar plate. However, it is easy to transfer a part of each colony onto a membrane by replication, and then lyse the colony so that the DNA it contains is fixed to the membrane. This produces a pattern of DNA spots on the membrane in positions corresponding to the colonies on the original plate (Figure 3.6), which can then be hybridized to the labelled probe to enable identification, and recovery, of the required colony. Hybridization, using labelled DNA or RNA probes, is an important part of many other techniques that we will encounter in subsequent chapters.

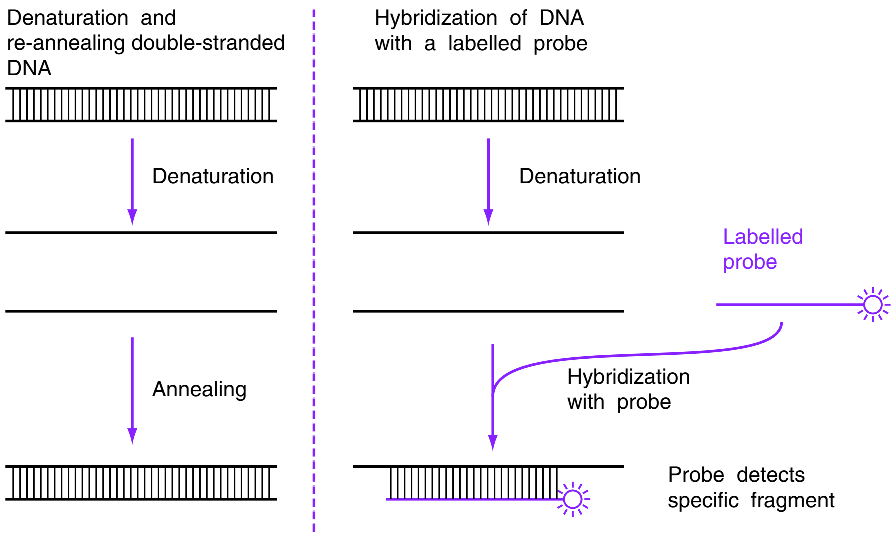

Figure 3.5 Hybridization and gene probes

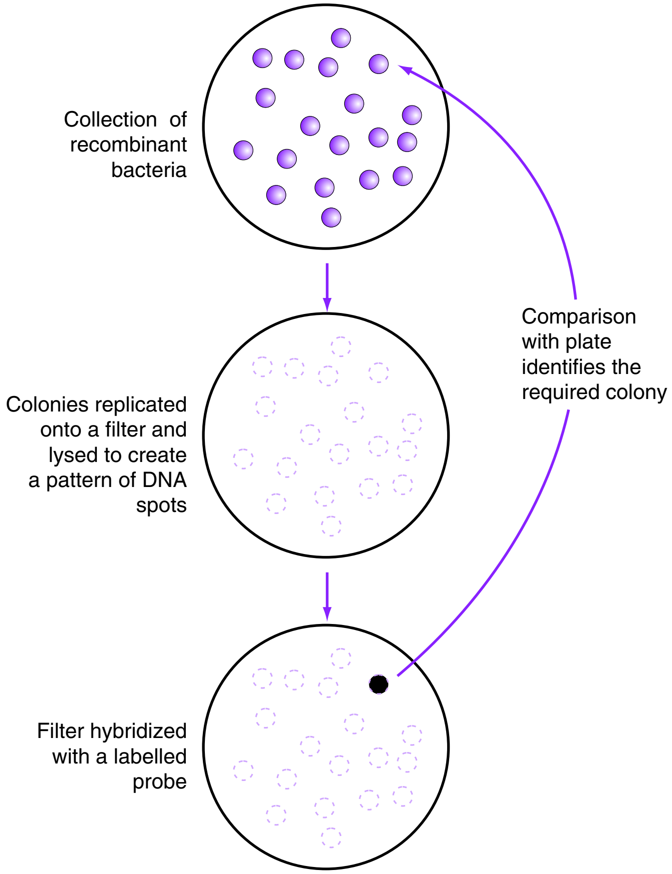

Figure 3.6 Colony hybridization

3.5 Polymerase Chain Reaction

The technique known as the polymerase chain reaction (PCR) often provides an alternative to gene cloning and gene libraries as a way of obtaining usable quantities of specific DNA sequences. PCR requires the use a pair of primers that will anneal to sites at either side of the required region of DNA (Figure 3.7). DNA polymerase action will then synthesize new DNA strands starting from each primer. Denaturation of the products, and re-annealing of the primers, will allow a second round of synthesis. Repeated cycles of denaturation, annealing and extension will give rise to an exponential amplification of the DNA sequence between the two primers, with the amount of product doubling in each cycle, so that after say 20 cycles there will (theoretically) be

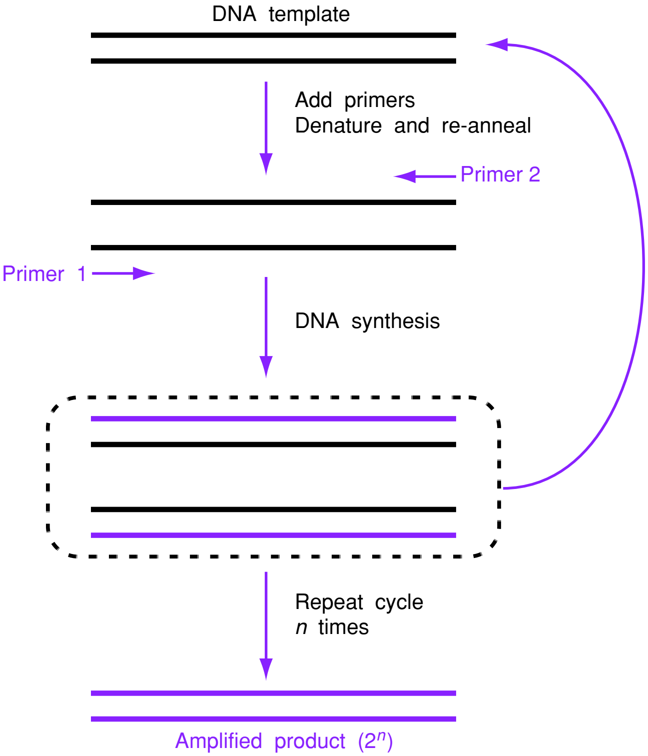

Figure 3.7 Polymerase chain reaction

a million-fold increase in the amount of product. This enables the amplification of a specific region of the DNA, and the product can then be cloned directly. The polymerase chain reaction, and some of the many applications, are described more fully in Chapter 9.

In this chapter we have provided a brief overview of the principal methods used in gene cloning. These procedures, and some of the main alternative strategies, are described more fully in subsequent chapters.

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4 Purification and Separation of Nucleic Acids

4.1 Extraction and Purification of Nucleic Acids

The first step for most of the procedures referred to in this book is to extract the DNA (or for some purposes RNA) from the cell and to purify it by separating it from other cellular components. Although recent technological advances have made this much less of a challenge than it once was, the quality of the starting material remains a crucially important factor for most purposes. For some applications, such as PCR (see Chapter 9) or hybridization analyses (see Chapter 8), less pure material may be acceptable. In this chapter, we review the concepts underlying the most commonly used methods of purifying and fractionating nucleic acids; for further experimental details, you will need to consult a laboratory manual (see Appendix A).

4.1.1 *Breaking up cells and tissues*

Although there are many different methods for purifying nucleic acids, they have a number of basic features in common. Firstly, we need the starting material. This could be a culture of bacterial or eukaryotic cells, which would simply need to be separated from the growth medium (for example by centrifugation), or a more complex tissue sample, which first needs to be homogenized so that the individual cells can be lysed. Wherever possible, the material should be freshly harvested or frozen until ready to use, to avoid degradation by enzymes present in the cell extract.

The cells then need to be lysed to release their components. The nature of the treatment will vary widely according to the cell type. Bacterial cells have walls that have to be broken before the cell contents can be released. This is usually accomplished by using *lysozyme* (an enzyme naturally present in egg white and tears for the very purpose of breaking down bacterial cell walls), often in conjunction with EDTA and a detergent such as SDS (sodium dodecyl sulphate). EDTA eliminates divalent cations and thus destabilizes the outer membrane in bacteria such as *E. coli*, and also inhibits DNases that would otherwise tend to degrade the DNA, while the detergent will solubilize the membrane lipids.

Plant and fungal cells have cell walls that are different from those in bacteria, and require alternative treatments, either mechanical or enzymatic, while animal cells (which lack a cell wall) can usually be lysed by more gentle treatment with a mild detergent.

After breaking up cell walls, and plasma membrane, we find ourselves with a mixture of that material and the intracellular components which have now been released – a complex solution of DNA, RNA, proteins, lipids and carbohydrates. Note that the sudden lysis of the cell will usually result in some fragmentation of chromosomal DNA. In particular, the bacterial chromosome, which is usually circular in its native state, will be broken into linear fragments. Where it is necessary to obtain very large (even intact) chromosomal DNA, more gentle lysis conditions are necessary (see the description of pulsed field gel electrophoresis in Chapter 12). Bacterial plasmids, however, are readily obtained in their native, circular, state by standard lysis conditions.

The next step in the procedure is to separate the desired nucleic acid from these other components.

4.1.2 Enzyme treatment

Removal of RNA from a DNA preparation is easily achieved by treatment with ribonuclease (RNase). Since RNase is a very heat-stable enzyme, it is easy to ensure that it is free of traces of deoxyribonuclease (DNase) that would otherwise degrade your DNA, simply by heating the enzyme before use. Removal of DNA from RNA preparations used to be less easy, since it requires DNase without any RNase activity. However, it is now possible to buy RNase-free DNase (as well as DNase-free ribonuclease). Protein contamination can be removed by digestion with a proteolytic enzyme such as proteinase K.

These treatments are applied if necessary in different nucleic acid purification protocols. However, in some protocols they are omitted, either because the contamination is unimportant for a specific purpose, or because the contaminants will be removed anyway through subsequent steps, as described below.

4.1.3 Phenol–chloroform extraction

Removal of proteins is particularly important as the cell contains a number of enzymes that will degrade nucleic acids, as well as other proteins that will interfere with subsequent procedures by binding to the nucleic acids. A

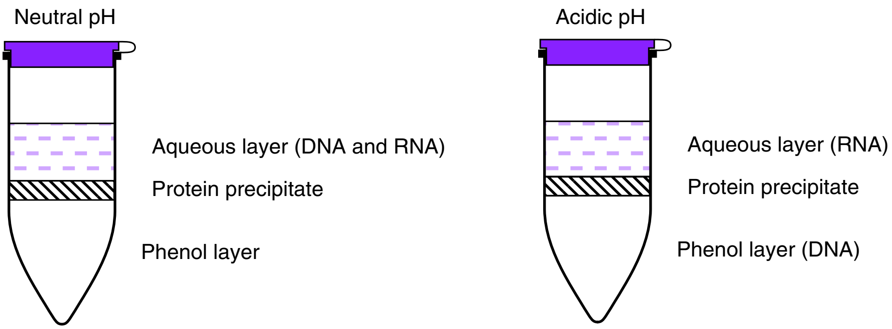


Figure 4.1 Phenol extraction

classical, and still very frequently employed way of removing proteins is by extraction with liquefied phenol, or preferably a mixture of phenol and chloroform. Phenol and chloroform are (largely) immiscible with water, and so you will get two layers (phases) when added to your cell extract. When the mixture is vigorously agitated, the proteins will be denatured and precipitated at the interphase (Figure 4.1). If you are using phenol that has been equilibrated with a neutral or alkaline buffer (as is normally the case), the nucleic acids (DNA and RNA) will remain in the aqueous layer. On the other hand, if you carry out the extraction with acidic phenol, DNA will partition into the organic phase, allowing you to recover RNA from the aqueous phase. Phenol is naturally acidic, so equilibration with water, or the use of an acidic buffer, will produce the appropriate conditions.

Phenol extraction is also useful in subsequent stages of manipulation when it is necessary to ensure that all traces of an enzyme have been removed before proceeding to the next step. (*Note*: phenol is highly toxic by skin absorption, and gloves *must* be worn.)

4.1.4 Alcohol precipitation

Following phenol extraction, you will have a protein-free sample of your nucleic acid(s). However, it will probably be more dilute than you want it to be, and furthermore it will contain traces of phenol and chloroform. Phenol in particular does have a significant degree of solubility in water, and could lead to denaturation of enzymes in subsequent steps. The answer is normally to concentrate (and further purify) the solution by *precipitating* the nucleic acid. This is done by adding an alcohol, either isopropanol or (more frequently) ethanol; in the presence of monovalent cations (Na^+, K^+ or NH_4^+) a nucleic acid precipitate forms which can be collected at the bottom of the test tube by

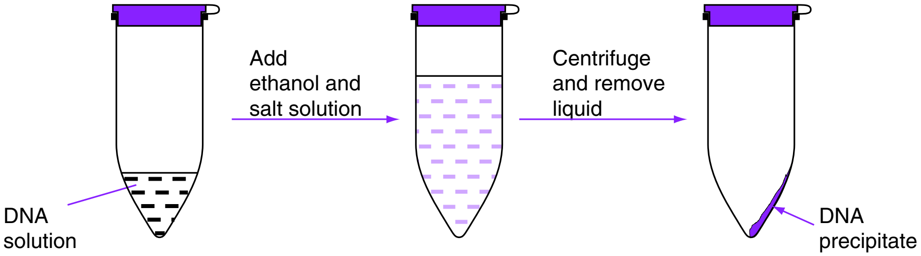


Figure 4.2 Ethanol precipitation

centrifugation (Figure 4.2). Some of the salt will precipitate as well and is removed by washing with 70% ethanol.

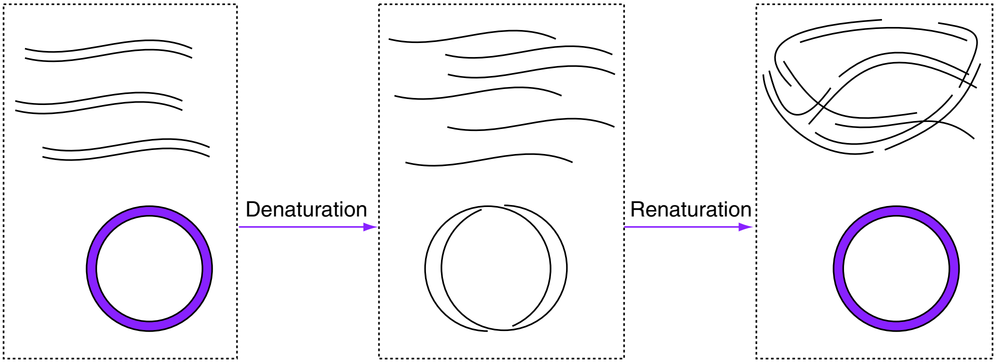
The details of alcohol precipitation procedures vary according to the nature of the nucleic acid; for example, low molecular weight DNA is much less readily precipitated. This is not a major factor in the purification of DNA from cell extracts, but does need to be considered at later stages, such as when purifying small fragments of DNA.

4.1.5 Gradient centrifugation

Centrifugation at moderate speeds is frequently used in DNA purification for the separation of particulate matter from a solution, whether it be the removal of cellular debris or the recovery of precipitated nucleic acids. It is also frequently employed in various column purification methods (see below). Apart from these methods, an old method for separating nucleic acids, or indeed any type of macromolecule, is to subject them to *ultracentrifugation* in a density gradient. This includes the use of caesium salt gradients, usually with the addition of ethidium bromide, for the separation of plasmid DNA from bacterial genomic DNA, or of RNA from DNA. In addition, sucrose gradients can be used for the size-selection of large DNA fragments when constructing a genomic library. In these systems, DNA molecules are separated because of differences in size and/or configuration (i.e. supercoiled versus relaxed). These methods are still in use, but have to a large extent been replaced by far faster and less cumbersome methods; therefore we will not consider them in detail here.

4.1.6 Alkaline denaturation

This is a widely used procedure for separating plasmids from chromosomal DNA in bacterial cell extracts. In a bacterial cell extract, chromosomal DNA



Chromosomal DNA as linear fragments; plasmid as covalently closed circular DNA

Denatured plasmid DNA strands remain interlocked; linear chromosomal fragments dissociate

Interlocked plasmid strands snap together; chromosomal fragments aggregate

Figure 4.3 Alkaline denaturation procedure for plasmid purification

exists as linear fragments, due to the fragmentation that occurs during lysis of the cell. Raising the pH to about 12 will disrupt the hydrogen bonds and allow the linear strands to separate. Plasmids (especially the relatively small ones used for gene cloning) are tougher and are not disrupted by cell lysis; they remain as intact supercoiled circular DNA. Although the high pH will disrupt the hydrogen bonds, the two circular strands will not be able to separate physically, and will remain interlinked. When the pH is reduced, the interlinked plasmid strands will snap back to reform the double-stranded plasmid (Figure 4.3). On the other hand, the separated linear chromosomal fragments cannot do this; instead they will aggregate into an insoluble network that can be removed by centrifugation, leaving the plasmids in solution. Other cell components, including cell wall debris and many proteins, are also removed by this procedure, so phenol extraction may not be needed (although it increases the stability of the extract). The plasmid preparation obtained by this method is pure enough for many purposes (including restriction digestion); when further purification is necessary this can be achieved using affinity matrices (see below).

4.1.7 Column purification

Two types of column purification are frequently used when purifying nucleic acids, often by the aid of centrifugation (*spin columns*). In *size-selection chromatography*, a sample is passed through a matrix of small porous beads. Smaller molecules, such as salts and unincorporated nucleotides, will enter the beads, whereas larger molecules such as longer nucleic acid chains will pass right through the column. This type of purification is a valuable tool and often a fast and simple alternative to purification by alcohol precipitation.

In *affinity chromatography* purification, the macromolecules in your sample will bind to the resin in the column. This could be an anionic resin, which binds to the negatively charged phosphate groups in the nucleic acid backbone, or more sophisticated ones such as resins coated with oligo-dT sequences, which specifically bind to the poly-A tails of eukaryotic mRNA molecules (see Chapter 7). In both cases, undesirable molecules can be washed from the column, after which the stringency conditions are changed and the bound nucleic acids eluted into a small volume of water or buffer.

4.2 Detection and Quantitation of Nucleic Acids

If your DNA preparation is reasonably pure (i.e. free of other materials that absorb ultraviolet radiation, including RNA, free nucleotides, and proteins), then you can estimate the DNA concentration by measuring the absorbance of the solution in a spectrophotometer at 260 nm. This is convenient, but not very sensitive: a solution of 50 μg/ml of double-stranded DNA will have an absorbance of 1. However, the presence of proteins or phenol will affect this estimate. Furthermore, UV absorbance gives you no check on the integrity of your DNA; it can be completely degraded and still give you a reading.

Dyes such as ethidium bromide are commonly used for both detecting and quantitating nucleic acids. Ethidium bromide has a flat ring structure which is able to stack in between the bases in nucleic acids; this is known as *intercalation*. The dye can then be detected by its fluorescence (in the red–orange region of the spectrum) when exposed to UV irradiation. This is the most widely used method for staining electrophoresis gels (see below), and can also be used for estimating the amount of DNA (or RNA) in your sample, by comparing the intensity of the fluorescence with a sample of known concentration run on the same gel.

Note that ethidium bromide is mutagenic, and precaution must be taken to eliminate health risks. Alternative, less hazardous, dyes are increasingly being used for this purpose.

4.3 Gel Electrophoresis

Gel electrophoresis is a crucial technique for both the analysis and the purification of nucleic acids. When a charged molecule is placed in an electric field, it will migrate towards the electrode with the opposite charge; nucleic acid molecules, being negatively charged, will move towards the positive pole (anode). In a gel, which consists of a complex network of pores, the rate at which a nucleic acid molecule moves will be determined by its ability to penetrate through this network. For linear fragments of double-stranded DNA within a certain size range, this will reflect the size of the molecule (i.e. the length of the DNA). We do not have to consider the amount of charge that the molecule carries (unlike some other applications of electrophoresis), since all nucleic acids carry the same amount of charge per unit size.

The effective size range of a gel is determined by its composition. We can use agarose gels for separating nucleic acid molecules greater than a few hundred base pairs, reducing the agarose concentration to obtain effective separation of larger fragments, or increasing it for small fragments. For even smaller molecules, down to only a few tens of base pairs, we would use polyacrylamide gels.

4.3.1 Analytical gel electrophoresis

We can therefore use agarose gel electrophoresis for analysing the composition and quality of a nucleic acid sample. In particular, it is invaluable for determining the size of DNA fragments from a restriction digest or the products of a PCR reaction (see Chapter 9). For this purpose it is necessary to calibrate the gel by running a standard marker containing fragments of known sizes; in Figure 4.4, the standard marker is provided by a *Hin*dIII digest of DNA from the lambda bacteriophage. It can be seen that over much of the size range, there is a linear relationship between the logarithm of the fragment size and the distance it has moved. From this calibration graph, you can then estimate the size of your unknown fragment(s) – assuming they are linear double-stranded DNA (see below).

The reason for the non-linearity of the curve with larger DNA molecules is that these molecules move through the gel in a different way. Although these molecules are large, they are also very thin, and they can in effect slither through the gel end-on. It takes some time for them to become lined up, but once they are, then the rate at which they move is largely independent of their size. So for a particular gel, all molecules above a certain size will have virtually the same mobility. In the gel shown, all DNA molecules larger than about 20 kb will not be separated, but the use of gels with a lower agarose concentration will extend the size range. Special techniques, involving frequent switching of the direction of the electric field, are available for separating very large DNA molecules – see the description of pulsed-field gel electrophoresis (PFGE) in Chapter 12.

If a more precise confirmation of the nature of the sample is required, the gel is *blotted* onto a membrane support, and *hybridized* with a nucleic acid probe (see the section on Southern blotting in Chapter 8).

Polyacrylamide gel electrophoresis offers a much sharper size-separation of nucleic acid molecules, down to the separation of fragments that only differ in size by one single base. This is used in methods such as *primer extension* analysis

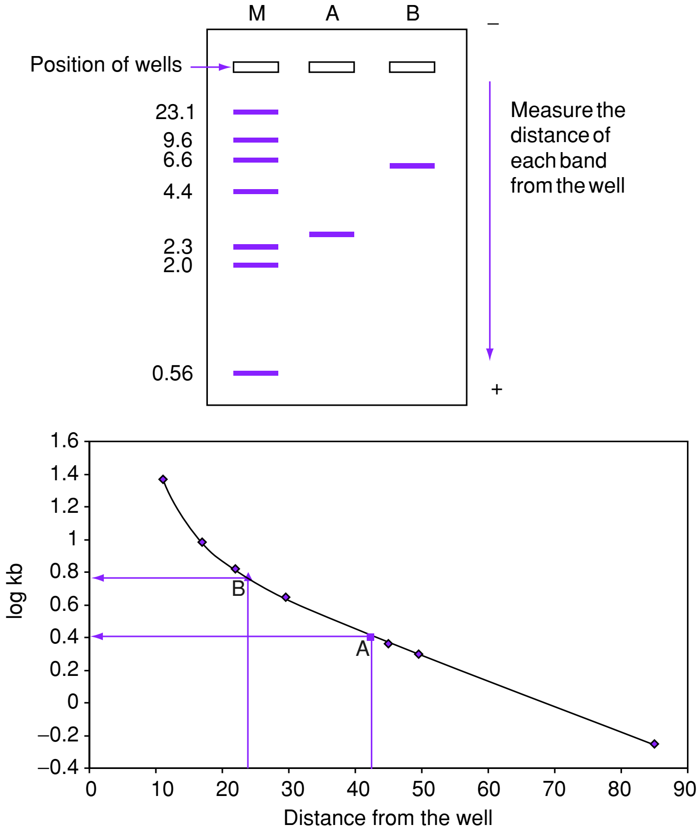


Figure 4.4 Analytical gel electrophoresis; using the standard marker to provide a calibration curve, the size of fragment A is estimated as 2.5 kb and fragment B as 6.0 kb

and *gel retardation* (or *band shift*) assays (see Chapter 13), and has formed the basis for the development of DNA sequencing methods (Chapter 10).

Not all DNA molecules are linear. Native plasmids are supercoiled circular molecules, but if a plasmid is nicked (i.e. one of the strands is broken) the loose ends are free to rotate, and it adopts a relaxed, open circular form. A double strand break will produce a linearized plasmid (see Figure 4.5). Although all three forms are the same size (in terms of the number of base pairs), they will move differently in a gel, with the open circular form moving more slowly than either the linear or the supercoiled DNA. The relative mobility of the latter two forms is more difficult to predict, and will depend on the size and the electrophoretic conditions. It is therefore quite normal for a purified plasmid preparation to show two or three bands in a gel; it does not necessarily mean that there is more than one plasmid. (A further complication is that there may be dimeric or multimeric forms of the plasmid present.)

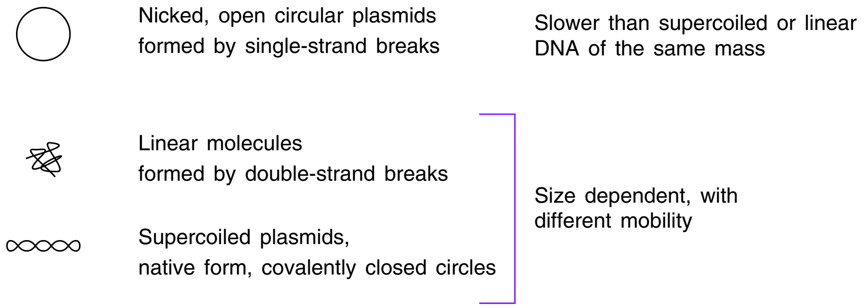


Figure 4.5 Electrophoretic mobility of forms of plasmid DNA

Most RNA molecules you will encounter are single-stranded, and in some cases (such as in DNA sequencing) we will come across single-stranded DNA as well. These need special consideration. As described in Chapter 2, single-stranded nucleic acids will tend to fold up into complex secondary structures, so as to remove the hydrophobic bases from the aqueous environment. The migration of the molecule will be greatly influenced by the way it folds. If we want to get a true picture of its size, we have to make sure that it remains in an unfolded state. To do this, we use *denaturing gels*, in which denaturing agents such as urea or formaldehyde are included.

4.3.2 Preparative gel electrophoresis

Gel electrophoresis is also an important tool in the purification of a specific nucleic acid fragment from a complex mixture. In this case, using a low-melting-point preparation of agarose makes it easier to recover the DNA fragment. After separating the sample, it is visualized with ethidium bromide. With the gel still on the transilluminator, and using suitable equipment to protect yourself from the UV radiation, the band(s) that need to be purified are excised using a razor blade or scalpel. The DNA can then be recovered and purified from the gel fragment, using standard DNA purification procedures.

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5 Cutting and Joining DNA

5.1 Restriction Endonucleases

Restriction endonucleases, the enzymes that are used to cut DNA molecules in specific places, derive their name from the phenomenon of host-controlled restriction and modification. This can occur when a bacteriophage preparation grown using one bacterial strain is used to infect a different strain. For example, phage grown using *E. coli* strain C will infect *E. coli* strain K very inefficiently. *E. coli* K *restricts* the growth of the phage obtained from *E. coli* C. The reason for this restriction of phage growth is that *E. coli* K produces an endonuclease, which is therefore known as a *restriction endonuclease*, that cuts DNA into pieces, so that the incoming phage DNA is rapidly broken down and only occasionally escapes to produce phage progeny (Figure 5.1). The host DNA must be protected against the action of the endonuclease, and this is achieved by a second enzyme that modifies DNA, by methylation, so that it is not attacked by the endonuclease. Although this phenomenon was initially demonstrated with bacteriophage infection, and this is the easiest way of studying it, it can also occur whenever DNA is transferred between one bacterial strain and another, including plasmid transfer – or genetically engineered DNA. If the receiving strain possesses a restriction/modification system that is not present in the originating host, then the yields of transformants or recombinants will be very low.

The *E. coli* K restriction enzyme is not actually very useful for our purposes, since it does not generate specific fragments. It recognizes specific sequences in the DNA but does not cut them. Instead it tracks along the DNA for a variable distance, sometimes as far as 5 kb, before breaking the DNA strand. Enzymes of this type are known as Type I restriction endonucleases. The enzymes that are commonly used, known as Type II restriction endonucleases, typically recognize and cut within (or immediately adjacent to) specific target sequences and therefore generate specific fragments. A small number of Type II restriction enzymes cut the DNA at a defined distance (usually only a few bases) away from the recognition site. The applications of such enzymes are limited and they will not be considered further.

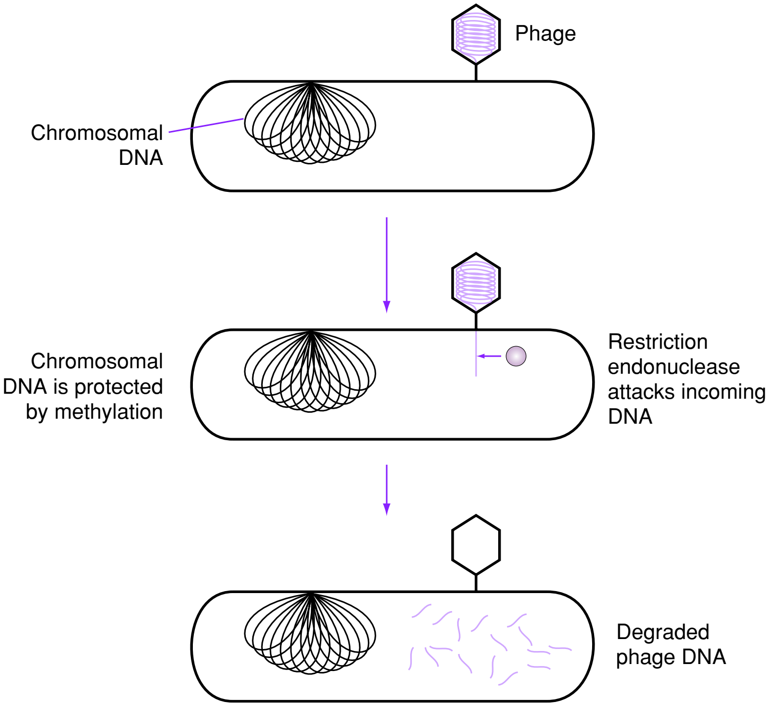


Figure 5.1 Bacteriophage restriction

5.1.1 Specificity

A very large number of Type II restriction endonucleases have been characterized and are used in DNA manipulations. These are identified firstly by the name of the organism from which they are obtained, using the first letter of the genus and the first two letters of the species name, together with a suffix indicating the specific enzyme from that species. Thus *Pst*I indicates a specific enzyme obtained from the bacterium *Providencia stuartii*, and *Hae*I, *Hae*II, and *Hae*III indicate three different enzymes, with different specificities, from *Haemophilus aegyptius*. The convention is to write the first part (derived from the name of the source organism) in italics, just as you would with the species name.

Some examples are shown in Box 5.1, together with their recognition sequences and the position of the site at which they break the DNA. One of the key parameters that affects the way in which we use these enzymes is the length of the recognition site, which will affect the frequency with which they cut DNA, and hence the average size of the fragments generated. For example, take the enzyme *Sau*3A, which has a 4-base recognition site, GATC. If we assume, for the moment, that there is an equal proportion of all four bases, and

Box 5.1 Examples of restriction endonucleases

Enzyme	Recognition site	Number of bases	Ends generated	Original source of enzyme
*Eco*RI	G/AATTC	6	5′ sticky	*Escherichia coli* RY13
*Bam*HI	G/GATCC	6	5′ sticky	*Bacillus amyloliquefaciens* H
*Bgl*II	A/GATCT	6	5′ sticky	*Bacillus globigii*
*Pst*I	CTGCA/G	6	3′ sticky	*Providencia stuartii*
*Xma*I	C/CCGGG	6	5′ sticky	*Xanthomonas malvacearum*
*Sma*I	CCC/GGG	6	blunt	*Serratia marcescens*
*Sau*3A	/GATC	4	5′ sticky	*Staphylococccus aureus* 3A
*Alu*I	AG/CT	4	blunt	*Arthrobacter luteus*
*Not*I	GC/GGCCGC	8	5′ sticky	*Nocardia otitidis-caviarum*
*Pac*I	TTAAT/TAA	8	3′ sticky	*Pseudomonas alcaligenes*

Only one strand of the recognition site is shown, with a slash (/) showing the position of the cleavage site. All the examples shown are palindromic, so the sequence of the second strand, read as the reverse complement, and the position of the cleavage site, will be the same as that shown. Thus the reverse complement of 5′-GAATTC-3′ is also 5′-GAATTC-3, and both strands are cut by *Eco*RI between G and A.

also that the bases are randomly distributed, then at any position on the chromosome there is a one in four chance that it is a G. Then there is a one in four chance that the next base is an A; the chance of having the sequence GA is the product of the two, or 1 in 4^2 (1 in 16). Extending the argument, the chance of the sequence GAT occurring is 1 in 4^3, and that of the 4-base sequence GATC is 1 in 4^4, or 1 in 256. So this enzyme (and any others with a 4-base recognition site) would cut DNA (on these assumptions) on average every 4^4 bases, and so would generate a set of fragments with an *average* size of 256 bases. The actual sizes of the fragments will be distributed quite widely on either side of this average value. A six-base site such as that recognized by *Eco*RI (GAATTC) would occur every 4^6 bases (or 4096 bases). To give some perspective to this calculation, a moderately sized protein might have about 300 amino acids and would therefore be coded for by a DNA sequence of 900 bases in length (ignoring the possible presence of introns). So a four-base cutting enzyme would often give fragments much smaller than a whole gene. On the other hand a bacterial genome of perhaps 4×10^6 bases (4 Mb) would be expected (given the same assumptions) to be cut into about 1000 fragments by an enzyme such as *Eco*RI.

Of course the assumptions in this calculation are not necessarily valid. Firstly, we assumed an equal proportion of all four bases. If we consider both strands of a double-stranded DNA, the number of G and C residues is equal, since for every G on one strand there is a C on the other, and vice versa. Similarly the number of A residues is the same as the number of T residues. (This equality of G and C, and of A and T, does not apply in the same way to each strand taken individually – but they are in fact usually nearly balanced.) So we can simplify the examination of base composition by comparing the number of G+C bases to the number of A+T residues. This is commonly expressed as the percentage of (G+C), or GC%. This parameter can vary widely from one species to another. For *E. coli*, our assumption is valid, as the GC content *is* actually 50% , but other fully-sequenced genomes range from 37% G+C (*Staphylococcus aureus*) to 72% G+C (*Streptomyces coelicolor*). For an organism with a high proportion of G+C bases in its DNA, the predicted number of *Eco*RI sites (GAATTC) will be lower than in the calculation above.

Furthermore, the sequence of bases is not random; for various reasons some combinations of bases occur much less often than would be expected. Some sites are therefore significantly under-represented in the genome as a whole. The occurrence of a specific site may even vary from one region of the genome to another.

A further factor that will affect the ability of restriction endonucleases to cut DNA is methylation of the DNA. We have already seen that each restriction endonuclease, in its original host, will be accompanied by a modifying enzyme that protects the chromosomal DNA against cleavage by the endonuclease, by methylation of the same sequence. In addition, most laboratory strains of *E. coli* contain two site-specific DNA methyltransferases (methylases). The Dam methylase catalyses methylation of the adenine residues in the sequence GATC, while the Dcm methylase modifies the internal cytosine in the sequences CCAGG and CCTGG. Methylation at these positions may make the DNA resistant to attack by restriction enzymes that cut the DNA at sites containing these sequences (see Box 5.1). Methyltransferases also occur in eukaryotes, especially the CpG methylase which modifies the cytosine in some sites containing the dinucleotide CpG. Although these are not known to be involved in restriction/modification systems, they can affect the ability of restriction enzymes to cut certain sites in DNA from higher eukaryotes. A further complication is that many *E. coli* strains have restriction systems (McrA, McrBC, Mrr) that attack DNA that is methylated at CpG sites, and the recovery of cloned DNA from mammals and higher plants can be reduced in such strains. The full implications of methylation of DNA are too complex to be dealt with here, beyond saying that it may be necessary to consider these factors when selecting restriction enzymes and *E. coli* host strains.

Although most of the commonly used enzymes recognize either four base or six base sites, (or in some cases five bases) there are also important roles for enzymes that cut even less frequently, such as *Not*I and *Pac*I (see Box 5.1). These both have eight base recognition sites, which might be expected to occur (on average) every 4^8 bases (about 65 kb), but the sequences are far from random (being composed entirely of G+C or A+T respectively) so a given DNA sequence may be assumed to contain very few sites (or even none at all) for one or the other of these enzymes, depending on the base composition.

5.1.2 Sticky and blunt ends

Another important parameter that affects the use we make of restriction enzymes is the position of the cut site within the recognition sequence. In this context it should be noted that most (but not all) restriction endonuclease recognition sites are termed *palindromic*, although the term is not strictly accurate. A verbal palindrome, for example 'radar' reads the same from left to right as from right to left. A restriction site 'palindrome', such as GAATTC, is a bit different. At first glance it does not seem to be the same when read in the other direction, but you have to remember that the two strands of DNA lie in opposite directions. When we write GAATTC we are looking at the sequence of the 'top' strand, which runs 5′ to 3′ when read from left to right – so we should write the sequence as 5′GAATTC3′. On the 'bottom' (complementary) strand, we have to read from right to left to see the 5′ to 3′ sequence, which would also be 5′GAATTC3′ (see Figure 5.2).

Within this sequence, the restriction enzyme *Eco*RI will cut the DNA between the G and the A on each strand, and will hence produce fragments with four bases unpaired at the 5′ end (Figure 5.3). These four bases (AATT) are the same on all fragments generated with this enzyme, and these ends are complementary to one another. They will thus tend to form base pairs, and so help to stick the fragments together. They are therefore referred to as *cohesive* or *sticky* ends. It should be noted that the base pairing formed with a sequence of four bases is very weak and would not be stable, so we need to use DNA ligase to finally join the fragments together with a covalent bond to form a recombinant

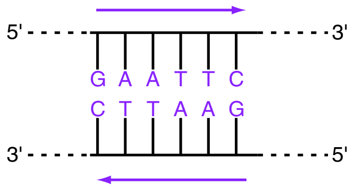


Figure 5.2 Reading a palindromic sequence

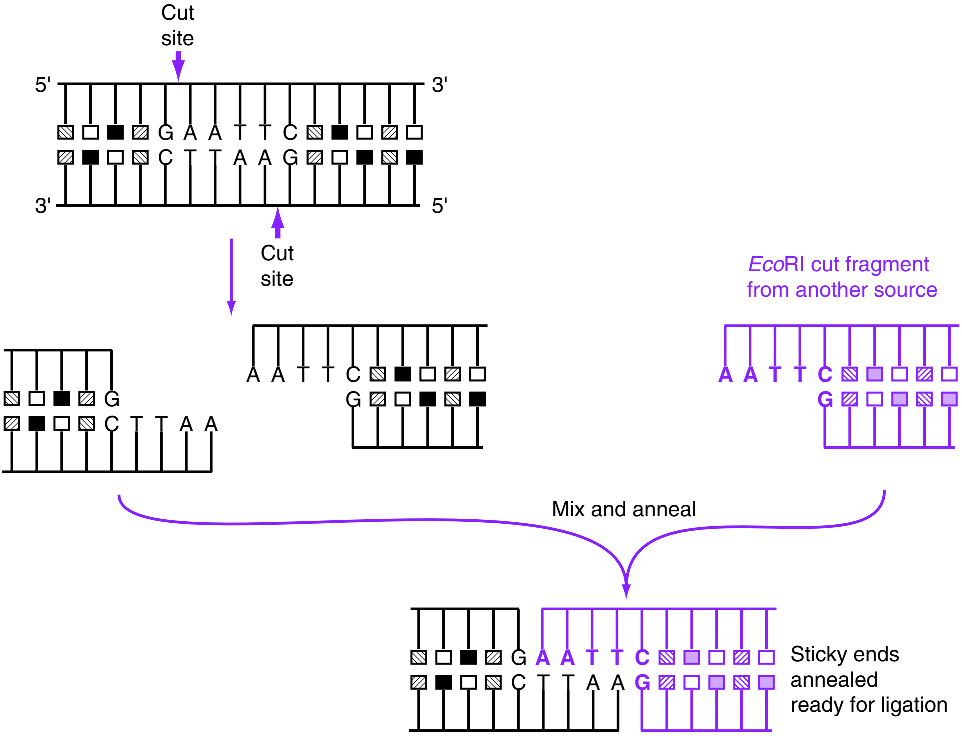


Figure 5.3 Sticky ends generated by *Eco*RI

molecule (see below). Nevertheless, the limited cohesiveness of these ends does make the ligation process much more efficient.

Some enzymes, such as *Pst*I, also form cohesive ends, but by cutting asymmetrically within the right-hand part of the recognition site; they thus generate sticky ends with unpaired single-strand sequences at the 3′ ends of the fragment (Figure 5.4).

Although enzymes that generate cohesive ends are useful for gene cloning because of the efficiency of the ligation process, there is a limitation. You can only join together fragments with compatible ends. So two *Eco*RI fragments can be ligated to each other, or two *Bam*HI fragments can be joined, but you cannot ligate an *Eco*RI fragment directly to a *Bam*HI fragment. However, there are circumstances in which compatible ends can be generated by different restriction enzymes. For example, the restriction enzymes *Bam*HI and *Bgl*II recognize different sequences (see Box 5.1), but they both generate the same sticky ends (with unpaired GATC sequences) and these can be joined.

More flexibility can be achieved by using an enzyme such as *Sma*I, which recognizes the six base sequence CCCGGG and cuts symmetrically at the centre position (see Figure 5.4); it generates blunt ends, which although much

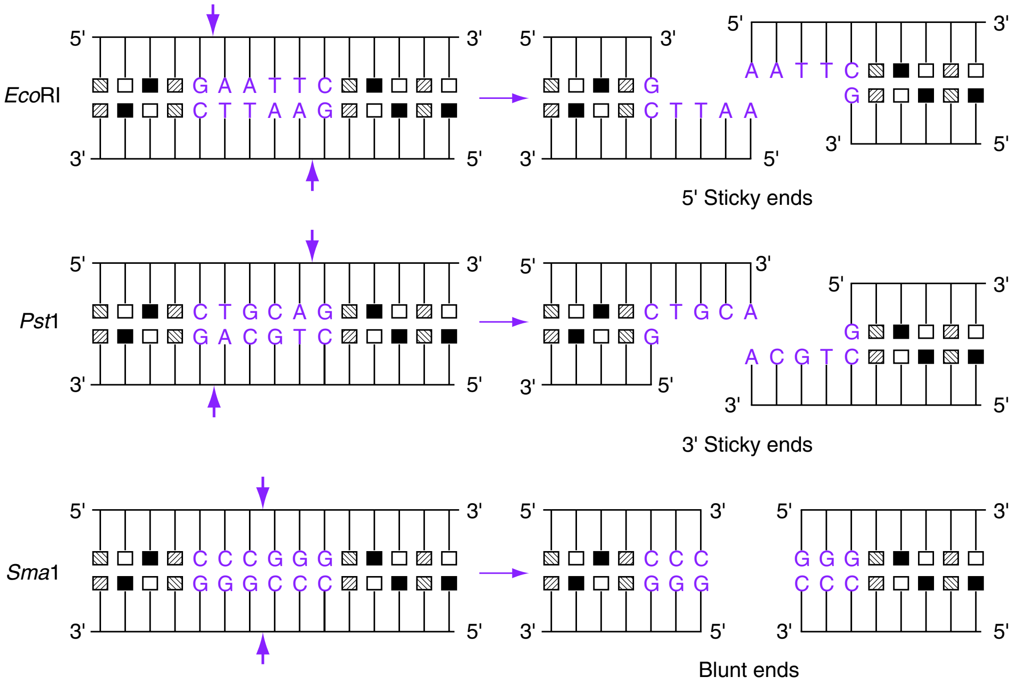


Figure 5.4 Restriction fragment ends

less efficiently ligated have the advantage that they can be joined to any other blunt-ended fragment.

5.1.3 Isoschizomers

An additional element of flexibility arises from the existence of several enzymes that recognize the same sequence of bases. These are known as *isoschizomers* (Greek *iso*, equal; *skhizo*, to split). In many cases, isoschizomers not only have the same recognition site, but cut in the same place within that recognition sequence. Although this can be of some use, for technical reasons we don't need to go into, more important are examples of isoschizomers which recognize the same sequence but cut in a different position within that sequence. For example, *Acc*65I and *Kpn*I both recognize the sequence GGTACC (Figure 5.5), but cut it at a different place. They therefore generate different sticky ends. This gives you the option of obtaining virtually the same fragment of DNA but with different sticky ends that can be ligated to other fragments. Another example is the pair of isoschizomers *Xma*I and *Sma*I. *Xma*I cuts asymmetrically and produces sticky ends that can be ligated to other *Xma*I

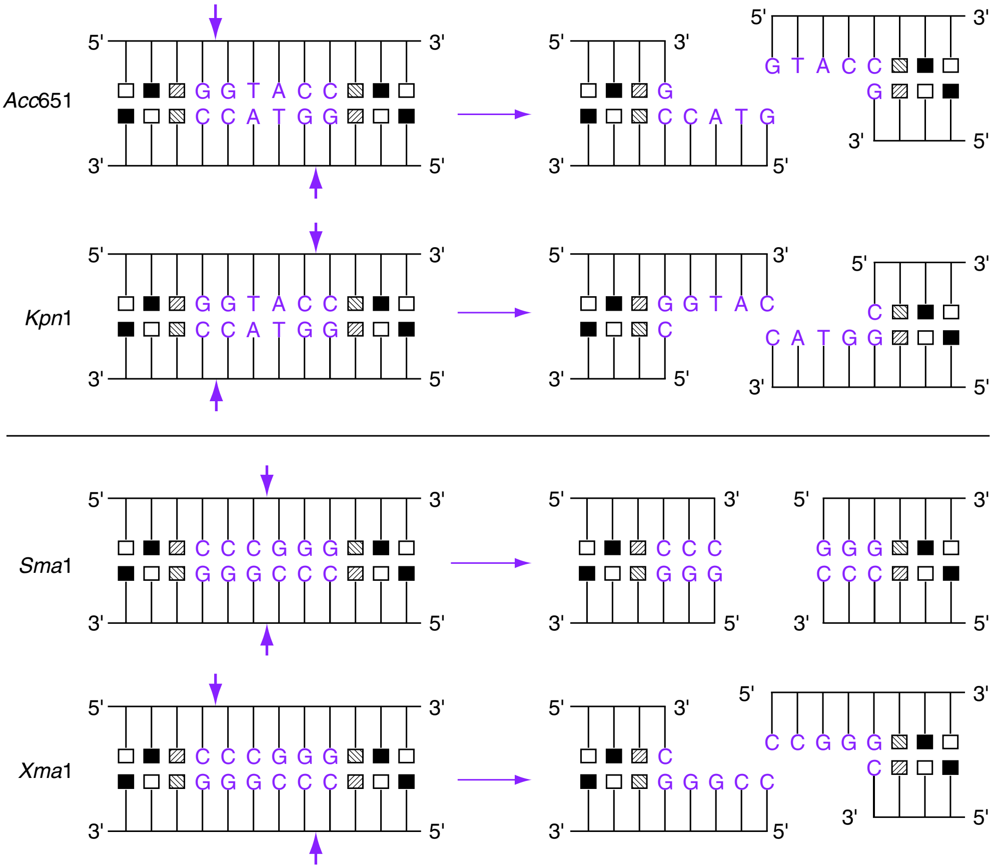


Figure 5.5 Isoschizomers

fragments, while *Sma*I, as mentioned above, will generate blunt-ended fragments at the same site, allowing you to ligate the fragment to other blunt-ended DNA sequences. (You could object that these enzymes are not literally ‘same-cutters’, which is true if you look at the actual site where the break is made. However, the fragments generated by cutting a DNA molecule with *Xma*I, for example, will be the same size as those generated by *Sma*I, so you will see identical patterns on an agarose gel, even though the ends of the fragments are different.)

5.1.4 *Processing restriction fragments*

After a restriction digestion, if a digest produces more than one fragment, the fragments will normally need to be separated on a low-melting-point agarose gel, and the desired fragment excised and purified (see Chapter 4). Alternatively, if the digest produces only one fragment, or if a complex mixture is being digested for the production of a library (see Chapter 7), it is normally sufficient to inactivate the enzyme before continuing with the next stage. Normally, this is accomplished by heat-inactivation; the efficiency of this will have been specified by the supplier in their product information sheet. If the enzyme is heat-stable, phenol extraction is necessary to ensure inactivation.

Later in this chapter we will look at ways in which the ends of DNA fragments generated by restriction endonuclease cleavage can be modified to make the cloning process easier and more versatile. This includes converting sticky ends to blunt ends, by filling in the missing bases complementary to the unpaired ends or by digesting away the single-stranded regions. We will also consider the use of linkers and adaptors – short synthetic DNA fragments that add new restriction sites to the end of a fragment, thus enabling fragments generated in different ways to be joined together.

We also need to be aware that DNA fragments for ligation can be generated in ways other than by cleavage with restriction endonucleases, ranging from the non-specific generation of truly random fragments (by mechanical shearing or digestion with non-specific nucleases) through reverse transcription of mRNA into DNA (see Chapter 7) to the highly specific amplification of a chosen piece of DNA by PCR (Chapter 9) and the use of synthetic DNA (Chapter 15).

5.2 Ligation

As outlined in Chapter 3, the next stage in cloning a gene is to join the DNA fragment to a vector molecule, such as a plasmid or bacteriophage, that can be replicated by the host cell after transformation. The joining, or ligation, of DNA fragments is carried out by an enzyme known as *DNA ligase*.

The natural role of DNA ligase is to repair single-strand breaks (nicks) in the sugar–phosphate backbone of a double-stranded DNA molecule, such as may occur through damage to DNA (or following the repair of such damage), as well as the joining of the short fragments produced as a consequence of replication of the ‘lagging strand’ during DNA replication. The action of the ligase requires that the nick should expose a $3'$-OH group and a $5'$-phosphate (Figure 5.6). Digestion with restriction endonucleases cuts the DNA in this way, i.e. it leaves the phosphate on the $5'$ position of the deoxyribose. The unstable pairing of two restriction fragments with compatible sticky ends can therefore be considered as a double-stranded DNA molecule with a nick in each strand, very close together, and is therefore a substrate for DNA ligase action.

Some DNA ligases, such as T4 DNA ligase (encoded by the bacteriophage T4), are also capable of ligating blunt-ended fragments, albeit much less

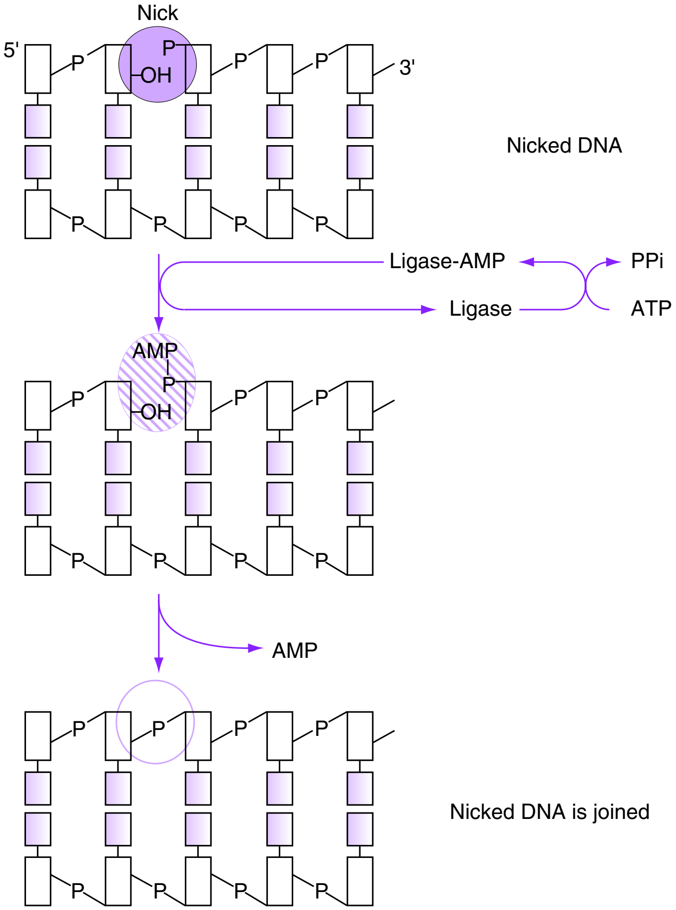


Figure 5.6 Action of T4 DNA ligase

efficiently, while others (notably the *E. coli* DNA ligase) are not; they require pairing of overlapping ends. To keep things simple, we will only consider the action of T4 DNA ligase, which is by far the most extensively used one. We will refer to this enzyme as T4 ligase (or just 'ligase') although there is also a (much less commonly used) T4 RNA ligase.

The T4 ligase requires ATP as a co-substrate. In the first step, the ligase reacts with ATP to form a covalent enzyme-AMP complex, which in turn reacts with the 5′-phosphate on one side of the nick, transferring the AMP to the phosphate group. The final stage is the attack by the 3′-OH group, forming a new covalent phosphodiester bond (thus restoring the integrity of the sugar–phosphate backbone) and releasing AMP. The absolute requirement for the 5′-phosphate is extremely important; by removing the 5′-phosphate we can prevent the occurrence of unwanted ligation (see below).

5.2.1 Optimizing ligation conditions

Ligation can be one of the most unpredictable (and often frustrating) steps in the cloning process. Factors that may compromise the success of ligation include the presence of inhibitory material contaminating our DNA preparations and degradation of the enzyme or the DNA (including loss of the 5′-phosphate). In addition, the conditions need to be adjusted correctly to achieve the optimum effect. One of the most controversial of these conditions is temperature. Originally, it was considered that since ligation was in effect a repair of single strand nicks in a double-strand molecule, we should optimize the pairing of the fragments. Therefore, much of the early work used ligation at 10°C (or even 4°C), which needs long incubation since the enzyme activity is low at that temperature. Fashions change, and many protocols now recommend 16°C, but this is inconvenient unless you have a cooled water bath to hand; room temperature is often used as a compromise. Technological advances have also meant that buffers are commercially available that allow much faster and more efficient ligations.

Since the reaction we want normally involves two different molecules of DNA (*inter*molecular ligation), we would expect it to be extremely sensitive to DNA concentration. It is therefore important to use high concentrations of DNA; but which DNA component? We have two components: the vector and the insert (ignoring for the moment the fact that the insert may itself be a heterogeneous mixture of fragments). There are therefore a variety of possible reactions that can occur (Figure 5.7), and by adjusting the relative amounts of the components, as well as the overall concentration of DNA, we can influence the likelihood of these different reactions.

At low concentrations of DNA, we are more likely to get the two ends of the same molecule joining (an *intra*molecular reaction), since the rate of a reaction involving one component will be linearly related to its concentration, whereas a reaction involving two different components will be proportional to the product of the two concentrations. (Or for a reaction involving two molecules of the same substrate, the rate will be proportional to the square of the concentration.)

If we increase the concentration of the vector but not the insert, we will get a greater increase in the ligation of two vector molecules together (which we don't want) than in the production of the recombinant vector–insert product. Conversely, increasing insert concentration will give increased levels of insert–insert dimers (which we also do not want, although they are less of a problem as they will not give rise to transformants).

So we need not only to keep the overall DNA concentration high, but also to create an optimum vector–insert ratio. It is not easy to predict reliably what that ratio should be but typically it would range from 3:1 to 1:3. Note that these are molar ratios and have to take account of the relative size of the vector and

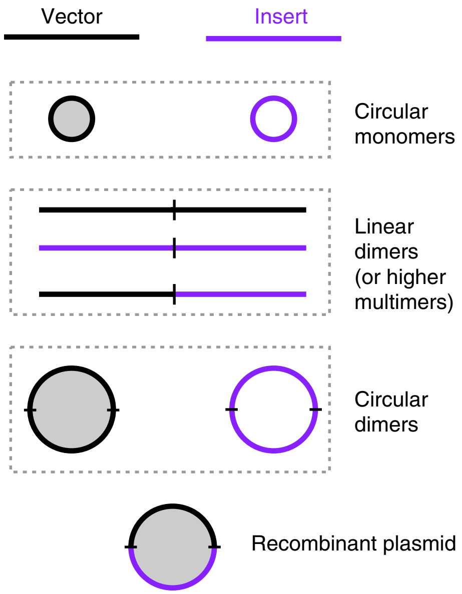


Figure 5.7 Ligation – some of the potential products; only the shaded circles are expected to be replicated after transformation

the insert. For example, if the vector is 5 kb and the insert is 500 bases, then a 1:1 molar ratio would involve 10 times as much vector, by weight, as insert (e.g. 500 ng of vector and 50 ng of insert). Conversely, for the same 5 kb vector but an insert size of 50 kb, if you used 500 ng of vector you would need 5 μg of insert to achieve a 1:1 molar ratio. In general, to convert the amount of DNA by weight into a value that can be used to calculate the molar ratio, divide the amount used (by weight) by the size of the DNA. Thus, if W_V and W_I are the weights used of vector and insert DNA respectively, and S_V, S_I are the sizes of vector and insert (e.g. in kilobases), then the vector:insert ratio is W_V/S_V: W_I/S_I.

A further complication arises if we are working with a heterogeneous collection of potential insert fragments, as would be the case if we were making a gene library (see Chapter 7). Loading more insert DNA into the ligation mixture will increase the possibility of obtaining multiple inserts. In other words, we may produce recombinant plasmids that carry two or more completely different pieces of DNA. This is not a good idea. It can lead to seriously misleading results when we come to characterize the clones in the library and try to relate them to the structure of the genome of our starting organism.

So adjusting the relative amount of the vector and insert will not only influence the success of ligation but will also have an effect on the nature of the products formed. Intramolecular ligation of vector molecules, or formation of vector dimers, will result in transformants that do not carry our insert, while putting in too much insert will result in insert dimers (which will not give transformants) or recombinant plasmids carrying multiple inserts. Fortunately we do not have to rely entirely on adjusting the levels of DNA in order to obtain the result that we want. In the next section, we will look at the use of alkaline phosphatase to remove 5′-phosphate groups (thus preventing some types of ligation), and in Chapter 6 we will consider designs of vector that allow us to distinguish between recombinant and non-recombinant transformants.

5.3 Alkaline Phosphatase

As described above, the ligation process depends absolutely on the presence of a 5′-phosphate at the nick site. If this phosphate group is removed, that site cannot be ligated. Removal of 5′-phosphate groups is achieved with an enzyme known as alkaline phosphatase (because of its optimum pH) – the most commonly used enzyme is calf intestinal phosphatase (CIP). Treatment of the vector molecule with CIP before ligation will remove the 5′-phosphates, and so make it impossible for self-ligation of the vector (either intramolecular or intermolecular) to occur. Ligation of vector to insert can occur, however, as the insert still has its 5′-phosphates. It is of course important to remove the CIP before ligation, and this is best done by phenol extraction and subsequent ethanol precipitation as heat inactivation is not sufficiently reliable. If you are using the same vector repeatedly, it is possible to carry this out on a larger scale and store the unused treated vector for further experiments. This is desirable as CIP treatment is not entirely reliable, and a bulk preparation of dephosphorylated vector that you have already used successfully is a considerable asset in subsequent experiments. If you are using a standard vector, you can buy ready-made and tested dephosphorylated vector.

However, further inspection of the situation (Figure 5.8) will reveal that it is not quite that simple. At each junction between two DNA fragments there are *two* nicks that need repairing. At one of these, the 5′-phosphate is supplied by the insert, and that can be ligated normally. However, at the other nick, there should be a 5′-phosphate on the *vector*, and that has been removed. So the second nick cannot be mended. How can we deal with this situation?

Actually it is not a real problem. The recombinant plasmid will have two unrepaired nicks in its (circular) DNA (one at each end of the inserted fragment) – but it will hold together very stably by virtue of the base pairing all along the inserted DNA fragment. So we have hundreds, or thousands, of base pairs holding the two strands together. It will be maintained stably at 37°C, as a double-stranded molecule.

When this nicked molecule is introduced into a bacterial cell by transformation, enzymes within the cell will rapidly repair the remaining nicks, by adding

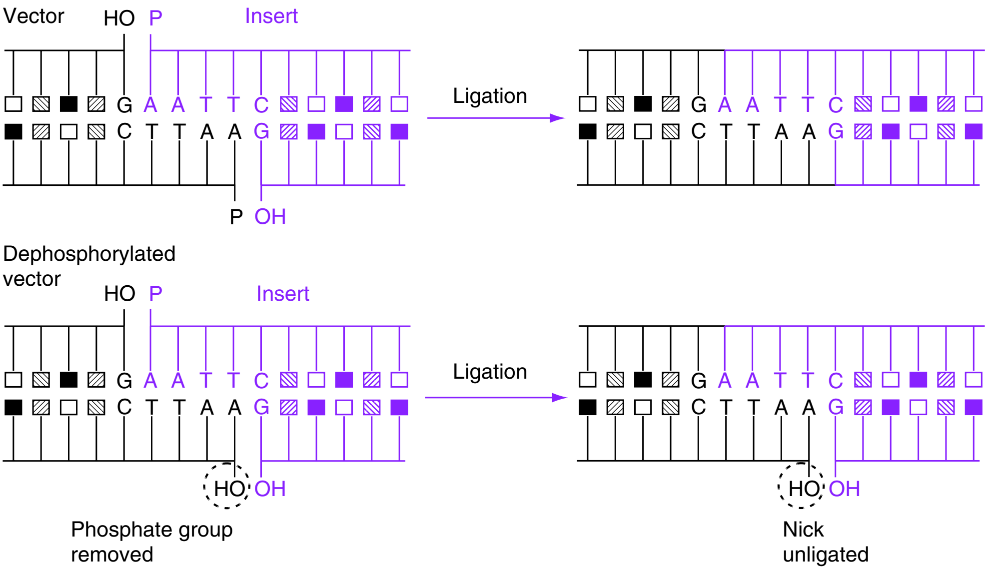


Figure 5.8 Ligation – effect of dephosphorylation of vector

the missing phosphates and ligating the broken ends. The plasmid will be replicated without any difficulty.

Potentially some of the other problems still remain. We could still get insert dimers, or multiple inserts. However, since we no longer have to worry about self-ligation of the vector, we can increase vector concentration relative to the insert and thus drive the reaction in the direction we want.

Even so, if we are making a gene library, when the possibility of multiple inserts is at its most serious as a problem, we may want to turn this strategy on its head. Phosphatase treatment of the insert rather than the vector will prevent multiple inserts, and we can prevent the occurrence of non-recombinant transformants (carrying self-ligated vector rather than vector–insert recombinants) by using special vectors that are unable to produce clones unless they carry an inserted DNA fragment (see Chapter 6).

5.4 Double Digests

There are good reasons to try to avoid using alkaline phosphatase if possible. The treatment easily overdigests, not only removing the phosphate group but modifying and disabling the sticky end. In addition, the required phenol extraction and ethanol precipitation are time-consuming (and can also result in loss of, or damage to, the DNA). If you are simply trying to make a specific recombinant plasmid, and have efficient ways of differentiating self-ligated vectors, a perfectly optimal ligation is not essential. You will only need one clone eventually, and as long as you can identify this without trawling through hundreds of others you will be content.

However, there are circumstances when it is necessary to be able to ensure that a high proportion of the colonies obtained are genuine recombinants. For example, if you are trying to create a representative DNA library, self-ligated vectors and multiple inserts are a real problem. Even simple binary ligations can sometimes be problematic, in particular if you happen to do a ligation where there is no easy way of rapidly differentiating recombinant plasmids from self-ligated vectors (see Chapter 6).

If you want to avoid the use of alkaline phosphatase, one alternative method of avoiding self-ligation of vector and insert is to cut both components with two different restriction enzymes. Indeed, you may want to do this for other reasons anyway. Most modern vectors have multiple cloning sites (see Chapter 6) so you can cut the vector with, for example, *Eco*RI and *Bam*HI. Provided that both enzymes have cut efficiently (and that you have removed the small fragment between the two sites), then vector re-ligation will be impossible. If your insert fragment has been digested with the same two enzymes, then virtually all the colonies obtained will be recombinant, i.e. they will contain the insert fragment. There are still some other possible reactions, such as ligation of two vector molecules together, but these events will be comparatively rare.

There are two sorts of problem with double digests. The first is to find conditions that allow both enzymes to work. Suppliers' catalogues often contain tables of recommended buffers for a variety of combinations of restriction enzymes. Enzymes that require widely different temperatures are more difficult, and you may have to use the enzymes sequentially. The second problem occurs if the two sites are close together. Some restriction enzymes do not cut very efficiently at a recognition site that is near to the end of a DNA molecule. Again, some suppliers' catalogues provide tables that allow you to determine if this is a problem. However, even if you do everything by the book, you may still find that you get incomplete digestion, and it is necessary to carry out control ligations to test the efficiency of the double digest.

5.5 Modification of Restriction Fragment Ends

Restriction fragments with sticky ends are useful as they can be readily ligated. However, there is a limitation on their usefulness, as they can only be ligated to another fragment with compatible ends. So an *Eco*RI fragment can be ligated to another *Eco*RI fragment, but not to one generated by *Bam*HI. Not only do you lose the advantage of the cohesive ends, but the unpaired DNA gets in the way, making you better off with blunt-ended fragments.

This is a potential nuisance, as the vector you are using will only have a limited number of possible sites into which you can insert DNA (and you may specifically want to put your insert in a particular position), and it may not be possible to generate a suitable insert with the same enzyme. In this section we will look at ways in which you can change the ends of a DNA fragment to enable it to be ligated with other sites.

5.5.1 Trimming and filling

One strategy that enables a more flexible use of restriction fragments is to convert the sticky ends into blunt ends, either by filling in the complementary strand, or by trimming back the unpaired sequence (see Figure 5.9). If the restriction fragment has a 5′ overhang (such as those generated by *Eco*RI), the 3′ OH group provides a primer site that can be extended by DNA polymerases, thus filling in the recessed end and converting it to double-stranded. However, some DNA polymerases (such as *E. coli* DNA polymerase I) also have 5′–3′ exonuclease activity, which would remove the 5′ overhang (and maybe more); these enzymes are not suitable for filling in the ends. Therefore, for this purpose you would use an enzyme lacking this 5′–3′ exonuclease activity such as the Klenow fragment of *E. coli* DNA polymerase I (a proteolytic product of *E. coli* DNA polymerase I in which the region responsible for the 5′– 3′ exonuclease activity has been removed).

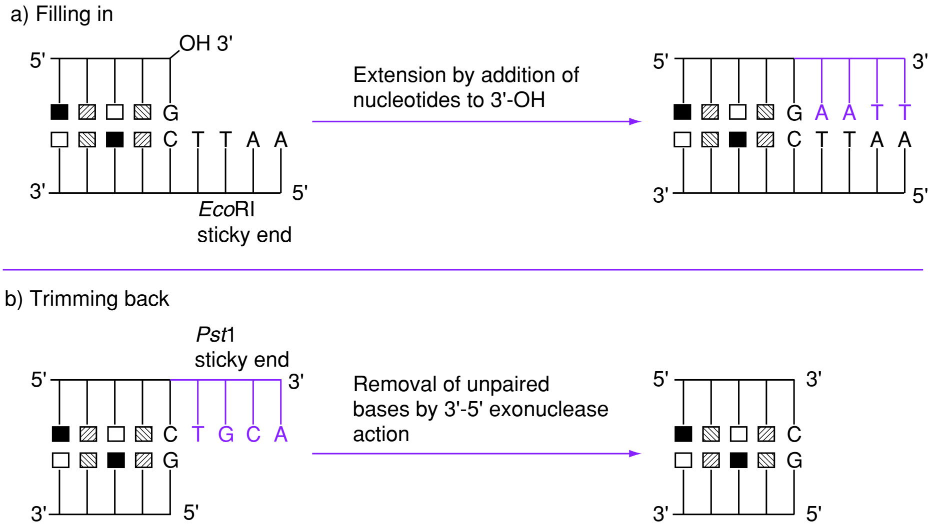


Figure 5.9 Converting sticky ends to blunt ends

Restriction fragments with 3′ sticky ends, such as those produced by *Pst*I, cannot be filled in, but the unpaired sequences can be trimmed back, using the 3′– 5′ exonuclease activity of an enzyme such as T4 DNA polymerase.

5.5.2 Linkers and adapters

Both of the above procedures, filling in or trimming back, result in conversion of sticky ends to blunt ends, which can then be ligated to any other blunt-ended fragment, however produced. This is a start, but it is still some way short of the complete versatility that is our goal, especially as blunt-end ligation is so inefficient, and requires a much higher concentration of DNA (which may be difficult to come by). The process can be made much more efficient, and much more versatile, by the use of *linkers*. These are short synthetic pieces of DNA that contain a restriction site. For example, the sequence CCGGATCCGG contains the *Bam*HI site (GGATCC). Furthermore it is self-complementary, so you only need to synthesize (or buy) one strand; two molecules of it will anneal to produce a double-stranded DNA fragment 10 base pairs long (see Figure 5.10). If this is joined to a blunt-ended potential insert fragment by blunt-end ligation, your fragment now will have a *Bam*HI site near each end. Cutting this with *Bam*HI will generate a fragment with *Bam*HI sticky ends, that can be ligated with a *Bam*HI-cut vector. If your insert already has an internal *Bam*HI site, this is not possible. The alternative is to use *adapters* rather than linkers (see below).

You may object that this still requires blunt-end ligation, to join the linker to the potential insert. However, the efficiency of blunt-end ligation can be markedly improved by using high concentrations of at least one of the components. In this case, you can easily produce and use large amounts of the linker. Furthermore, since the linker is very small (e.g. 10 bases), and it is the *molar* concentration that is important, even modest amounts of the linker by mass will represent an enormous excess of linker in molar terms. For example, if you use 100 ng of a 1 kb insert, then 10 ng of linker will represent a 10:1 linker:insert ratio. The high molar concentration of the linker will drive the reaction very effectively. Of course, this efficient ligation is likely to add multiple copies of the linker to the ends of your insert, but this is not a problem – the subsequent restriction digestion will remove them.

Further versatility can be obtained by the use of *adapters*. These are pairs of short oligonucleotides that are designed to anneal together in such a way as to create a short double-stranded DNA fragment with different sticky ends (or with one sticky and one blunt end). For example the sequences 5′-GATCCCCGGG and 5′-AATTCCCGGG will anneal as shown in Figure 5.11 to produce a fragment with a *Bam*HI sticky end at one end and an *Eco*RI sticky end at the other, without needing to be cut by a restriction enzyme. Ligation of this adaptor to a restriction fragment generated by *Bam*HI

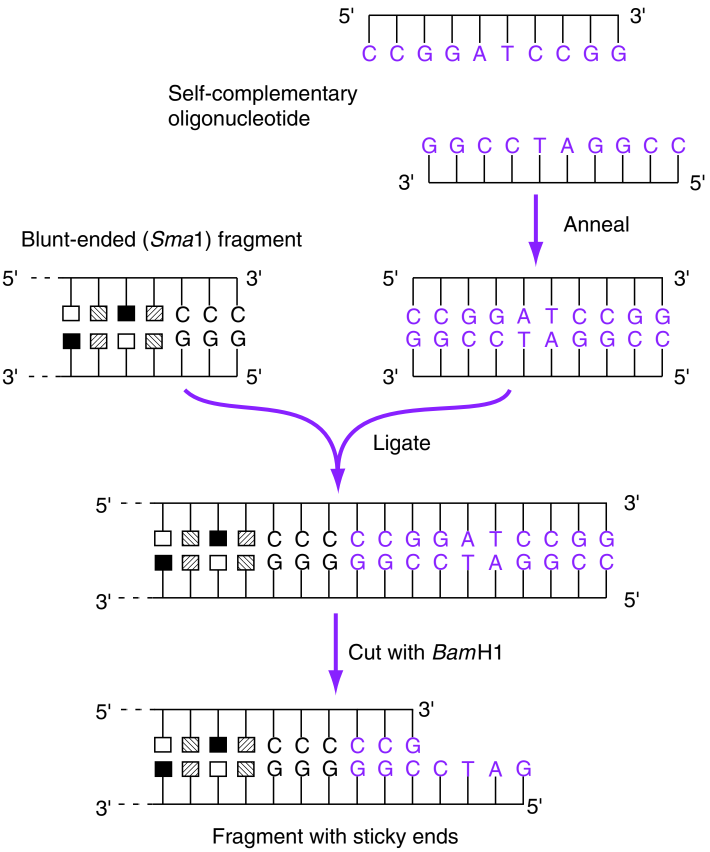


Figure 5.10 Linkers

digestion will produce a DNA fragment with *Eco*RI ends that can now be ligated with an *Eco*RI cut vector.

Alternatively, an adaptor with one sticky end and one blunt end can be used to convert blunt-ended DNA fragments, such as those generated by cDNA synthesis (see Chapter 7), into fragments with a sticky end, which increases the cloning efficiency substantially. As with linkers, you can use high molar concentrations of adaptors to drive ligation very efficiently, and the use of adapters with non-phosphorylated sticky ends ensures that you will not get multiple additions of the adaptor to the end of your DNA fragment.

5.5.3 Homopolymer tailing

An alternative way of adding sticky ends to DNA molecules is to use the enzyme *terminal deoxynucleotide transferase* (or *terminal transferase* for

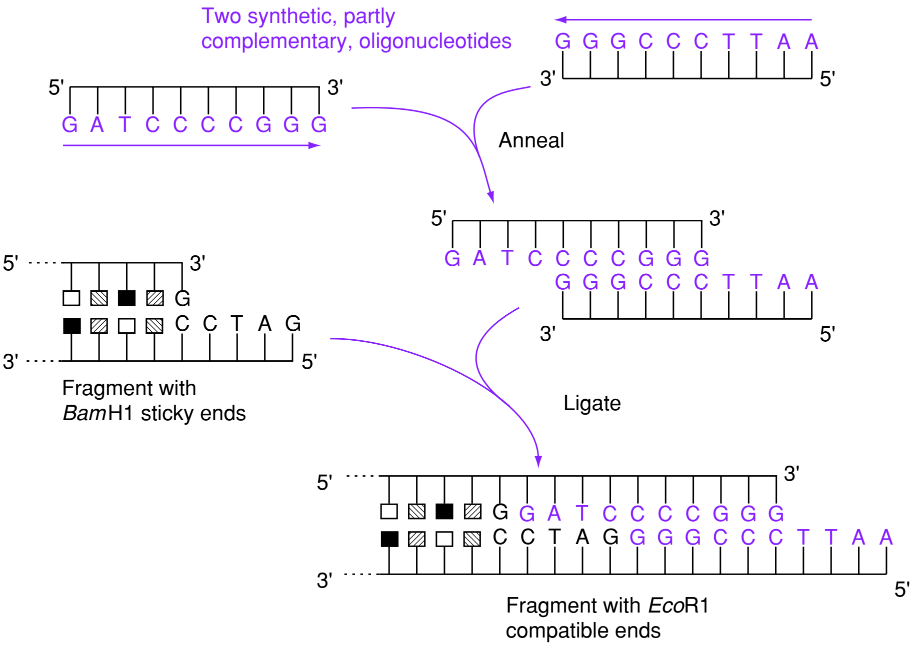


Figure 5.11 Adaptors

short). When supplied with a single deoxynucleotide triphosphate (say dGTP), this enzyme will repetitively add nucleotides to the 3′OH end of a DNA molecule (Figure 5.12). This enzyme is different from DNA polymerases in that it does not require a template strand, so this reaction will produce a molecule with a single-stranded run of G residues at each 3′ end, hence the term homopolymer tailing. If the vector is treated in this way, and the insert fragment(s) are treated with terminal transferase and dCTP (generating a tail of C residues), the two tails are complementary and will tend to anneal to one another (see Figure 5.13).

It is not possible to ensure that the tails are all exactly the same length, so when the molecules anneal together there will be gaps in one of the sequences. This does not matter. If the tails are longer than about 20 nucleotides, then the pairing of the tails will be strong enough to be stable at room temperature, and

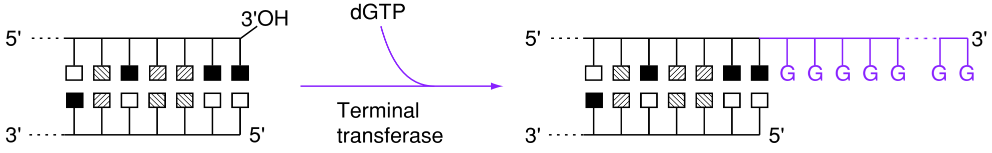


Figure 5.12 Tailing with terminal transferase

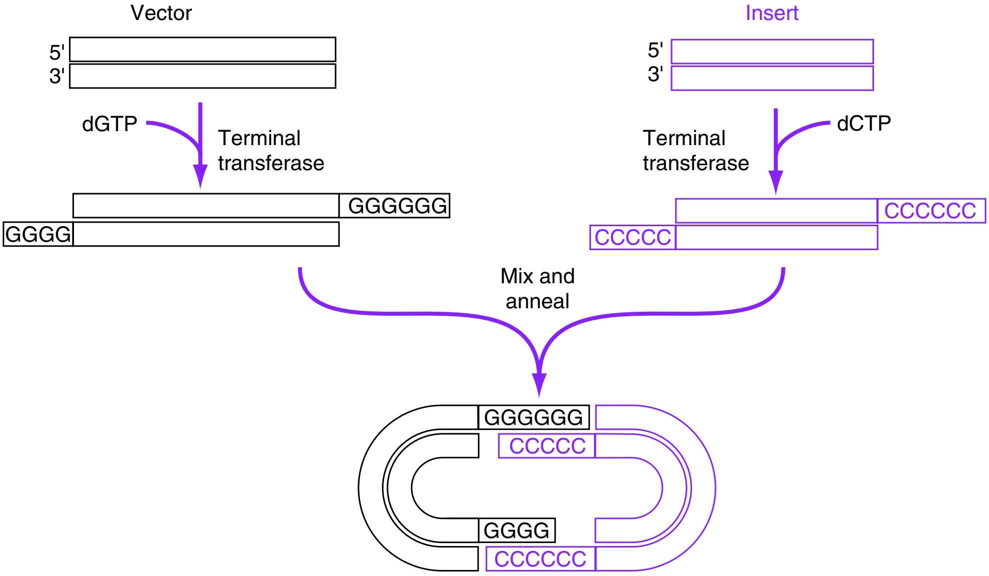


Figure 5.13 Cloning using homopolymer tailing

the product can be used for transformation without repairing the gaps, or even without sealing the nicks with ligase. Both gaps and nicks will be repaired within the host cell after transformation.

One advantage of this strategy is that it is not possible for the vector to reform without an insert: the ends of the vector are not complementary to one another. The downside is that you have constructed a recombinant plasmid that contains a variable number of GC base pairs at either end of the insert, so it lacks the precision associated with formation of a recombinant using the other methods described in this chapter. This is a disadvantage if you want to recover the insert from the recombinant vector, for example to reclone it in another vector. However, you can use restriction sites in the flanking region of the vector to release the insert. Or you can sequence the ends of the insert and use that information to design PCR primers (see Chapter 9) to allow you to specifically amplify the insert.

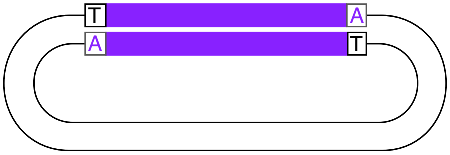
5.6 Other ways of joining DNA molecules

5.6.1 *TA cloning of PCR products*

Some of the polymerases used in PCR amplification (see Chapter 9) also have a (very limited) terminal transferase action: they add a single adenosine residue to

PCR product with 3' A overhang

Prepared cloning vector



Recombinant plasmid

Figure 5.14 TA cloning

the 3′ ends of the synthesized strands. This can cause problems with cloning, as the ends are not truly blunt, so blunt-end cloning is likely to be unsuccessful. However, it provides an opportunity as well as a problem. There are commercially available ‘TA vectors’, which are supplied in linearized form with a single 3′-T ‘overhang’ which is compatible with the unpaired 3′-A on the PCR product (see Figure 5.14). This concept can be combined with topoisomerase cloning (see below) to form a highly efficient way of cloning such PCR products.

Alternative strategies include designing the PCR primers to incorporate a restriction site; the product can then be digested with the appropriate enzyme before ligation in the conventional manner. The strategies for cloning PCR products are considered further in Chapter 9.

5.6.2 DNA topoisomerase

Although DNA ligase is the most commonly used enzyme for joining two DNA molecules, it is not the only enzyme that can carry out this action. DNA topoisomerase I is another example. Topoisomerases in general are responsible for controlling the degree of supercoiling of DNA (see Chapter 2); type I topoisomerases achieve this by cutting one DNA strand, which is then free to rotate (thus reducing the supercoiling), and subsequently rejoining the cut ends of the DNA strands. The enzyme remains covalently attached to the phosphate group at the end of the broken strand after cutting it, thus retaining the bond energy and being in place for the subsequent re-joining (see Figure 5.15). In one commercially available system, a linearised vector is supplied with *Vaccinia* virus topoisomerase I covalently attached to phosphate groups at the 3′ ends. When the prepared vector is mixed with the DNA fragment to be cloned, the enzyme transfers the phosphate linkages to the 5′ ends of the fragment, thus joining the insert to the vector. Since the topoisomerase is already attached to the vector, the reaction only requires two molecules (the vector and the insert) to come into contact, and is therefore quicker than conventional ligation which needs simultaneous contact between *three* molecules (vector, insert, and ligase).

Note that, unlike the action of DNA ligase, the insert in topoisomerase ligation should not have a 5′-phosphate group. This system is mainly applied to the cloning of PCR products (Chapter 9), where the 5′ end (derived from the primers used in amplification) is not normally phosphorylated, unless you specifically order a 5′-phosphorylated primer.

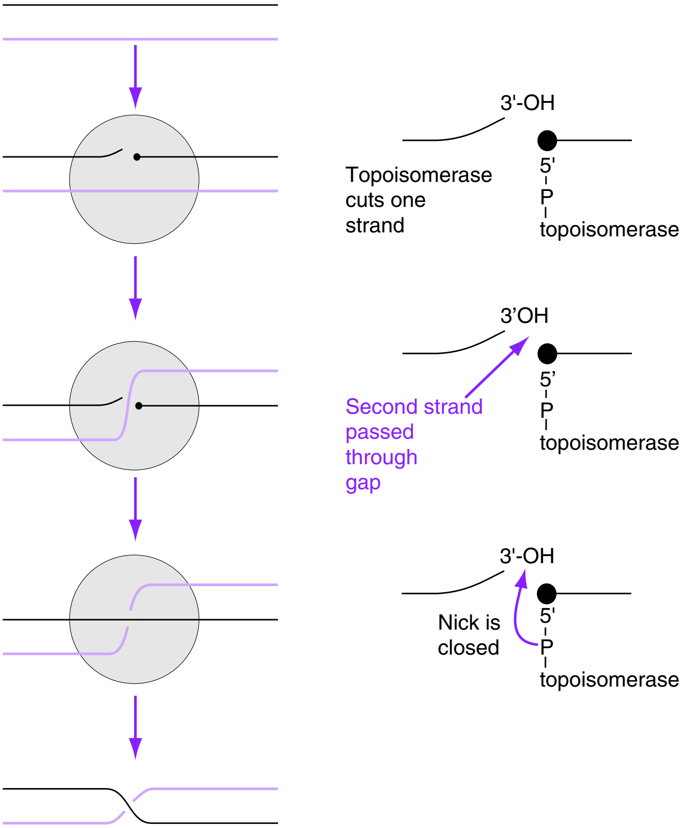


Figure 5.15 Action of topoisomerase

As mentioned above, PCR products often have a single unpaired A residue added at the 3′ end. Some of the topoisomerase vectors are designed to be compatible with such a product, as the topoisomerase is covalently attached to an unpaired 3′-T residue; the 3′-A on the PCR product will then pair with this T, enhancing the efficiency of the reaction.

Other enzyme systems are available for concerted breakage and rejoining of DNA strands allowing, for example, a one-step transfer of a cloned gene from one plasmid to another. One example is the site-specific recombinase of bacteriophage lambda (the natural role of which is to insert lambda DNA into the bacterial chromosome, see Chapter 6). A second example is the Cre-*lox* system from the P1 bacteriophage. Cre is a highly site-specific recombinase, which will act only on the specific sequence known as the *lox* site. In both cases the enzyme will carry out recombination between two plasmids carrying the appropriate recognition sequences, leading to transfer of a cloned gene between two plasmids, or fusion of the plasmids.

5.7 Summary

In this chapter we have seen how restriction endonucleases can be used to cut DNA, how ligase can be used to join fragments together, and some of the ways in which these processes can be manipulated to improve the efficiency and versatility of the process. In the next chapter, we will look at the nature of the vectors into which the required fragment can be inserted, and how the resulting DNA can be introduced into a bacterial cell.

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Jeremy W Dale and Malcom von Schantz

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6 Vectors

In the previous chapter, we looked at the ways in which a potential insert can be ligated with a vector molecule, to enable that insert DNA to be replicated after insertion into a bacterial cell. We now need to consider further the nature of the vectors that can be used. Once again, we will initially be concentrating on the vectors that are used for cloning in bacteria (principally *E. coli*), and subsequently looking at the vectors needed for cloning in eukaryotes.

6.1 Plasmid Vectors

6.1.1 Properties of plasmid vectors

Plasmids are by far the most widely used, versatile, and easily manipulated vectors. They are the work-horses of the molecular biology laboratory. They are naturally occurring extrachromosomal DNA molecules, usually circular, double-stranded and supercoiled. Plasmids occur widely in nature, and are found in most bacterial species. They vary considerably in size, from a few thousand base pairs up to several hundred kilobases, although plasmids used as gene cloning vectors are usually small (typically 2–5 kb). Most of the commonly used ones are based on (or are closely related to) a naturally occurring *E. coli* plasmid called ColE1. Later in this chapter we will look at other types of vectors such as bacteriophages.

The most notorious property of plasmids lies in their ability to disseminate antibiotic resistance genes. They are responsible to a large extent for the spread of antibiotic resistance – although it should be noted that the plasmids used for gene cloning are nearly always unable to spread from one bacterium to another, and there are restrictions on experimental protocols to ensure that these experiments do not add new antibiotic resistance genes to clinically important pathogenic bacteria. Antibiotic resistance is not the limit of the ability of plasmids, nor the reason for their existence. Interest tends to focus on antibiotic resistance because of its importance in medical microbiology, and because of the ease with which resistance genes can be isolated and studied. However, many naturally occurring plasmids code for other properties, or even for none at all, or at least none that we can discern. Plasmids exist because they can replicate within bacteria, and sometimes spread from one bacterium to another. That is all. They are a form of DNA parasite. Any advantage they confer on the host bacterium is a bonus that helps the plasmid to survive.

Plasmid replication

In genetic engineering, we make use of this ability of plasmids to be replicated, as it enables us to insert pieces of DNA which are then copied as part of the plasmid, and hence passed on to the progeny when the cell replicates. The most fundamental property of a plasmid, whether we are considering it as a cloning vector or as a natural phenomenon, is therefore the ability to replicate in the host bacterium. Most, or all, of the enzymes and other products needed for this replication are already present in the host cell; the amount of information that the plasmid has to supply may be only a few hundred base pairs. This region of the plasmid that is necessary for replication is generally referred to as the origin of replication, although literally the origin, or the site at which replication starts, is one specific base.

Plasmids that use the origin of replication from ColE1 or its relatives are multi-copy plasmids. Wild-type ColE1 is present at about 15 copies per cell, while most of the engineered vectors used today are present in numbers running into many hundreds of copies per cell. This is convenient in some ways as it makes it easier to purify large amounts of the plasmid, and if you want to express a cloned gene you also get a gene dosage effect. The presence of so many copies of the gene in the cell is reflected in higher levels of the product of that gene (see also Chapter 15). However, this can also be a disadvantage. Even without expression of the cloned gene, the large amount of plasmid DNA may make the cell grow more slowly. If the gene or its product is in any way harmful to the bacterium, it can sometimes be very difficult to isolate the required clone. For some specific purposes therefore it is desirable to use alternative vectors that exist at low copy number (or to use different vectors altogether that do not require continued viability of the cell, such as some types of bacteriophage vector – see below).

Some plasmids are able to replicate in a wide variety of bacterial species (broad host-range plasmids), but most of those that are used for gene cloning are rather more restricted in their host range. In one way this is useful: if there is any question about potential health hazards or environmental consequences associated with cloning a specific fragment of DNA, then using a narrow host-range plasmid makes it very unlikely that the gene will be transmitted to other organisms.

On the other hand, you may wish to carry out genetic manipulations in a bacterium other than *E. coli*, especially if your interest lies in studying the behaviour of specific bacteria rather than simply using them to clone pieces of DNA. It will then usually be necessary to isolate or construct new vector plasmids, based on a replication origin that is functional in your chosen species. The host range of your new vector will probably also be limited, and it may well be unable to replicate in *E. coli*. This is a disadvantage, because you are likely to want to use *E. coli* as an intermediate host for the initial cloning and for studying the structure and behaviour of the gene that you have cloned. However, it is possible to insert two origins of replication into your plasmid, so that it will be replicated in *E. coli* using one origin, and in your chosen host using the alternative replication origin. Such a vector is known as a *shuttle plasmid*, because it can be transferred back and forth between the two species. We can also use shuttle vectors to transfer cloned genes between *E. coli* and a eukaryotic organism. We will be coming across various applications of shuttle vectors in subsequent chapters.

Therefore, the first essential characteristic of a plasmid cloning vector is the origin of replication, usually designated as *ori* in plasmid maps.

Cloning sites

The second characteristic that is necessary for a plasmid to be useful as a cloning vector is a *cloning site*. This is a unique restriction site, so that the enzyme concerned will cut the plasmid once only. If a circular molecule is broken at one position, it is converted into a linear molecule, and it is relatively simple to join the ends together to reform an intact circle. If an enzyme cuts more than once, the plasmid will be cut into two or more pieces, and joining them up again to make an intact plasmid will be much more complicated. A basic plasmid used as a cloning vector may contain only one or two such unique restriction sites, which must of course be located in a region of the plasmid that is not essential for replication or any other functions that we need.

With such a plasmid you are limited not only in your choice of restriction fragments that can be inserted and in the position of insertion, but also in the number of different fragments that can be inserted. This is because in most cases ligation of two restriction fragments, generated with the same enzyme, recreates the original restriction site. Thus when you insert say a *Bam*HI fragment into a site on the vector that has been cut with *Bam*HI, the resulting recombinant plasmid will have two *Bam*HI sites: one at each end of the inserted fragment (see Figure 6.1). This is the basis of a common test for the presence of such an insert. Digesting the supposed recombinant plasmid with, in this case, *Bam*HI will release a DNA fragment that should be the size of the insert you are trying to clone – see Chapter 8. However, since the recombinant plasmid now has two *Bam*HI sites, it would be difficult to clone further *Bam*HI fragments into it.

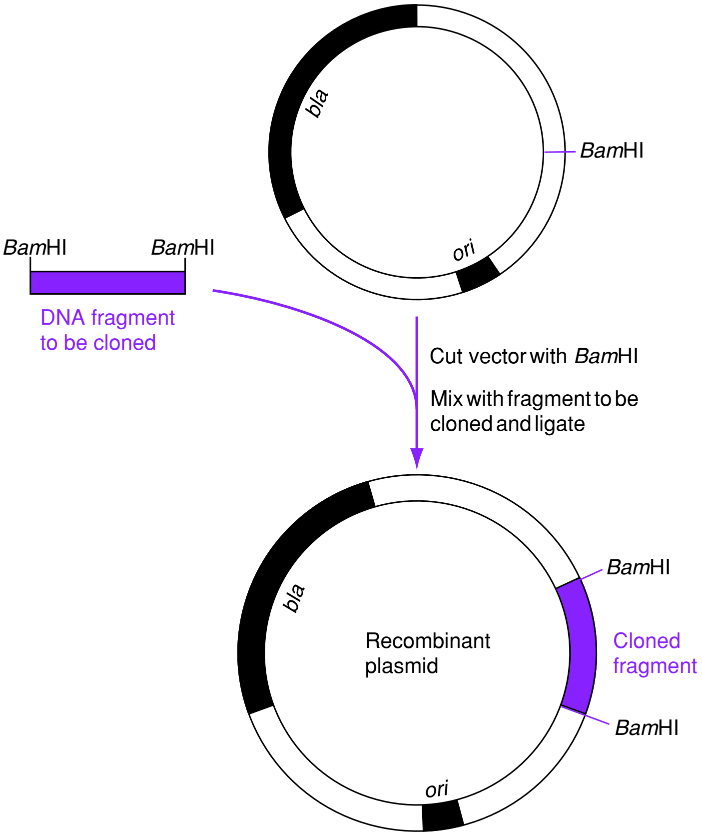
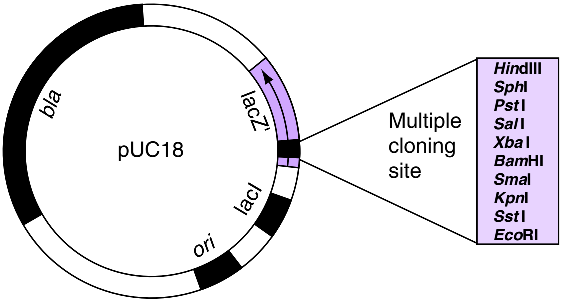


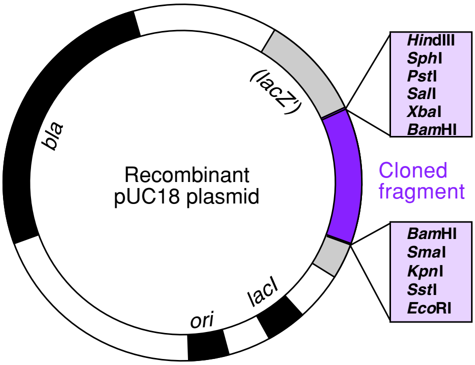
Figure 6.1 Cloning with a plasmid vector: *bla* = beta-lactamase (ampicillin resistance) selective marker; *ori* = origin of replication

The problem here is that in many cases we *do* want to insert several fragments into the same plasmid. We may want to combine the expression signals of one gene with the coding region of another, or we may want to insert additional markers that can be used to identify the presence of the plasmid. Or we may, as described above, want to insert the replication origin from another plasmid so as to create a shuttle vector, and still leave sites available for further inserts. The best way around this problem is to create a *multiple cloning site* (MCS), i.e. a short DNA region that contains recognition sites for a number of different enzymes. This is done by synthesizing a short piece of DNA with the required restriction sites, and inserting that into the plasmid in the usual way. Figure 6.2 shows the structure of pUC18, one of a family of similar plasmids that are commonly used as cloning vectors, and you will see that pUC18 contains such a multiple cloning site. Insertion of a fragment into the *Bam*HI site, as in Figure 6.3, will still leave a selection of other sites available for further inserts.



bla = beta-lactamase (ampicillin resistance); selective marker
ori = origin of replication
lacZ' = beta-galactosidase (partial gene)
lacI = repressor of *lac* promoter

Figure 6.2 Structure of the plasmid cloning vector pUC18



The *lacZ* gene has been disrupted by insertion of a DNA fragment, resulting in white colonies on X-gal plates

bla = beta-lactamase (ampicillin resistance); selective marker
ori = origin of replication
lacZ' = beta-galactosidase (partial gene)
lacI = repressor of *lac* promoter

Figure 6.3 Use of the plasmid cloning vector pUC18

Selectable markers

So we have a plasmid with a replication origin and one or more restriction sites. One further feature is essential for a functionally useful vector, and that is a selectable marker. The need for this arises from the inefficiency both of ligation and of bacterial transformation. Even with the high efficiency systems that are now available for *E. coli*, the best yield available, using native plasmid DNA, implies that only about 1 per cent of the bacterial cells actually take up the DNA. In practice the yields are likely to be lower than this – and if you are using a host other than *E. coli*, many orders of magnitude lower. Therefore in order to be able to recover the transformed clones, it is necessary to be able to prevent the non-transformed cells (i.e. those cells that have not taken up the plasmid) from growing. The presence of an antibiotic resistance gene on the plasmid vector means that you can simply plate out the transformation mix on an agar plate containing the relevant antibiotic, and only the transformants will be able to grow. In Figure 6.2, you will see that pUC18 carries a β-lactamase gene (*bla*), coding for an enzyme that hydrolyses β-lactam (penicillin-like) antibiotics such as ampicillin (and hence often referred to as Amp^R, for ampicillin resistance).

Insertional inactivation

In Figure 6.3 you will see a further feature of pUC18. The multiple cloning site is located near to the 5′ end of a β-galactosidase gene (*lacZ*). The synthetic oligonucleotide that creates the multiple cloning site was designed so that it did not affect the reading frame of the *lacZ* gene; it merely results in the production of β-galactosidase with some additional amino acids near to the amino terminus of the protein. This does not affect the function of the enzyme; it is still able to hydrolyse lactose. More accurately, we should say that pUC18 carries a part of the *lacZ* gene; we use *E. coli* strains that carry the remainder of the gene. The product of the host gene is unable to hydrolyse lactose by itself, and so the host strain without the plasmid is Lac^-, i.e. it does not ferment lactose. When pUC18 is inserted into the host, the plasmid-encoded polypeptide will associate with the host product to form a functional enzyme. We say that pUC18 is capable of *complementing* the host defect in *lacZ*.

We can easily detect the activity of the β-galactosidase by plating the organism onto agar containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (universally known as *X-gal*), together with the inducing agent isopropyl thiogalactoside (*IPTG*). The X-gal substrate is colourless but the action of β-galactosidase releases the dye moiety, resulting in a deep blue colour. Colonies carrying pUC18 are therefore blue when grown on this medium. However, if we are successful in inserting a DNA fragment at the cloning site, the gene will (normally) be disrupted and the resulting *E. coli* colonies are referred to as ‘white’. The advantage of this *insertional inactivation* is that we can tell not only that the cells have been transformed with the plasmid (since they are able to grow in the presence of ampicillin), but also that the plasmid is a recombinant, and not merely the original pUC18 self-ligated. An insertional inactivation marker such as this is not an essential feature of a cloning vector but it does provide a useful way of monitoring the success of the ligation strategy and overcoming some of the problems referred to in the previous chapter.

One word of caution: if the insert is relatively small, and if it happens to consist of a multiple of three bases, transcription and translation of the *lacZ* gene may still occur and the enzyme may still have enough activity (despite the addition of still more amino acids at the N terminus) to produce a detectable blue colour. Conversely, a white colony is not a guarantee of cloning success as the deletion of even a single base at the cloning site, or the insertion of undesirable junk fragments (in other than multiples of three bases), will put the *lacZ* gene in the wrong reading frame and thus inactivate it.

The advantage of plasmid vectors, compared with the other vectors described subsequently in this chapter, is that they are small and easy to manipulate; also they are conceptually simple and universal. You can make and use plasmid vectors for a wide range of organisms without a detailed knowledge of the molecular biology of the host or the vector. On the other hand, the basic plasmid vectors that we have been considering so far are limited in their cloning capacity, i.e. the size of the insert they can accommodate. Later in this chapter, we will look at other vectors that will accommodate larger inserts, but first we need to consider the ways in which we can introduce the recombinant plasmids into host bacterial cells.

6.1.2 Transformation

Bacterial transformation was discovered in 1928 with the demonstration by Fred Griffith that cultures of the pneumococcus (*Streptococcus pneumoniae*) that had lost virulence could have their pathogenicity restored by addition of an extract of a killed virulent strain. It was the identification, many years later (by Avery, MacLeod and McCarty), that the ‘transforming principle’ is DNA that resolved the question of the chemical nature of the genetic material.

This experiment rests on the natural ability of the pneumococcus to take up ‘naked’ DNA from its surroundings. This ability is known as *competence*. Competence develops naturally in some bacterial species, but although the range of species that exhibit natural competence is much wider than was thought for many years to be the case, it is still too limited in scope (or too inefficient or too selective) to be of much use for genetic engineering. In particular, *E. coli* does not seem to exhibit natural competence. It was therefore necessary to develop alternative ways of introducing plasmid DNA into bacterial cells. Although these methods are radically different, they are still referred to as transformation, which is defined as the uptake of naked DNA, to distinguish it from other methods of horizontal gene transfer, namely *conjugation* (direct transfer by cell to cell contact) and *transduction* (which is mediated by bacteriophage infection).

The breakthrough came with the demonstration that competence in *E. coli* cells could be induced by washing them with ice-cold calcium chloride, followed by adding the plasmid DNA and subjecting the mixture to a brief, mild heat shock (e.g. 2 min at 42°C). It is then necessary to dilute the cells in growth medium and incubate for a while (30–60 min) to allow the bacteria to recover and to express the resistance marker introduced on the plasmid, before plating them onto a selective medium containing the appropriate antibiotic. Although this simple basic process represented a major, and essential, step forward, it was very inefficient with yields of perhaps 10^4 transformants per μg of pure, supercoiled plasmid DNA (and less with ligation mixtures or non-supercoiled DNA). Gradually, over the years, improvements have been made in the transformation process, both by modifying the preparation of competent cells (e.g. using salts other than calcium chloride) and also by selecting *E. coli* strains with mutations that make them easier to transform. With the best of these systems it is now possible to obtain transformation frequencies in excess of 10^9 transformants per μg of plasmid DNA. Where such high yields are required, it is cost-effective to purchase pre-prepared competent cells of a strain with high transformation efficiency. Note that although transformation frequencies are generally quoted in these terms (number of transformants per μg of DNA), there is not a linear relationship between the number of transformants and the amount of DNA used. Transformation works best with low levels of DNA, and the efficiency with which bacterial cells take up DNA falls off as the concentration of DNA is increased. If you increase the amount of DNA too much, you might even decrease not just the efficiency but the actual numbers of transformants.

Reference back to the discussion of ligation in Chapter 5 will disclose a quandary here. Ligation works best with high concentrations of DNA. The following step, transformation, works best with small amounts of DNA. The resolution is clear, although unpalatable: use only a small proportion of your ligation mix in the transformation step. If you really need very large numbers of transformants, scaling up the transformation step does not work very well – it is usually much better to carry out several separate small-scale transformations.

Transformation based on induced competence and heat shock can be used for bacterial species other than *E. coli*, but you immediately lose all the advantages that have been gained by optimization of transformation conditions for selected strains of *E. coli*. At best, therefore, transformation is likely to be very inefficient – and in most cases simply using an *E. coli* procedure will not work at all. Therefore laboratories that are interested in manipulating other bacterial species have had to develop alternative methods of transformation.

Electroporation is the most versatile transformation procedure. Bacterial cells, washed with water to remove electrolytes from the growth medium, are mixed with DNA and subjected to a brief pulse of high-voltage electricity. This appears to induce temporary holes in the cell envelope through which the DNA can enter. The cells are then diluted into a recovery medium before plating on a selective medium in the same way as above. Although it is comparatively easy to obtain *some* transformants with a wide range of bacteria (or other cells), there are many parameters that need to be adjusted to obtain optimum performance, including the conditions under which the cells are grown, the temperature of the suspension, and the duration and voltage of the electric pulse.

Since the added DNA seems to simply diffuse through the holes created (briefly) by the electric pulse, the effect is not specific for DNA; other substances, notably RNA or proteins, can also be introduced into bacterial cells by electroporation. Nor is it directionally specific. Material within the cell can diffuse out as well, and the procedure has been used for isolating plasmid DNA from bacterial cells. It follows from this that, since the plasmid that comes out of one cell can enter another one, electroporation can be used to transfer plasmids from one strain to another, simply by applying it to a mixture of the two strains.

Other methods that are used more commonly with animal and plant cells, including microinjection, biolistics, and protoplast transformation are considered in Chapter 17.

6.2 Vectors Based on the Lambda Bacteriophage

6.2.1 *Lambda biology*

Plasmid vectors are at their best when cloning relatively small fragments of DNA. Although there is probably no fixed limit to the size of a DNA fragment that can be inserted into a plasmid, the recombinant plasmid may become rather less stable with larger DNA inserts, the efficiency of transformation is reduced, and the plasmid will give a much smaller yield when grown and purified in *E coli*. Vectors based on bacteriophage lambda allow efficient cloning of larger fragments, which is important in constructing gene libraries. The larger the inserts, the fewer clones you have to screen to find the one you

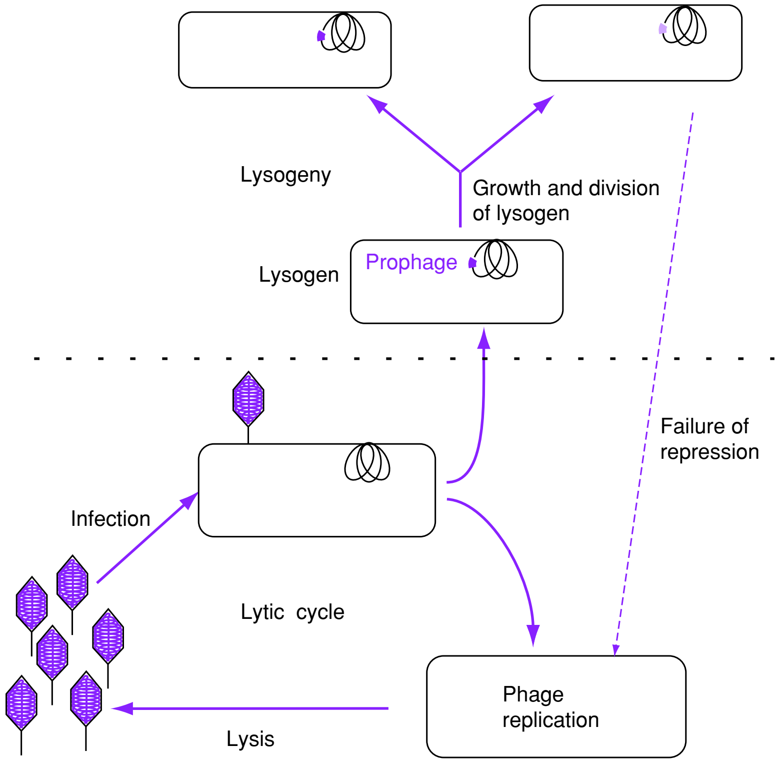


Figure 6.4 Lytic cycle and lysogeny

want (see Chapters 7 and 8). Lambda vectors also have advantages in gene library construction, as it is much easier to screen large libraries when using bacteriophage vectors; the results with bacteriophage plaques are much cleaner than those obtained with bacterial colonies.

In order to understand the nature and use of lambda cloning vectors, some knowledge of the basic biology of bacteriophage lambda is necessary. While we hope you are familiar with this, a recap of the salient features (summarized in Figure 6.4) will be useful.

Lysogeny

Lambda is a temperate bacteriophage, i.e. on infection of *E. coli* it may enter a productive lytic cycle, resulting in lysis of the cell and liberation of a number of phage particles, or it may enter a more or less stable relationship with the host known as *lysogeny*. In the lysogenic state, expression of almost all of the phage genes is switched off by the action of a phage-encoded repressor protein, the product of the *cI* gene. The expression of this gene during the establishment of lysogeny requires two other genes, *cII* and *cIII*. The proportion of infected cells going down each route is influenced by environmental conditions, as well as by the genetic composition of the phage and the host. Some phage mutants will only produce lytic infection, and these give rise to clear plaques, while the wild-type phage produces turbid plaques due to the presence of lysogens which are resistant to further attack by lambda phage (known as *superinfection immunity*). On the other hand, some bacterial host strains carrying a mutation known as *hfl* (high frequency of lysogenization) produce a much higher proportion of lysogens when infected with wild-type lambda – which can be useful if we want a more stably altered host strain, for example if we are studying the expression of genes carried by the phage. Generally, when using lambda vectors we are more interested in the recombinant phage carrying the cloned genes, and the lytic cycle is the more relevant one in such cases.

Although the lysogenic state is relatively stable, that stability is not absolute. A culture of a bacterial lysogen will normally contain phage particles in the supernatant, due to a low level of spontaneous failure of the repression mechanism. This rate of breakdown of repression can be increased by treating the culture with agents that damage the DNA, such as UV irradiation; the DNA damage induces the production of repair enzymes which amongst other things destroy the *cI* repressor protein, allowing initiation of the lytic cycle. Some widely used lambda vectors carry a mutation in the *cI* gene which makes the protein more temperature-sensitive (*cI857* mutation). A lysogen carrying such a mutant phage can be grown as a lysogen at a reduced temperature and the lytic cycle will be induced by raising the temperature, due to inactivation of the repressor protein.

In the lysogenic state, lambda is normally integrated into the bacterial chromosome, by site-specific recombination at a specific position, and is therefore replicated as part of the bacterial DNA. Induction of the lysogen requires excision from the chromosome. However, this integration, although common amongst temperate phages, is not an essential feature of lysogeny. Lambda can continue to replicate in an extrachromosomal, plasmid-like state; with some bacteriophages (including P1, which we will encounter later in this chapter) this is the normal mode of replication in lysogeny.

Particles of wild-type bacteriophage lambda have a double-stranded linear DNA genome of 48 514 base pairs, in which the 12 bases at each end are unpaired but complementary (see Figure 6.5). These ends are therefore 'sticky' or 'cohesive', much like the ends of many restriction fragments – but the longer length of these sticky ends makes the pairing much more stable, even at 37°C. The ends can be separated by heating lambda DNA, and if it is then cooled rapidly you will get linear monomeric lambda DNA. At low temperatures, the ends of the molecule will move slowly, and therefore the re-annealing of the sticky ends will take a long time. Eventually, however, it will resume a circular (although not covalently joined) structure. When lambda infects a bacterial cell

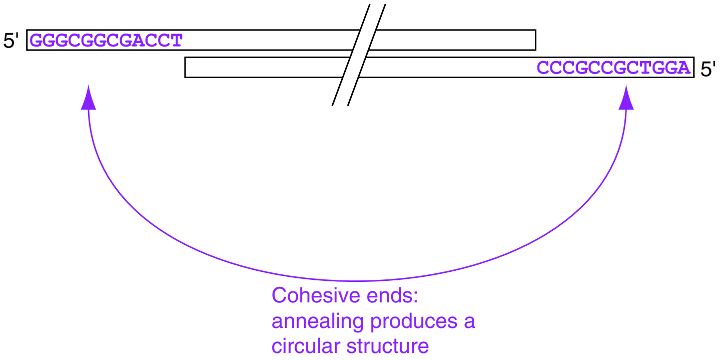


Figure 6.5 Cohesive ends of lambda DNA

and injects its DNA into the cell, it will therefore form a circular structure, with the nicks being repaired *in vivo* by bacterial DNA ligase. At about this time, a complex series of events occur that affect subsequent gene expression, determining whether the phage enters the lytic cycle or establishes lysogeny. We do not need to consider the details of the *lytic–lysogenic decision*, except to emphasize that it is essentially irreversible so that once started on one or the other route, the phage is committed to that process. However, we do need to consider the events in the lytic cycle.

Lytic cycle

In the lytic cycle, this circular DNA structure is initially replicated, in a plasmid-like manner (*theta* replication), to produce more circular DNA. Eventually, however, replication switches to an alternative mode (*rolling-circle replication*) which generates a long linear DNA molecule containing a large number of copies of the lambda genome joined end to end in a continuous structure (see Figure 6.6). While all this is going on, the genes carried by the phage are being expressed to produce the components of the phage particle. These proteins are assembled first of all into two separate structures: the head (as an empty precursor structure into which the DNA will be inserted), and the tail (which will be joined to the head after the DNA has been packaged).

The packaging process involves enzymes recognizing specific sites on the multiple-length DNA molecule generated by rolling-circle replication, and making asymmetric cuts in the DNA at these positions. These staggered breaks in the DNA give rise to the cohesive ends seen in the mature phage DNA; these sites are known as *cohesive end sites* (*cos* sites). Accompanying these cleavages, the region of DNA between two *cos* sites – representing a unit length of the lambda genome – is wound tightly into the phage head. This process is known

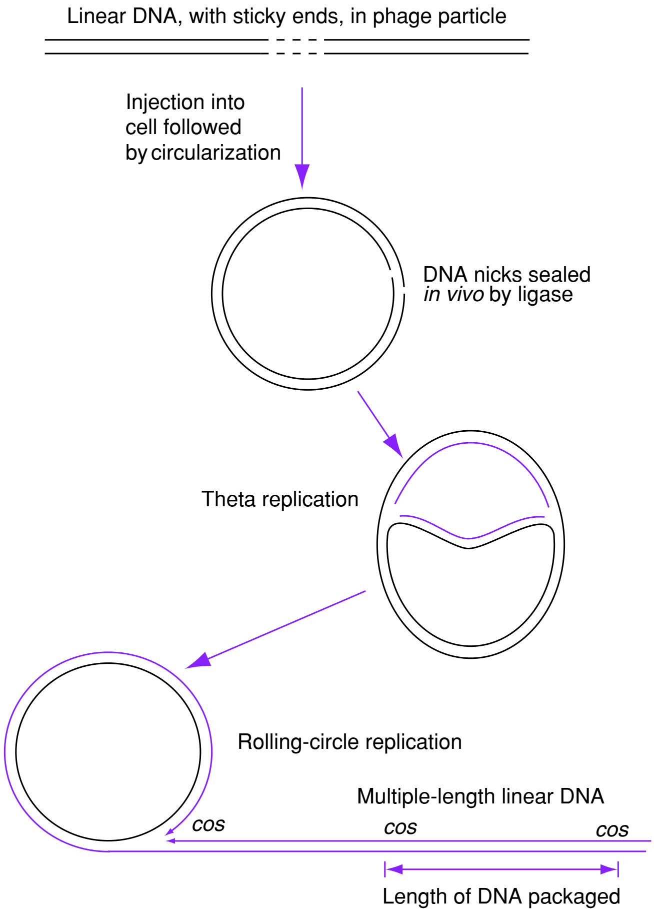


Figure 6.6 Replication of bacteriophage lambda DNA

as *packaging*. Following successful packaging of the DNA into the phage head, the tail is added to produce the mature phage particle, which is eventually released when the cell lyses.

Lysis of the bacterial cell is accomplished largely through the action of a phage-encoded protein, the product of gene *S*. Mutations in this gene can cause a delay or failure of lysis – which can be advantageous in increasing the yield of bacteriophages, as the replication of the phage will therefore continue for a longer time instead of being interrupted by lysis of the host cell. Many lambda vectors have such a mutation.

One of the most important features of this process from our point of view is that the length of DNA that will be packaged into the phage head is determined by the distance between two *cos* sites. If we insert a piece of DNA into our lambda vector, we will increase that distance, and so the amount of DNA to be packaged will be bigger. However, the head is a fixed size, and can only accommodate a certain amount of DNA. It can take somewhat more than is present in wild-type lambda (up to about 51 kb altogether, which is about 5 per cent more than wild-type). As one of the reasons for using lambda is to be able to clone large pieces of DNA, this would be a serious limitation. The way round it is to delete some of the DNA that is normally present. This is possible, because the lambda genome contains a number of genes that are not absolutely necessary – especially if we only need lytic growth, when we can delete any genes that are solely required for the establishment of lysogeny. However, we cannot delete too much. The stability of the phage head requires a certain amount of DNA so, even though there are more genes that are not required, we cannot delete all that DNA. To produce viable phage, there has to be a minimum of 37 kb of DNA (about 75 per cent of wild-type) between the two *cos* sites that are cleaved.

The existence of these *packaging limits* is a very important feature of the design and application of lambda vectors, and also of cosmids which we will discuss later.

6.2.2 In vitro *packaging*

Naked bacteriophage DNA can be introduced into a host bacterial cell by transformation (often referred to as *transfection* when talking about phage DNA), in much the same way as we described for a plasmid. The big difference is that in this case instead of plating on a selective agar and counting bacterial colonies, we would mix the transfection mix with a culture of a phage-sensitive indicator bacterium in molten soft agar and look for *plaques* (zones of clearing due to lysis of the bacteria) when overlaid onto an agar plate. Note that in this case we do not need an antibiotic resistance gene as a selective marker.

However, the large size of most bacteriophage DNA molecules, including that of lambda, makes transfection an inefficient process compared with plasmid transformation, and not suitable for the generation of gene libraries which is the principal application of lambda vectors. However, there is a more efficient alternative. Some mutant lambda phages, in an appropriate bacterial host strain, will produce empty phage heads (as they lack a protein needed for packaging the DNA), while others are defective in the production of the head, but contain the proteins needed for packaging. The two extracts are thus complementary to one another. Use of the mixture allows productive packaging of added DNA, which occurs very effectively *in vitro* (including the addition of the tails). The resulting phage particles can then be assayed by addition of a sensitive bacterial culture and plating as an overlay, as above. Since *in vitro* packaging of lambda DNA is much more effective than transfection, it is the method that is almost always used.

One feature of this system that is markedly different from working with plasmid vectors is that the packaging reaction is most efficient with multiple-length DNA. The enzyme involved in packaging the DNA normally cuts the DNA at two different *cos* sites on a multiple-length molecule; monomeric circular molecules with a single *cos* site are packaged very poorly. So whereas with plasmid vectors the ideal ligation product is a monomeric circular plasmid consisting of one copy of the vector plus insert, for lambda vectors it is advantageous to adjust the ligation conditions so that we *do* get multiple end-to-end ligation of lambda molecules together with the insert fragments. The stickiness of the ends of the linear lambda DNA means this happens very readily.

6.2.3 Insertion vectors

The simplest form of lambda vectors is that known as an *insertion vector*. These are similar in concept to a plasmid vector, in that they contain a single cloning site into which DNA can be inserted. However, wild-type lambda DNA contains many sites for most of the commonly used restriction enzymes; you cannot just cut it with say *Hin*dIII and ligate it with your insert DNA. *Hin*dIII has seven sites in normal lambda DNA, and so will cut it into eight pieces. (Note the difference between a circular DNA molecule such as a plasmid, and a linear molecule like lambda: cut a circular DNA molecule once and you still have one fragment; cut linear DNA once and you have two fragments.) It would be almost impossible to join all these fragments (and your insert) together in the right order. To circumvent this, all lambda vectors have been genetically manipulated to remove unwanted restriction sites. In some cases this has been done by deleting regions of DNA carrying these sites (and hence also increasing the cloning capacity); another strategy is to select mutants with alterations in their sequence that result in the loss of the unwanted restriction sites.

In Figure 6.7, we see one example of a lambda vector, known as lambda gt10. In this vector, there is only a single site at which *Eco*RI will cut the DNA. The deletions and other manipulations that this phage has undergone have removed some of the unwanted sites, and have also reduced the overall size of the phage DNA to 43.3 kb (which is still large enough to produce viable phage particles, and still contains the genes that are needed for viability), and hence allows the insertion of foreign DNA up to a maximum of 7.6 kb.

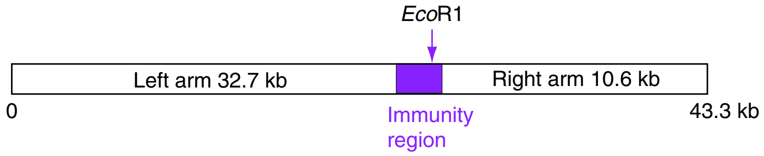


Figure 6.7 Lambda insertion vector gt10

Cutting this vector with *Eco*RI will produce two DNA fragments, referred to as the left and right arms. Although it therefore appears that the insert would have to be ligated to two different pieces of DNA, in practice this does not complicate the ligation as much as might be imagined. One end of each of the two fragments is derived from the cohesive ends of the lambda DNA, and will therefore anneal quite stably at 37°C – so although not covalently joined they can be considered as a single DNA fragment.

Lambda gt10 also provides us with another example of how insertional inactivation can be used to distinguish the parental vector (which may form by religation of the arms without an insert) from the recombinants. The *Eco*RI site is found within the repressor (*c*I) gene, so the recombinant phage, which carry an insert in this position, are unable to make functional repressor. As a consequence, they will be unable to establish lysogeny and will give rise to clear plaques, whereas the parental gt10 phage will give rise to turbid plaques. The distinction can be made even more marked, by using a host strain carrying the *hfl* (high frequency of lysogenization) mutation. In such a strain, any parental phage will establish lysogeny extremely efficiently, rather than entering the lytic cycle, with the result that few, if any, plaques will be obtained. This does not affect the recombinants, which are unable to make functional repressor, and therefore do not establish lysogeny. So you can achieve substantial enrichment of recombinant phage over the religated vector without having to resort to dephosphorylation with alkaline phosphatase.

Another example of a lambda insertion vector, lambda gt11, is used in a rather different way. Since it allows expression of the cloned fragment, it is considered later in this chapter, together with other expression vectors.

The packaging limits for lambda DNA are between 37 kb and 51 kb, as described above. In other words, we cannot make an insertion vector smaller than 37 kb, or we will be unable to grow it to produce the DNA that we need. Also, we cannot insert a DNA fragment so big that it would make the product larger than 51 kb; the recombinant DNA would be unable to be packaged into the phage heads. It follows from this that the maximum cloning capacity for an insertion vector is (51 minus 37) kb = 14 kb. This is larger than we would normally clone comfortably in a plasmid vector, but still smaller than we would like for some purposes. In order to increase the available cloning capacity, we have to turn to a different type of lambda vector, known as a *replacement vector*.

6.2.4 Replacement vectors

The packaging limits that restrict the cloning capacity of insertion vectors are imposed by the physical requirements of the phage head rather than by the nature of the genes needed. There are more genes that are not essential for lytic growth and could be deleted, except that it would make the phage DNA too small to produce viable progeny. That provides a clue to an alternative design of lambda cloning vectors. Instead of merely inserting extra DNA, arrange the vector so that a piece of DNA can be removed and replaced by your insert – hence the term *replacement vector*.

Figure 6.8 shows an example of a lambda replacement vector, EMBL4. Instead of being cut just once by the restriction enzyme of choice (in this case *Bam*HI), there are two sites where the DNA will be cleaved. The vector DNA will therefore be cut into three fragments: the left and right arms (which will anneal by virtue of their cohesive ends) and a third fragment which is not needed (except to maintain the size of the DNA) and can be discarded. Since the only purpose of this fragment is to help to fill up the phage head it is known as a *stuffer fragment*.

In use, therefore, this vector would be cut with *Bam*HI and the fragments separated, e.g. by gel electrophoresis. The stuffer fragment would be thrown away, and the arms mixed with the restriction fragments to be cloned. These could be generated by *Bam*HI digestion, or by cleavage of the target with another enzyme that produces compatible ends, e.g. *Sau*3A (see Chapter 5). Ligation of the mixture would produce recombinant phage DNA that would be packaged into phage heads by *in vitro* packaging. The cloning capacity of the vector is thus considerably increased; in this case the size of the arms combined comes to 29 kb and thus you can clone fragments up to (51 minus 29) kb = 22 kb.

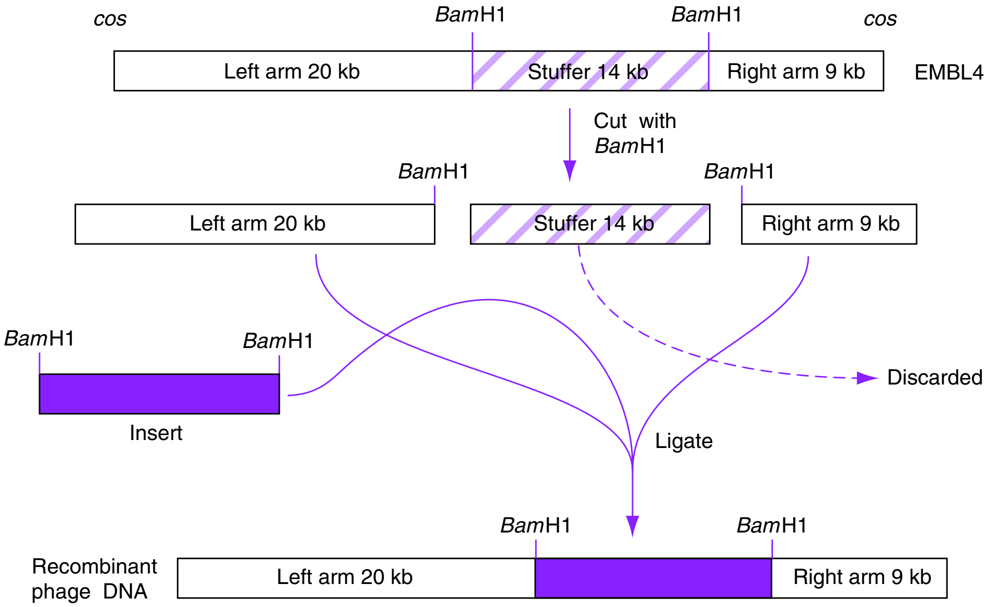


Figure 6.8 EMBL4

There is a further advantage to such a vector. The combined size of the arms is only 29 kb, which is less than the minimum required for packaging. Any pairs of arms that are ligated without an insert will therefore be too small to produce viable phage particles. Such viable particles will only be produced if ligation results in an insert of at least (37–29) kb = 8 kb. The vector thus provides a positive selection for recombinants as opposed to parental phage, and furthermore for recombinants that contain an insert of at least 8 kb. The gene library will therefore be free of non-recombinant phage.

In Chapter 5 we discussed strategies, with plasmid vectors, for ensuring that we obtained recombinant progeny rather than parental vector molecules, including the use of alkaline phosphatase treatment of the vector to prevent recircularization. This is not necessary with replacement vectors. Since we do not have to treat the vector with phosphatase, we have another possibility – dephosphorylation of the *insert*. In the production of a gene library, the insertion of more than one fragment into the same vector molecule is a problem that can give rise to anomalies in characterizing the insert in relation to the genome it came from. Phosphatase treatment of the insert will prevent insert–insert ligation, and hence will ensure that all of the recombinants carry only a single insert fragment.

There is yet another useful feature that can be built into a replacement vector. Since the stuffer fragment is not necessary for phage production (apart from filling up the phage head), it does not have to be lambda DNA. It can be anything we want, so we could for example put in a fragment carrying a β-galactosidase gene. Then any plaques formed by phage that still carry the stuffer fragment would be blue (on a medium containing X-gal). Of course ideally there should not be any; but there may be some phage DNA molecules that have not been cut completely, or there may be some stuffer DNA contaminating the preparation of the vector arms, which could then be ligated back into the vector. Any plaques containing the stuffer will be blue – so you have an immediate check that everything has gone according to plan, or not.

So we see that lambda vectors provide a highly versatile and efficient system for primary cloning of unknown fragments, especially in the construction of genomic and cDNA libraries (see Chapter 7). They extend the cloning capacity over that readily obtainable with plasmid vectors, and can easily generate the very large numbers of recombinants that are required for a gene library. However, some people do not like working with lambda systems, mainly because it requires a different set of techniques for growing, assaying and maintaining phage preparations. There is nothing really difficult about it; it is just unfamiliar. The only real disadvantage to lambda cloning systems is the size of the vector DNA. With an insertion vector, your recombinant may contain 5 kb of insert and 45 kb of vector. This makes it more difficult to analyse or manipulate your insert than would be the case with a plasmid recombinant – especially as the lambda vector will contain a substantial number of recognition sites for different restriction enzymes. The normal procedure therefore, having identified the recombinant clone of interest, would be to reclone the insert (or part of it) into a plasmid vector for further analysis and manipulation.

Although lambda phages are the most widely used phage vectors, there are other phages, or vectors based on them, that are used for specific purposes, including P1 and M13. These are discussed further in subsequent sections in this chapter; but first we need to look at a special class of vector that combines some of the features of lambda and plasmid vectors, and enables the cloning of even larger pieces of DNA. These are the *cosmids*.

6.3 Cosmids

The lambda packaging reaction has two fundamental requirements: the presence of a *cos* site, and the physical size of the DNA. Cosmids exploit this to provide cloning vectors with a capacity larger than can be achieved with lambda replacement vectors.

Basically, a cosmid is simply a plasmid which contains a *cos* site. As with all plasmid vectors, it has an origin of replication, a selectable marker (usually an antibiotic resistance gene), and a cloning site. Digestion, ligation with the potential insert fragments, and subsequent purification of recombinant clones, are carried out more or less as for a normal plasmid vector. However, instead of transforming bacterial cells with the ligation mix, as you would normally with a plasmid, you subject the ligation mixture to *in vitro* packaging as described above for lambda vectors. Since the cosmid carries a *cos* site, it can be a substrate for *in vitro* packaging, but only if it is big enough. The vector itself is quite small – in the example shown in Figure 6.9, it is 5.4 kb, which is much too small for successful packaging. The packaging reaction will only be successful if you have inserted a DNA fragment between about 32 kb and 45 kb

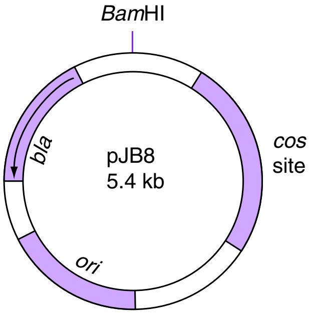


Figure 6.9 Structure of a cosmid

in size. So you not only have an increased cloning capacity, but also a positive selection for an insert, and for an insert that is quite large.

There is one difference in the ligation procedure between cosmids and plasmids. The *in vitro* packaging reaction requires two separate *cos* sites and therefore works best with multimeric structures resembling the normal lambda DNA substrate. The efficiency of cosmid cloning can therefore be enhanced by adjusting the ligation conditions to favour multimer formation, or by using more complex cosmid designs and/or more sophisticated digestion and ligation strategies that ensure that only multimeric ligation products are made. Note, however, that although the ligation results in multimeric structures, the *in vitro* packaging reaction will ensure that each recombinant clone contains only a monomeric cosmid.

Of course the products of the packaging reaction, although they are phage particles, will not give rise to more phages after infection of a host bacterium. They do not carry any of the genes that are needed for production of more phage particles, nor for lysis of the cell. So you will not get phage plaques. However, the cosmid will replicate as a plasmid, and hence will give rise to more cosmid-containing cells, and these can be selected as colonies on agar containing an antibiotic (in this case ampicillin).

For very large genomes, such as mammalian ones, the cloning capacity of cosmids is still rather too small for convenience, and alternative vectors with even greater capacity are now available (see below). However, for smaller and medium-sized genomes, cosmids can be extremely valuable. A complete bacterial genome can be covered by a selection of only a few hundred cosmids, which can be useful in genome mapping and sequencing projects.

Cosmids have some advantages over phage lambda, particularly in that they can be propagated and purified by conventional plasmid-oriented techniques, without having to become familiar with phage technology. Furthermore, with cosmids the vector is small compared with the insert, in contrast to lambda where more than half of the DNA of a recombinant phage is derived from the vector. Subcloning of fragments of your insert, to obtain fragments carrying just the gene you need, is therefore rather easier with cosmids than with lambda vectors. On the other hand, a lambda library can be easily stored as a pool of phage particles in a single tube.

6.4 M13 Vectors

Like lambda, M13 is a bacteriophage that infects *E. coli*. That is about as far as the resemblance goes, either biologically or in their use as cloning vectors.

M13 is a 'sex-specific' bacteriophage, or more accurately F-specific. It attaches to the tips of the pili that are produced on the surface of bacteria that carry an F-type plasmid, and is therefore unable to infect bacteria that do not carry such a plasmid. The very long, thin filamentous phage particles contain a circular, single-stranded DNA molecule of about 6 kb. After this DNA enters the cell, it is converted to a double-stranded molecule (the *replicative form, RF)* by synthesis of the complementary strand. This molecule is replicated by producing a circular single-stranded copy of one strand of the RF. This single-stranded DNA is again converted to a double-stranded form (see Figure 6.10). The production of the single-stranded intermediate requires a specific signal on the DNA, and is therefore completely strand-specific, i.e. it is always the same strand that appears in this form. This separation of the synthesis of the two strands is not unique to M13 but is found in some other bacteriophages and also some classes of plasmids. However, most of the phage DNA within the cell is double-stranded circular DNA (RF), and can be isolated by conventional plasmid purification methods.

Continued replication of the phage DNA leads to a build up of these plasmid-like DNA molecules within the cell. At the same time, expression of phage genes occurs, and the product of one of these genes binds to the single-stranded

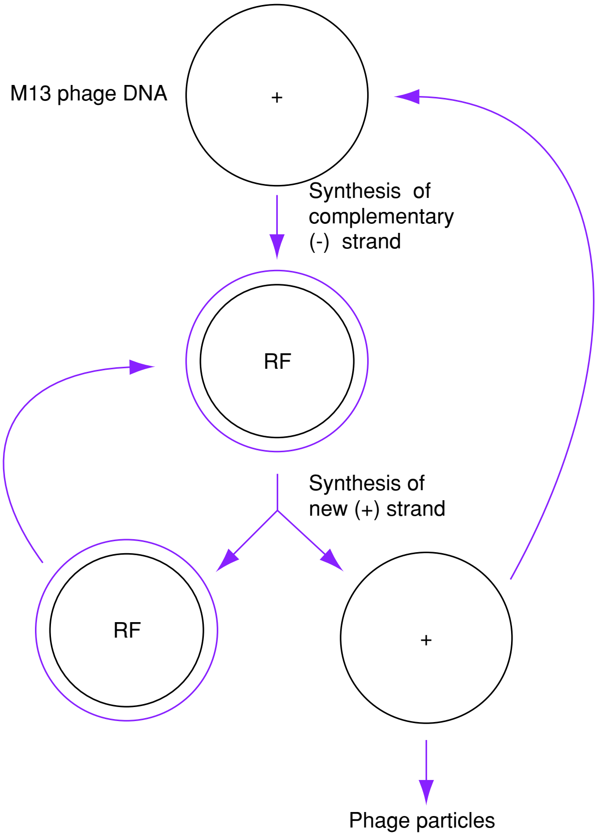


Figure 6.10 Replication of single-strand bacteriophages

product, initiating production of phage particles. This occurs by extrusion of the DNA through the cell membrane, during which process it becomes coated with phage proteins. The length of the filamentous phage particle is determined by the length of the DNA molecule, unlike lambda where the size of the particle is determined by the structure of the proteins of which it is composed. Hence, there are no absolute packaging limits for M13, although the phage does become increasingly fragile if large DNA fragments are inserted.

A curious, and significant, feature of M13 is that infection does not lead to bacterial lysis. Phage particles continue to be produced, and the cell remains viable, although it grows more slowly. Infection does result in the appearance of 'plaques' in a bacterial lawn, but these are zones of reduced growth rather than zones of lysis. As a consequence of the continuing viability of the host cell, very high titres of phage can be produced.

The main advantage of M13 is that it provides a very convenient way of obtaining single-stranded versions of a gene, which would be difficult to do in any other way. Single-stranded DNA obtained from M13 clones has been widely used for DNA sequencing (see Chapter 10). Although, nowadays, double-stranded DNA templates are commonly used for sequencing, single-stranded vectors are still preferred by some laboratories. Another application where single-stranded DNA can be advantageous (although not essential) is site-directed mutagenesis (Chapter 15). M13 vectors also have another role, not connected with the production of single-stranded DNA: this is the technique known as *phage display* (see Chapter 14).

Vectors based on M13 have been engineered to contain multiple cloning sites, and these vectors usually include a beta-galactosidase gene to distinguish recombinants from parental vector, as was described for pUC18 earlier in this chapter. DNA fragments can be cloned into such sites, using the plasmid-like replicative form; after transformation, the progeny are detected by a plaque assay, with 'white' plaques indicating a recombinant clone and blue plaques (on a medium containing X-gal) suggesting non-recombinant phage. As discussed earlier, in relation to pUC18, some blue plaques may occur through insertion of small fragments if they do not shift the reading frame of the *lac*Z gene.

If you then isolate phage particles from the supernatant of an infected culture, these will contain your gene in single-stranded form. Note that because of the specificity of the replication process, it is always the same strand that is found in all phage particles.

6.5 Expression Vectors

The above discussion has assumed that all you want to do is to clone a piece of DNA. It does not consider the possibility that you might want to obtain expression of the gene encoded by that DNA. If you take a DNA fragment from another organism and clone it in *E. coli*, there are many reasons why it may not be expressed. At the simplest level, these relate to the signals necessary for initiating transcription (a promoter) and translation (a ribosome-binding site and start codon). The basic way of encouraging (although not ensuring) expression of the cloned gene is to incorporate these signals into the vector, adjacent to the cloning site. This is then known as an *expression vector*.

Expression vectors are of two main types (see Figures 6.11 and 6.12). If the vector just carries a promoter, and relies on the translation signals present in the cloned DNA, it is referred to as a *transcriptional fusion* vector. On the other hand, if the vector supplies the translational signals as well (so you are inserting the cloned fragment into the coding region of a vector gene), then you have a *translational fusion*. Note that in this case the insert must be in frame with the start codon. The plasmid vector pUC18 that we looked at earlier is actually a translational fusion vector, although not often used as such. A better example is the lambda vector gt11 (see Figure 6.13). This is an insertional vector, 43.7 kb in length (making the maximum cloning capacity about 7 kb). It has been engineered to contain a β-galactosidase gene, and has a single *Eco*RI restriction site within that gene. This confers two properties on the vector. Firstly, insertion of DNA at the *Eco*RI site will inactivate the β-galactosidase gene, so that recombinants will give 'white' plaques on a medium containing X-gal. Secondly, the insert, if in the correct orientation and in frame, will give rise to a

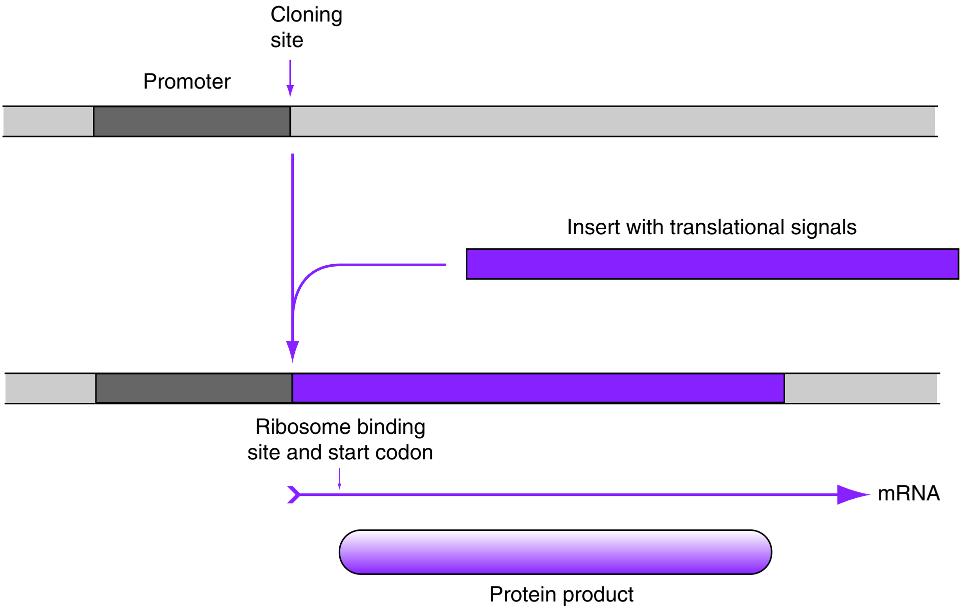


Figure 6.11 Expression vectors: transcriptional fusions

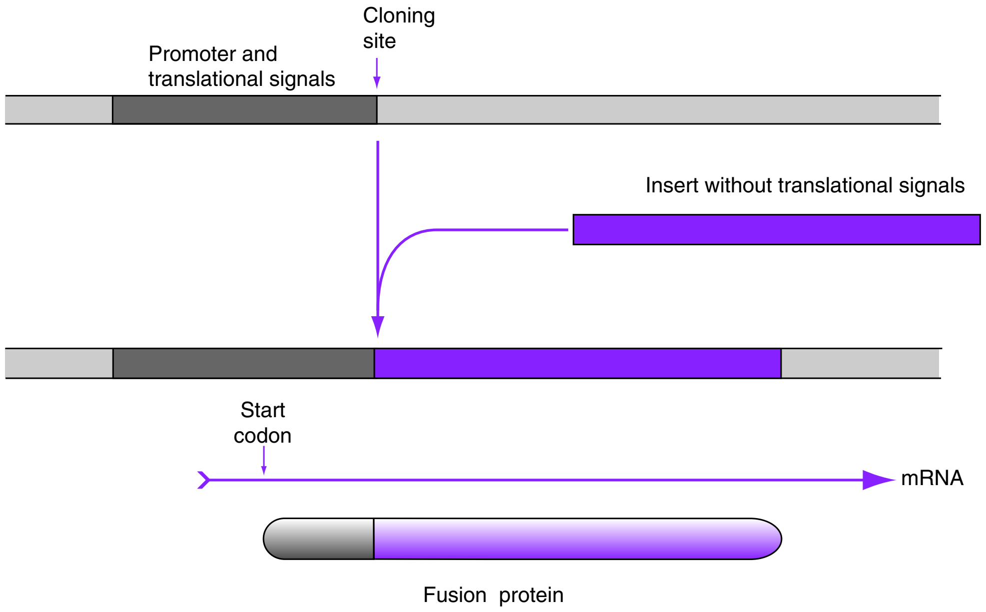


Figure 6.12 Expression vectors: translational fusions

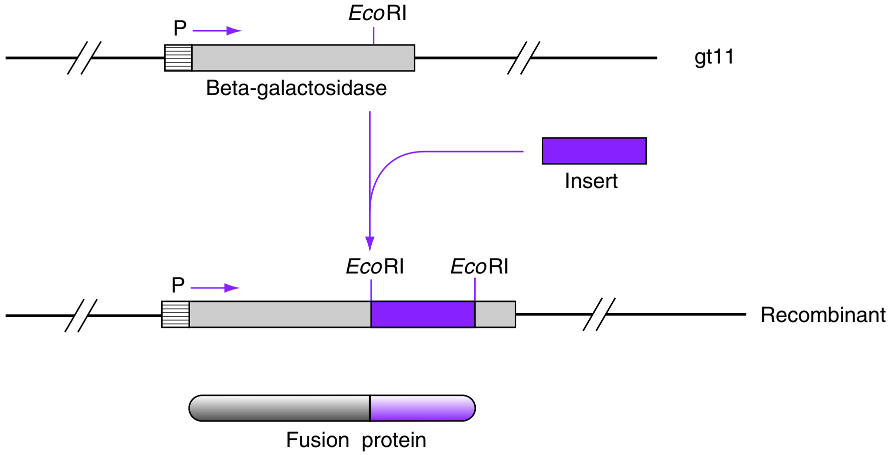


Figure 6.13 Use of lambda gt11 for generation of fusion proteins

fusion protein containing the product encoded by the insert fused to the β-galactosidase protein. This fusion protein is unlikely to have the biological functions associated with your cloned gene – it contains too much extraneous material – but that is not the point. It *is* reasonably likely to react with some antibodies to the natural product, which makes it a useful way of detecting the clone of interest, as we will see in Chapter 8.

To make full use of an expression vector you need to be able to choose whether to have expression on or off. The regulation of most inducible bacterial promoters, such as that of the *lac* operon, is rather 'leaky', i.e. there is still some expression even in the uninduced or repressed state. Firmer control can be achieved by the use of promoters from bacteriophages, notably one from the bacteriophage T7. In T7, this promoter controls the expression of the 'late' genes, i.e. the genes that are only switched on at a late stage of infection. This promoter is not recognized by *E. coli* RNA polymerase, but requires the T7 RNA polymerase, a product of genes expressed earlier in the infection cycle. So if we clone our DNA fragment downstream from a T7 promoter, using an 'ordinary' *E. coli* host (lacking a T7 polymerase gene) we will get no expression at all. This can be useful, as the product might be deleterious to the cell. Once we are satisfied that we have made the right construct, we can isolate the plasmid and put it into another *E. coli* strain that has been engineered to contain a T7 polymerase gene, and hence will allow transcription of the cloned gene. If the expression of the T7 polymerase gene is itself regulated, for example by putting it under the control of a *lac* promoter, then we can turn the expression of the T7 polymerase up (by adding IPTG) or down, so we still have control over the level of expression. Further devices can be included to inhibit the low level of T7 polymerase arising from the leakiness of the uninduced *lac* promoter.

The pGEM® series of vectors (see Figure 6.14) provide an example. In this case, there is a multiple cloning site adjacent to the T7 promoter, so any DNA inserted will be under the control of the T7 promoter. This is a transcriptional fusion vector, and it is often more useful for generating substantial amounts of an RNA copy of your cloned fragment, which can then be used as a probe for hybridization. The vector actually has a second specific promoter, derived from another bacteriophage (SP6), at the other side of the multiple cloning site, so if you provide an SP6 polymerase you will get an RNA copy of the other strand, the antisense strand. The usefulness of this will be apparent when we consider applications of antisense RNA, such as the RNase protection assay (Chapter 13).

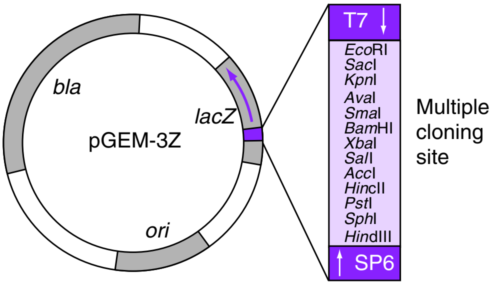


Figure 6.14 Structure of the expression vector pGEM-3Z: *bla* = beta-lactamase (ampicillin resistance); *ori* = origin of replication

There are also a variety of translational fusion vectors with T7 promoters, which are designed for obtaining high, controllable, levels of protein expression. We will return to the concept of expression vectors, and other factors that have to be considered for the optimization of protein production, in Chapter 15.

6.6 Vectors for Cloning and Expression in Eukaryotic Cells

Most primary cloning (i.e. the initial isolation of a gene or other DNA fragment from a target organism) is carried out using bacterial hosts (usually *E. coli*), because of the ease of manipulation and the range of powerful techniques that have been developed. Eukaryotic hosts are more commonly used for studying the behaviour of genes that have already been cloned (but in an environment more closely related to their original source), for analysing their effect on the host cell and modifying it, or for obtaining a product which is not made in its natural state in a bacterial host. There is therefore more emphasis with eukaryotic vectors in obtaining gene expression rather than making gene libraries or primary cloning (with the notable exception of YAC vectors, see below). There is a bewildering variety of vectors available for cloning in different eukaryotic hosts, and a full review of them is way beyond the scope of this book. In this chapter, we want to introduce some of the main concepts, many of which are similar in principle to those of bacterial cloning vectors although there are significant differences; we will consider further the use of eukaryotic hosts for product formation in Chapter 15, and the genetic modification of animal and plant cells (or whole animals and plants) in Chapter 17.

6.6.1 *Yeasts*

Microbiologically, ‘yeasts’ are single-celled fungi, as opposed to filamentous fungi, but the term is quite imprecise. Not all ‘yeasts’ are related taxonomically, and indeed some filamentous fungi can also grow in a unicellular form that is referred to as a yeast form. Although in common usage the term ‘yeast’ would be taken to mean the brewer’s/baker’s yeast *Saccharomyces cerevisiae*, even molecular biologists are starting to have to recognize the existence of other yeasts (especially members of the genus *Pichia*, which we will encounter again in Chapter 15). However, for the moment we will limit ourselves to *S. cerevisiae*.

The vectors that will be most familiar, after reading about bacterial cloning vectors, are the yeast episomal plasmids (YEp). These are based (Figure 6.15) on a naturally occurring yeast plasmid known as the 2 μm plasmid, and they are therefore able to replicate independently in yeast, at a high copy number

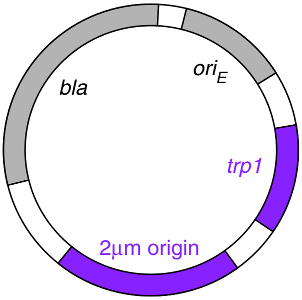


Figure 6.15 Structure of a yeast episomal vector: *bla* = beta-lactamase (ampicillin resistance); *ori* = origin of replication in *E. coli*; *trp1* = selectable marker in *S. cerevisiae* auxotrophs; 2 μm origin = origin of replication in *S. cerevisiae*

(25–100 copies per cell). As is usually the case with vectors for eukaryotic cells, these plasmids also have an *E. coli* origin of replication, enabling them to be grown and manipulated in an *E. coli* host (i.e. they are *shuttle vectors*). There is one point of detail in which they differ from bacterial cloning vectors, and that is the nature of the selectable marker. For bacterial vectors, we can exploit the large number of antibacterial antibiotics (and the correspondingly large number of antibiotic resistance genes) to enable us to select our transformants. There are fewer antibiotics available to which yeasts are sensitive (although some fungicides can be used), and therefore selection more commonly makes use of complementation of auxotrophic mutations in the host strain. For example, a host strain of *S. cerevisiae* with a mutation in the *trp1* gene will be unable to grow on a medium lacking tryptophan. If the vector plasmid carries a functional *trp1* gene, then transformants can be selected on a tryptophan-deficient medium. Other commonly used selectable markers include *ura3* (uracil), *leu2* (leucine), and *his3* (histidine). These vectors would usually also carry an antibiotic resistance marker for selection in *E. coli*.

Vectors that replicate as plasmids in *S. cerevisiae* are often rather unstable, in that they tend to be lost from the culture as plasmid-free daughter cells accumulate. This is due to erratic partitioning during mitosis. Newer versions of YEp vectors, taking advantage of a better understanding of the biology of the 2 μm plasmid, are more stable.

Autonomously replicating plasmids can also be constructed by inserting a specific sequence from a yeast chromosome; this sequence is known, unsurprisingly, as an *autonomously replicating sequence*, or *ars*. These plasmids are very unstable, but some constructs that also include a centromere are more stable (Figure 6.16). In contrast to the YEp vectors, these yeast centromere plasmids (YCp), are normally maintained at a low copy number (1–2 copies per cell), which can be advantageous if your product is in any way harmful to the cell, or if you want to study its regulation.

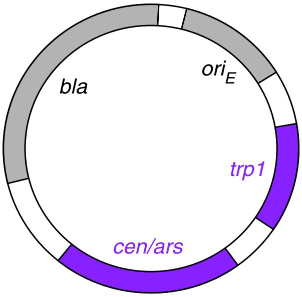


Figure 6.16 Structure of a yeast centromere vector: *bla* = beta-lactamase (ampicillin resistance); *ori* = origin of replication in *E. coli; trp1* = selectable marker in *S. cerevisiae* auxotrophs; *cen/ars* = centromere and autonomously replicating sequence, providing an origin of replication in *S. cerevisiae*

Since one of the purposes of using yeast as a host is to express the cloned gene, these vectors are commonly designed as expression vectors. The principles involved are similar to those described above for bacterial expression vectors, except that of course the expression signals involved are those applicable to *S. cerevisiae* rather than *E. coli*. If required, this can include signals for secretion, or for targeting to the nucleus or other cellular compartments. Note that *S. cerevisiae*, although eukaryotic, has very few introns, and is not the host of choice if you want to ensure correct excision of introns.

The vectors described are maintained in yeast as circular DNA molecules, much like a bacterial plasmid. Two other classes of vectors deserve a mention. First, there are the yeast integrating plasmids (YIp). These do not replicate independently but integrate into the chromosome by recombination (Figure 6.17). The frequency of transformation is very low, and it is difficult to recover the recombinant vector after transformation. The main advantage is that the transformants are much more stable than those obtained with the autonomously replicating plasmids.

Second, there are the yeast artificial chromosomes (YACs), which carry telomeres that enable their maintenance in *S. cerevisiae* as linear structures resembling a chromosome. The use of these vectors, for cloning very large pieces of DNA, is quite distinct from the uses of the vectors described above. YACs are considered further, along with other vectors used for the same purpose, in a later section of this chapter.

6.6.2 Mammalian cells

In bacteria, the cloning vectors used replicate separately from the chromosome, as plasmids or bacteriophages. As we have seen above, the same is true of many

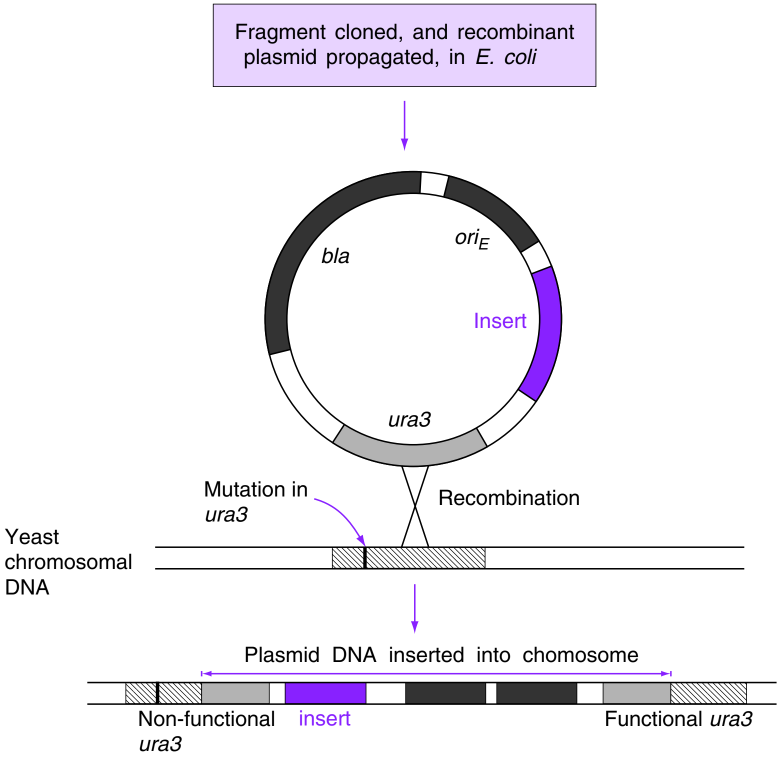


Figure 6.17 Structure and use of a yeast integrative plasmid (YIp)

types of vectors used in yeast. The situation with cloning in mammalian cells is somewhat different in that independent, plasmid-like, replication is often not sustained. Some vectors are capable of plasmid-like replication, especially those carrying the origin of replication from the virus SV40 (simian virus 40), which replicate episomally in some mammalian cells (such as COS cells). More stable clones are obtained by inserting the DNA into the chromosome, which happens readily in mammalian cells. In either case, the cloning vector enables you to organize your cloned gene in relation to a set of expression signals, many of which are derived from viruses such as SV40 or cytomegalovirus (CMV). The general features of such a vector are exemplified in Figure 6.18. A gene inserted at the multiple cloning site (MCS) enables high level constitutive expression from the CMV promoter, while the presence of a polyadenylation signal increases mRNA stability. The SV40 origin allows episomal replication in COS cells, and the neomycin phosphotransferase gene permits selection for resistance to the antibiotic G-418 (Geneticin®). Note that this is a shuttle vector, carrying an *E. coli* origin of replication and an ampicillin resistance gene (β-lactamase), so the construction can be carried out in *E. coli* before

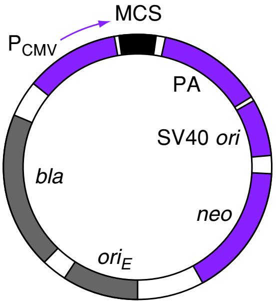


Figure 6.18 Structure of a basic episomal vector for gene expression in mammalian cells: P_{CMV} = CMV promoter – high level constitutive expression in mammalian cells; MCS = multiple cloning site; PA = polyadenylation signal; SV40 *ori* = origin of replication, episomal replication in *cos* cells; *neo* = neomycin phosphotransferase, resistance to G418 for selection in mammalian cells; ori_E = *E. coli* replication origin; *bla* = beta-lactamase (ampicillin resistance) for selection in *E. coli*

transferring the recombinant plasmid to a mammalian cell line. There is a wide variety of commercially available expression vectors for mammalian cells, with more sophisticated features than that shown. We will return to the topic of expression of cloned genes in mammalian cells in Chapter 15.

There are other types of vector available, based on various viruses, which can be used to transmit your cloned gene from one cell to another. Of these, the *retroviral* vectors deserve a special mention, and in order to understand these we need a brief account of retroviral biology. Retroviruses have an RNA genome. When a cell is infected, the RNA is copied into double-stranded DNA by the action of a viral protein, *reverse transcriptase*. This protein is present in the virion and enters the cell along with the RNA. (Reverse transcriptase is formally an RNA-directed DNA polymerase, and we will encounter it again in Chapter 7 where we consider its use in the production of cDNA from mRNA templates.) This DNA then circularizes and is integrated into the host cell DNA by the action of another virion protein known as *integrase*. The efficiency of integration of the DNA into the genome is one of the main attractions of this system for genetic manipulation of animal cells.

The integrated DNA is bounded by sequences known as *long terminal repeats* (LTR) which include a strong promoter for transcription of the integrated viral genes *gag*, *pol* and *env*. Full-length transcripts provide the viral RNA which is assembled into virus particles; one region of the virus, known as the *psi* site, is essential for this process. The packaged virus particles acquire envelope glycoproteins from the host cell membrane as they bud off from the cell, without lysis. These glycoproteins determine the type of receptors the virus uses to infect further cells.

Development of vectors based on retroviruses rests on the knowledge that most of these functions can be provided *in trans*, e.g. by genes from a defective helper virus already integrated into the genome of the host cell. The main features that are *cis*-acting, and therefore need to be located on the vector itself, are the LTR sequences and the *psi* site.

The basic features of the use of such a vector are outlined in Figure 6.19. The vector is a shuttle plasmid, so *E. coli* is used for construction of the recombinant plasmid by inserting the required gene at the multiple cloning site. This construct is then used to transfect a culture of a special cell line (*helper cells*) that contains the *gag*, *pol* and *env* genes required for virus production, integrated into the genome. The transfected cells will therefore be able to produce virus particles containing an RNA copy of your construct. These particles are able to infect other cells that do not contain the integrated essential genes; since

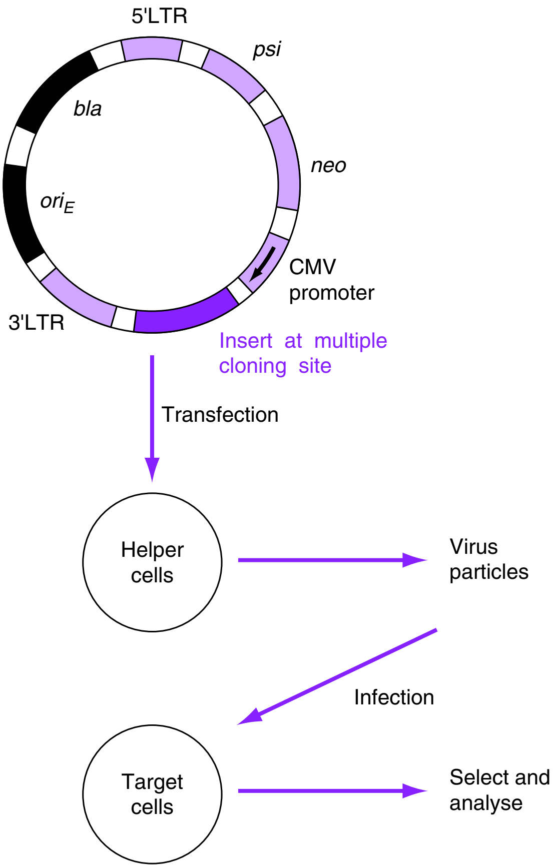


Figure 6.19 Structure and use of a retroviral vector: *bla* = selection in *E. coli*; ori_E = *E. coli* replication; *neo* = selection in infected cells; CMV promoter is for transcription of cloned gene; LTR, *psi* = essential *cis*-acting retroviral sequences (see text)

the viral particles carry preformed reverse transcriptase and integrase, the RNA will be copied into DNA in such cells, and the DNA will be efficiently integrated into the genome. However, since these cells do not carry the essential genes, no further production of viral particles will occur. However, your gene is now stably integrated into the chromosome and can be expressed from the adjacent promoter derived from the vector.

The specificity of the viral particles for other cells will be determined by the envelope gene carried by the helper cells. Replacing that gene by other genes for envelope glycoproteins from other viruses, in particular the VSV-G gene from vesicular stomatitis virus, enables a wider range of target cells to be used, not just mammalian cells but extending to, for example chickens, oysters, toads, zebrafish, mosquitoes – in fact cells from virtually all non-mammalian (and mammalian) species can be infected.

The expression of genes in mammalian cells, and the incorporation of foreign genes into the genome of whole animals (*transgenesis*) is considered further in Chapters 15 and 17 respectively.

6.7 Supervectors: YACs and BACs

Although cosmids were the first vectors that made the production and use of mammalian gene libraries feasible, their limited capacity would still not have sufficed for decoding the human genome. This was made possible by the development of novel supervectors that were able to carry 100 kb or more. The first one of these was the yeast artificial chromosome (YAC). Similarly to cosmids, these could be constructed through knowledge of what features were necessary to enable the vector to be carried by its host (in the case of yeast, telomeres, a centromere, and an origin of replication), as well as selectable markers and cloning sites (Figure 6.20). As with the shuttle plasmids we have referred to earlier, the YAC vector is propagated as a circular plasmid in *E coli*. Restriction enzyme digestion removes the stuffer fragment between the two telomeres, and cuts the remaining vector molecule into two linear arms, each carrying a selectable marker. The insert is then ligated between these arms, as in the case of phage lambda, and transformed into a yeast cell, with selection for complementation of both auxotrophic markers. This ensures that the recombinants contain both arms. Furthermore a successful recombinant must contain the *tel* sequences at each end, so that the yeast transformant can use these sequences to build functional telomeres. The titans amongst vectors, YACs are routinely used to clone 600 kb fragments, and specialized versions are available which can accommodate inserts close to 2 Mb. As such, they will not only easily accommodate any eukaryotic gene in its entirety, but also complete within their framework of three-dimensional structure and distant regulatory sequences. They have therefore been very useful in the production of transgenic organisms (see Chapter 17).

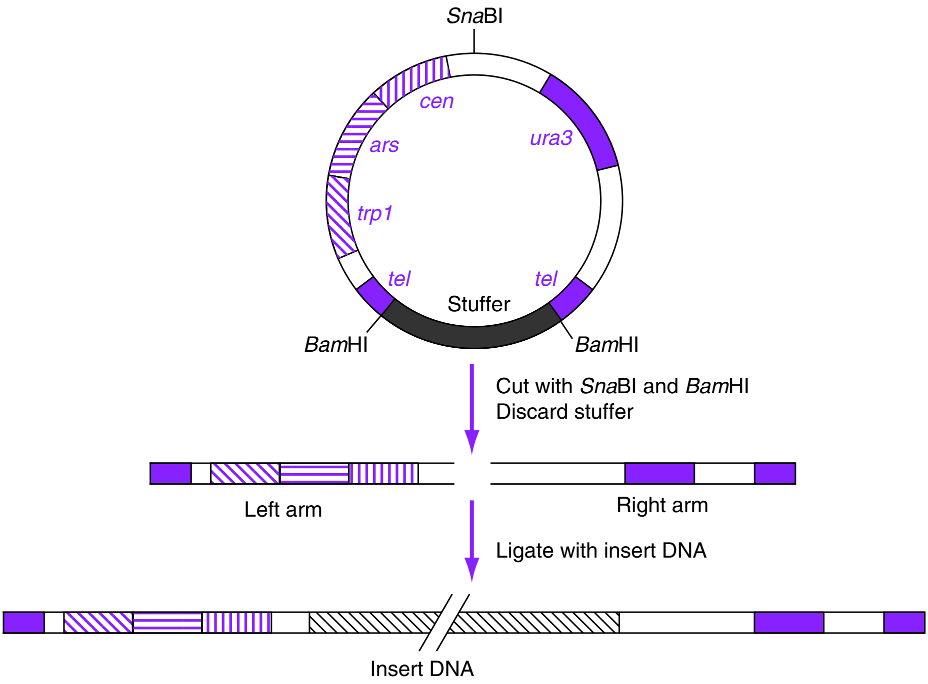


Figure 6.20 Structure and use of a yeast artificial chromosome vector: *trpl, ura3* = selectable markers; *cen/ars* = centromere and autonomously replicating sequence enabling replication in *S. cerevisiae; tel* = telomere; for simplification the *E. coli* origin of replication and selectable markers for *E. coli* are not shown

However, YACs have problems with the stability of the insert, especially with very large fragments which can be subject to rearrangement by recombination. Furthermore, apart from the fact that many laboratories are not set up for the use of yeast vectors, the recombinant molecules are not easy to recover and purify. Thus, larger bacterial vectors are used more than YACs even though their capacity is lower. These include vectors based on bacteriophage P1, which are able to accommodate inserts in excess of 100 kb, and bacterial artificial chromosomes (BACs), which are based on the F plasmid and can accommodate 300 kb of insert. These vectors lack the instability problems found in YACs, and play an important role in genome sequencing projects (see Chapter 10).

6.8 Summary

Earlier in this chapter we considered the advantage that can be gained in constructing gene libraries using vectors with higher cloning capacity such as lambda replacement vectors and cosmids. Even larger fragments can be cloned by employing other vector systems. For relatively small genomes, such as those of bacteria, there is not usually a need for such large inserts. If we simply find out that our gene is somewhere in a 300 kb insert, there is still a lot of work to do to find out exactly where it is. We might as well go straight to a library in a lambda replacement vector; with inserts of 15–20 kb, a library of a few thousand clones will provide adequate coverage of a bacterial genome. Screening a library of this size is quite straightforward. However, libraries of larger fragments are extremely useful for establishing physical maps of the chromosome and as an adjunct to genome sequencing projects (see Chapter 10).

However, for larger genomes, such as those of mammalian cells, a reasonably complete gene library in a lambda replacement vector would require hundreds of thousands of clones, and screening then becomes laborious. In these cases, the use of larger inserts is advantageous, and the use of additional systems such as yeast artificial chromosomes (YAC vectors) becomes appropriate.

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7

Genomic and cDNA Libraries

Gene libraries play a central role in gene cloning strategies, as will be apparent from the frequent references to them in previous chapters. Why are they so important? The answer to this question goes back to the basic concept of gene cloning, as outlined in Chapter 3. If you are trying to isolate a specific gene (or more generally a specific fragment of the genome), you will break the genome up into small enough pieces to be cloned using your chosen vector, and then screen that collection of clones to identify the one that contains the bit of DNA that you are looking for. (We will consider the ways of doing that in the next chapter.) You only want that one clone, so you could throw all the rest away; but then, if you want to clone another piece of DNA from the same source, you would have to go through the whole process again. So instead of discarding all the clones you do not want, you can store them away somewhere, pooled together, so that next time you can just screen the same collection to identify the clones that carry this other gene. You have made a gene library – a resource that can be used to retrieve any of the genes from your starting material. Even if you do not intend to keep it and re-screen it, you still call it a gene library.

A gene library in these terms is therefore a collection of clones which between them represent the entire genome of an organism. More specifically, we should refer to such a library as a *genomic* library, to distinguish it from a different sort of gene library which is constructed from DNA copies of the mRNA present in the originating cells at the time of isolation. These DNA copies of mRNA are referred to as *copy* or *complementary DNA* (*cDNA*), and hence such a library is referred to as a *cDNA library*. In this chapter we will look at the construction of these two types of libraries; ways of screening them are considered in Chapter 8.

7.1 Genomic Libraries

The first step in producing a genomic library is to fragment the genomic DNA into pieces of a suitable size for cloning in an appropriate vector. It might be considered that the simplest way to do this is to digest the DNA to completion with a restriction endonuclease such as *Eco*RI, but therein lies a problem. In Chapter 5, we saw that the average fragment size generated by *Eco*RI is about

4 kb (given certain assumptions about DNA composition), but this is only an average. Even if restriction sites are randomly distributed we would expect some fragments to be very much bigger, and some would be very small. Ligation tends to work best with smaller fragments, so these would be over-represented in the library, while some of the largest fragments may be too big to be cloned at all. The problem will be worse if restriction sites are not randomly distributed. In that case, we can end up with large regions of the genome having no sites at all for that enzyme.

That is only part of the problem. Identifying a clone carrying a specific DNA fragment is often not the end of the story. For various reasons, notably for genome sequencing, we are likely to want to be able to isolate the adjacent DNA as well. This would enable us to piece together all the small bits of DNA represented by individual clones so as to build up a bigger picture. If we make a library of, say, *Eco*R1 fragments, then we have no way of knowing how they fit together. There is no information in the library that connects one clone with another. To provide that information, we need a library of overlapping fragments. Figure 7.1 shows how these overlapping fragments enable the identification of clones on either side of the one that we originally selected. These clones could then be used to identify further overlapping clones, and so we can move along the chromosome in either direction. This is the basis of a technique known as *chromosome walking*, which we will come back to in Chapter 14.

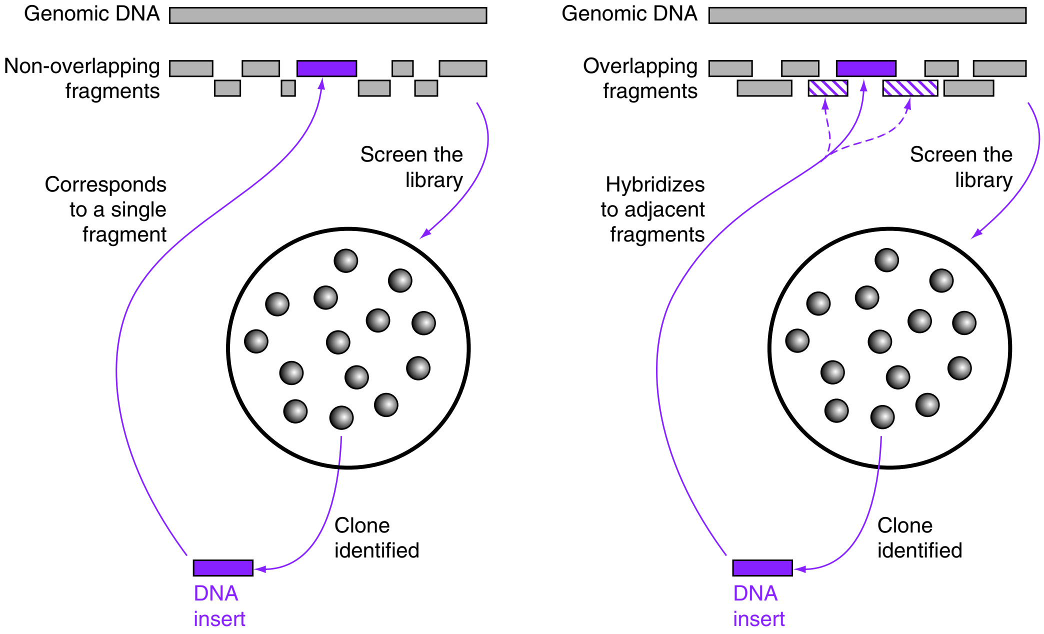

Figure 7.1 Genomic libraries: overlapping and non-overlapping fragments

7.1.1 Partial digests

One way to construct a library of overlapping fragments is to use partial digestion. This means using conditions, such as short digestion times, that result in only a small proportion of the available sites being cut. A similar effect can be obtained by using very small amounts of enzyme, or by incubating the digest at a reduced temperature. The digested material is then fractionated by electrophoresis to obtain fragments of the required size range before cloning in an appropriate vector. If there is an equal probability of cutting at any site, the result will be a series of overlapping fragments (Figure 7.2), which would overcome the difficulties referred to above. If we do this with an enzyme, such as *Eco*RI, that recognizes a six base sequence, the average size of fragments in a partial digest will be too large for cloning in typical plasmid or lambda vectors, but vectors that can accommodate large inserts (such as BAC or YAC vectors, see Chapter 6) can be used for generating a genomic library of such fragments from a large genome. However, such libraries are not often produced or grown by individual investigators; they are more likely to be held in centralized facilities from which individual clones (identified by database searches or array screening) can be obtained. For smaller genomes, such as those of bacteria, we would tend to use partial digests with an enzyme that cuts more frequently, e.g. one such as *Sau*3A that has a four base recognition site (a 'four base cutter') , and clone the products using a lambda or cosmid vector.

A four base cutter such as *Sau*3A will produce fragments of 256 bp on average (assuming an even distribution of sites) and the fragments suitable for a lambda replacement vector would be 15–20 kb in length, implying that we

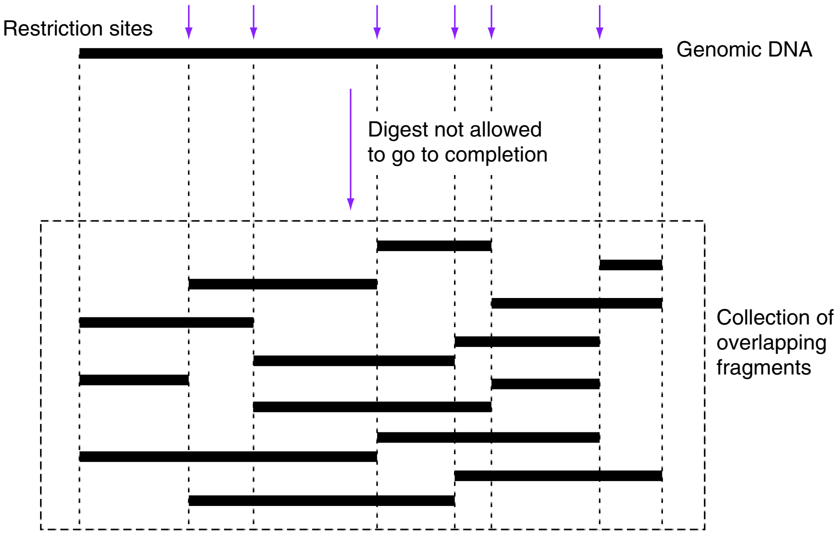

Figure 7.2 Using a partial digest to produce a collection of overlapping fragments

are cutting only a few percent of available sites. The reason for requiring such a low degree of digestion is not only to obtain overlapping fragments, but also because the distribution of restriction sites is not even. In some places there will be a number of sites very close together, and if we digest too much we will get only very small fragments from this region. These will be eliminated by size fractionation (or will not be suitable for cloning in the chosen vector), resulting in lack of representation of that region in the library. Thus, in order to minimize this problem, it is important to ensure that you get your partial digestion just right. The converse situation – lack of restriction sites in certain regions – is less likely to be an overwhelming problem in this case. There are unlikely to be any regions where neighbouring *Sau*3A sites are more than 20 kb apart, so there should be *some Sau*3A fragments in the appropriate size range (although the representation of different parts of the genome may be far from equal).

There is a further potential problem. We made the assumption that, in a partial digest, there is an equal probability of the enzyme cutting at any site, but this is often not true. In a partial digest, some sites may be cut more efficiently than others. The nature of the adjacent sequences, and the formation of secondary structures in the DNA, may cause some sites to be cut more rapidly than others. If this occurs, then our library of partial digest fragments will not be completely overlapping. When we come to try to fit the clones together we will find discontinuities in the map so that we will be unable to identify the adjacent gene (or the clone carrying it). One way of overcoming this is to use separate partial digests with different enzymes.

Even with a library made in this way, we have not completely guaranteed that all our problems are solved. There is still the possibility that some regions of the genome will be over-represented and other regions will occur less frequently in the library. The only way of avoiding this, and ensuring that all parts of the chromosome are equally represented, is to abandon the strategy of using restriction fragments, and instead to use a truly random way of fragmenting the genome. This is most commonly done by mechanical shearing, for example by passing the solution rapidly and repeatedly through a small syringe needle. This is an effective way of generating relatively large fragments, and has the advantage of requiring no special equipment, but the vulnerability of the DNA to shearing diminishes as the average fragment size decreases. A more elegant strategy is to use ultrasonication. You can monitor progress by electrophoresis of samples, and recover fragments in the selected size range by preparative electrophoresis.

Although the complete randomness of mechanical shearing makes it more attractive in principle than partial digests, most workers continue to use restriction digests. Not only is it easier to control the extent of degradation, but also the restriction digests can be directly ligated with the vector. In contrast, the fragments generated by mechanical shearing have either blunt or ‘ragged’ ends – that is they may contain variable lengths of single-stranded regions at the $5'$ or $3'$ ends. These are not suitable for ligation, and have to be converted to blunt ends by filling in or trimming back the ends (see Chapter 5). Then, since blunt-end ligation is a relatively inefficient process, the addition of linkers or adaptors (see Chapter 5) might be necessary in order to generate enough clones to constitute a representative library. However, mechanical shearing does have a role to play in generating the sets of overlapping fragments that are needed for *shotgun sequencing* (see Chapter 10).

7.1.2 Choice of vectors

Of the various types of vectors described in Chapter 6, which should we choose for constructing our genomic library? The principal factor influencing this choice is the size of the insert that these vectors can accommodate, since this will affect the size of the library that is necessary to obtain a reasonably complete representation of the entire genome. Obviously, the required size of the library will also be affected by the total size of the genome of the target organism. For any given library, there is a range of options, and the secondary consideration becomes the ease of making, growing, and screening the library with the resources available to you.

Superficially, you might think that if we start with a genome of 4 Mb (4×10^6 bases – a bacterial genome is of this order of magnitude) and produce a library of fragments which are 4 kb (4×10^3 bases) then you should be able to cover the entire genome with 1000 clones, since $(4 \times 10^6)/(4 \times 10^3) = 10^3$. However, this would only be possible if (a) all the clones are different, and (b) the clones are non-overlapping; but we are considering a library of random fragments, so the first condition is not met, and, as discussed above, we want the library to contain overlapping fragments, so the second condition does not hold either.

In a random collection of clones, the more clones we look at, the more likely it is that some of them will be completely or partially identical. In other words, the larger and more complete the library is, the more redundancy there will be. As we increase the size of the library it becomes less and less likely that each additional clone adds any new information; ultimately, it is not possible to produce a library that is *guaranteed* to carry all of the genetic information from the original genome, so we have to use probabilities. We can say there is a 90 per cent probability ($P = 0.9$) of having the gene that we want, or a 99 per cent probability ($P = 0.99$). The level at which we set this probability will affect the required size of the library.

More specifically, the number of independent clones needed can be calculated from the formula

$$N = \frac{\ln(1 - P)}{\ln(1 - f)}$$

where $N =$ required number of clones, $P =$ the probability of the library containing the desired piece of DNA, and $f =$ the fraction of the genome represented by an average clone, which is calculated by dividing the average insert size by the total genome size.

Note that this calculation refers to the number of *independent* clones, i.e. the number of bacteria that were transformed originally (or the number of phage particles arising from a packaging reaction). This is usually determined from the number of bacterial colonies or phage plaques produced. Once you have plated out the library, and resuspended the colonies or plaques, you have *amplified* the library, and each clone is represented by thousands of individual bacteria or phage in the tube containing your library. You cannot increase the size (or complexity) of the library by plating out larger volumes. If your original library contains 1000 clones, plating it out to produce 10 000 plaques will simply mean each clone is present (on average) 10 times. You are *not* screening 10 000 clones.

Box 7.1 shows that with the example of a bacterial genome of four million bases and a plasmid vector carrying inserts with an average size of 4 kb we will need a library of nearly 5000 clones to have a 99 per cent chance of recovering any specific sequence. (This assumes that all pieces of DNA are equally likely to turn up in the library, which is not entirely true; some fragments may be lethal, or may be difficult to clone for other reasons.)

Screening a library of 5000 plasmid clones is possible, but we can shorten the procedure by using vectors with a greater capacity. We can obtain a representative bacterial genomic library with about a thousand clones, using a lambda replacement vector. If we use a cosmid vector, the required size of the library is smaller – and we can reduce it even more by using vectors with a higher cloning capacity, but there is a trade-off. One of the main purposes of producing a gene library is to be able to identify clones carrying a specific gene (or more generally a specific fragment of DNA, which could include regulatory sequences or other features), so that we can isolate and characterize that gene. The larger the insert size, the more work we have to do subsequently to find out which bit of that insert carries the gene we are interested in. (This can be illustrated by extending the argument to the absurd limit: the smallest gene library would be represented by a single clone carrying the entire genome, which would get us no nearer to identifying the gene that we want!) Therefore, for bacterial genomic libraries, lambda replacement vectors are usually the best compromise (although other vectors such as cosmids are sometimes used).

Note, however, that genomic libraries can be used for other purposes as well, especially for mapping and sequencing genomes. For these purposes larger fragments, which are capable of bridging any gaps that might be present, can

Box 7.1 Estimates of the required size of genomic libraries

Organism	Genome size	Vector type	Insert size	*P*	Library size
Bacterium	4×10^6 bases	plasmid	4 kb	0.99	4.6×10^3
		lambda replacement	18 kb	0.99	1.0×10^3
		cosmid	40 kb	0.99	458
		BAC	300 kb	0.99	59
Mammal	3×10^9 bases	plasmid	4 kb	0.99	3.5×10^6
		lambda replacement	18 kb	0.99	7.7×10^5
		cosmid	40 kb	0.99	3.5×10^5
		BAC	300 kb	0.99	4.6×10^4

The values shown for the genome sizes of bacteria and mammals are examples for the purpose of this calculation. The actual genome sizes vary quite widely from one organism to another. The insert sizes for specific vectors will also vary.

be invaluable. Vectors with a larger cloning capacity, such as cosmids and BACs, are often used for this purpose (see Chapter 10).

With larger genomes, such as those of mammals, the situation is rather different. As can be seen from Box 7.1, a library created in a lambda replacement vector would have to consist of nearly a million clones to be reasonably representative and thus would be more laborious to screen. On the plus side, a lambda library is reasonably easy to construct in a small laboratory and without special knowledge or equipment, and a million clones can be screened on 20 large agar plates. However, wherever feasible, the use of vectors with larger cloning capacity can reduce the required size of the library to more manageable proportions.

A further factor that operates in favour of the use of larger inserts with mammalian (and other eukaryotic) genomes is that genes commonly contain introns. The overall size of the gene, as present in the genome, may therefore be too large to be contained within even a lambda replacement vector. (Although some bacterial genes do carry introns, they are relatively uncommon.) Therefore, if we need to obtain a clone carrying the entire gene, we will have to use a vector which can accommodate a large enough DNA fragment.

In the discussion so far, we have implicitly assumed that the library will be screened by replicating the clones to a filter, followed by hybridization with a labelled nucleic acid probe (see Chapter 8). Lambda vectors have a technical advantage over the other vectors in this process in that it is easier to screen phage plaques at high density than bacterial colonies. Not only do bacterial colonies tend to grow into one another if plated at high density, but also they tend to smudge when blotted, which can make it difficult to identify the required colony.

7.1.3 Construction and evaluation of a genomic library

The basis of the construction of a genomic library has been covered partly in earlier chapters and partly by this chapter so far. By way of a recap, the genomic DNA is fragmented, as randomly as possible, into suitable-sized pieces for insertion into your chosen vector. The vector is prepared by digestion with the appropriate enzyme and (for a lambda replacement vector) removing the stuffer fragment. It is often more convenient to buy a ready-prepared vector. The vector is then ligated with the complete mixture of genomic fragments. If you have chosen a lambda vector, or a cosmid, you will need to mix your ligation products with packaging extracts for the assembly of infectious phage particles (see Chapter 6). If you are using a plasmid vector, you will introduce the mixture of ligated DNA into a bacterial cell by transformation or electroporation. The library will then be obtained as bacterial colonies (if using plasmid or cosmid vectors, or BACs) or phage plaques on a bacterial lawn (with lambda vectors). A library in a yeast artificial chromosome vector (YAC) can be similarly electroporated into yeast cells.

For storage of the library, you would make a pooled suspension of the bacterial colonies, or of the phage harvested from the plate(s). Note that this library has already undergone an amplification. The original unamplified library is represented by the initial transformants, when each bacterial cell or phage particle is a unique product from a single molecular ligation event. Once you have plated them out to obtain colonies or plaques, each clone is represented many thousands of times in the library.

You then want to know how good your library is. First, you count the number of colonies or plaques (at an appropriate dilution so that you get countable colonies/plaques); if you do this using the original plates, it tells you the size (or complexity) of your library. If you determine the titre (the number of colonies or phage plaques) of the resuspended mixture, you will get a falsely elevated estimate of the size of the library. The amplification of gene libraries (Figure 7.3) is illustrated with a plasmid vector. The concept with a phage vector is the same, except that plaques are produced and the amplified

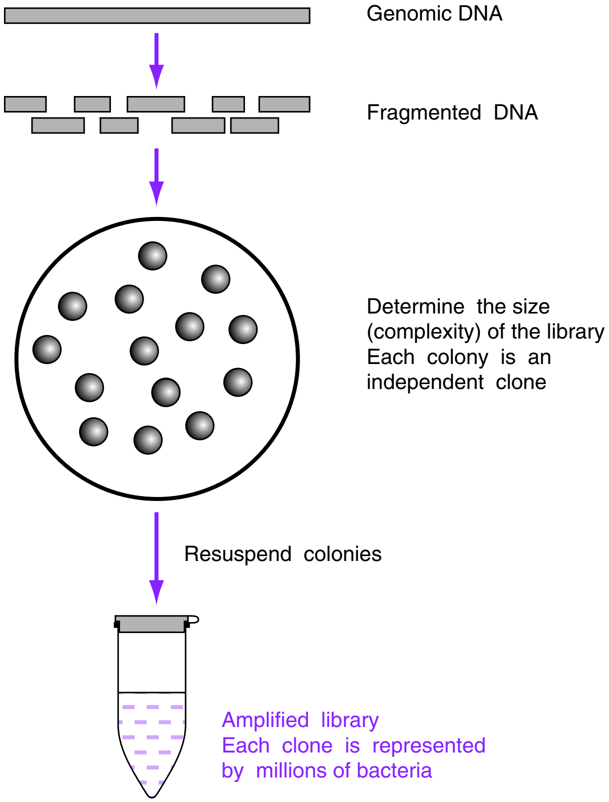

Figure 7.3 Amplification of gene libraries

library will consist of phage particles. The library would consist of thousands or millions of clones, rather than the few shown.

Second, you will want to determine the quality of the library, i.e. what proportion of the clones actually contain an insert, and how large are those inserts? Both of these questions can be answered by picking a number of clones, growing them up individually, extracting the plasmid or phage DNA and subjecting it to restriction digestion followed by agarose gel electrophoresis. The details will vary according to the vector and cloning strategy, but at the simplest level (insertion of restriction fragments into a plasmid) you will see that each clone has one band of constant size corresponding to the vector, and bands of various sizes which are your insert fragments (Figure 7.4). By determining the size of these fragments, you can estimate the average insert size in your library (assuming you have picked a representative sample). Those without an insert band (see track 5) are probably religated vector, so you can estimate what proportion of clones do not have an insert at all. Clearly

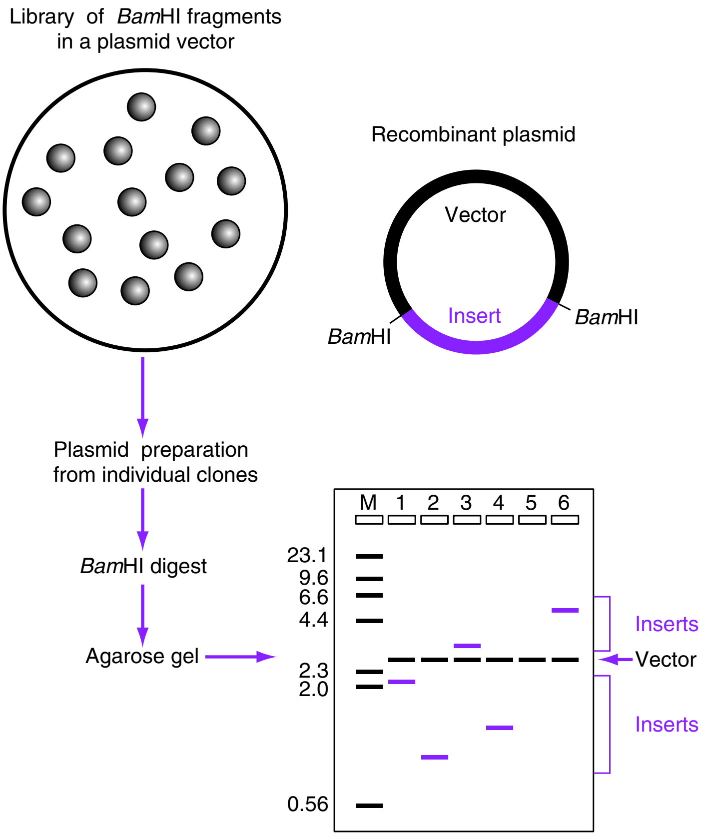

Figure 7.4 Assessing the quality of a gene library

you do not want too many of these, as it will reduce the efficiency of your screening.

In Chapter 6, we described how some vectors, such as lambda gt11 or plasmid vectors of the pUC family (such as pUC18), provide you with a more direct estimate of the proportion of clones lacking an insert. With these examples, the non-recombinant vectors (without an insert) will produce blue plaques/colonies on an X-gal/IPTG containing plate (due to the β-galactosidase gene in the vector). If you have successfully introduced an insert fragment into the vector, the insert will (usually) disrupt the β-galactosidase gene, giving ‘white’ clones. Any religated or intact vector will give blue plaques or colonies. Lambda replacement vectors should not produce any clones simply lacking an insert (as they would be too small to be packaged) but intact vector may persist, or the stuffer fragment may not have been completely removed. Many replacement vectors contain additional markers to enable further differentiation or selection of recombinant clones. Cosmids also provide positive selection for the presence of inserts, again because the religated empty vector is too small for the packaging reaction.

Ideally, each clone should contain a single insert fragment. Multiple inserts can cause problems later on, as they will provide misleading information about the relationship between different parts of the genome. With those vectors that provide positive selection for inserts (lambda replacement vectors and cosmids) it is possible to dephosphorylate the insert (using alkaline phosphatase) – rather than the vector – thus virtually eliminating the possibility of multiple inserts. If you do this with other vectors, you run the risk of obtaining an unacceptably high frequency of vector religation. It may be worth running this risk, especially if you are using a vector such as pUC18 or lambda gt11 when you can tell straight away what your insertion frequency is. If you get too many blue colonies/plaques, then you throw the library away and try again.

7.2 Growing and Storing Libraries

Once a library has been made, it represents a potentially useful resource for subsequent experiments, as well as for the initial purpose for which it was produced. You will therefore want to store it safely for future use. A random library will consist of a tube containing a suspension of pooled colonies from a plate (if you used a plasmid or cosmid vector), or pooled bacteriophage particles (for a phage vector). This would normally be kept at −80°C; bacterial cells in a plasmid library are protected from the adverse effects of freezing by glycerol. Phage libraries, by contrast, are kept as cell-free virus particles, cryoprotected by dimethyl sulphoxide (DMSO). When the library is to be screened, a small lump of the frozen stock is removed and thawed, leaving the rest of the aliquot frozen. Plasmid libraries in cells are simply spread out on agar plates containing the appropriate antibiotic, which ensures that the cells do not shed their plasmids. Phage libraries, on the other hand, first need to be mixed with prepared bacterial cells before being plated out.

If your library is a valuable one, or if you are likely to be using it repeatedly, it is preferable to divide it into aliquots before freezing it, so that each aliquot is only thawed once. This avoids the loss of clones that will accompany repeated freezing and thawing. The same principle applies to gridded and ordered libraries (see below), but even more strongly. The ideal arrangement is to have one master copy of the library which is kept for emergencies, and several sub-master copies, also stored at −80°C, which will each be used only a limited number of times to produce a working copy as and when required.

The first task is to determine the titre of the library, which almost inevitably will have dropped since the original stock was frozen. A dilution series is produced and each dilution is spread out on an agar plate and grown at 37°C overnight. This allows the investigator to calculate the titre of the library, and to determine how much is to be used for each plate in the actual screening. The number of clones required for the screen can be calculated using the formula described above. In a genomic library every gene is (ideally) represented equally often. Thus, the number of clones that need to be screened is dependent on (a) the size of the fragments in the library, and (b) the size of the genome.

In the next section, we describe the construction of a different sort of library, using cDNA produced by reverse transcription of mRNA. To determine the number of clones in a cDNA library that you need to screen, you have to take account of the abundance of the relevant mRNA. If you are searching for a cDNA clone for a reasonably abundant mRNA, you might have to screen 20 000 clones (10 plates), while for a very rare transcript, you might need 200 000 clones (100 plates).

7.3 cDNA Libraries

A genomic library represents all the DNA in the genome, whether it is expressed or not. However, very often it is really the genes that are being expressed that are our main target. This is likely to be quite a small proportion of the total DNA especially in; for example, mammalian cells. So if we base our library on the mRNA extracted from the target cells, rather than on their DNA, we will be able to focus our library more closely on the real target, and make the identification of the required clones much more efficient.

The advantages of cDNA libraries, and the contrast with genomic libraries, extend further than that. First of all, since the introns are removed by processing the mRNA, the cDNA clones will reflect only the coding regions of the gene (exons) rather than the much longer sequence contained in the genome. This is especially relevant if we want to try to express the gene in a bacterial host, which will be unable to splice the mRNA.

Secondly, in any organism, some or most of the DNA does not appear to code for anything, or to have any other identifiable purpose. This is often referred to as *junk DNA* – but we need to be careful in this interpretation, as some of these sequences may have a function that we have not yet ascertained. Some of this apparent junk represents integrated viral DNA (including bacteriophages in bacterial genomes), or the remains of integrated viruses that have lost the ability to produce functional viral particles. Genomes also commonly contain sequences which are in effect DNA parasites (such as insertion sequences and transposons), as well as other repetitive elements, which do not necessarily contribute anything to the overall properties of the cell. There are also *pseudogenes*, which appear to be non-functional copies of real genes, as they contain a number of changes in the sequence, such as stop codons, which preclude their expression. Although pseudogenes are (presumably) redundant gene copies so that loss of function can be tolerated, and might therefore be labelled as 'junk DNA', they nevertheless represent an evolutionary resource and may evolve later on into new genes with novel functions.

The amount of apparent junk in the genome is to some extent related to genome size. Bacterial genomes carry mobile elements and phages, and some other repetitive elements, but in most cases relatively few obvious pseudogenes (although there are many potential coding sequences to which we cannot ascribe any function). With larger genomes, such as those of mammals, the proportion of junk DNA is much higher.

A library based on mRNA, rather than a genomic library, will reflect only those genes that are actually expressed in a particular cell or tissue sample at a particular time. Hence we will eliminate all the real junk. However, it goes further than that. A cell will use only a part of its genetic capability at any one time. A bacterial cell will switch genes on or off, depending on its environment and its stage of growth; and in a multicellular organism, differentiation of cells into tissues and organs will be reflected in more or less permanent changes in the nature of the genes that are expressed. Some genes will be active only during specific developmental stages; others will be active during specific times of the day. You may also choose to make a library from a cancerous sample, or from an individual who suffers from a genetic disease. A library of this sort will reflect the nature of the cells from which the mRNA was obtained. As we will see later on, this not only reduces very substantially the number of clones needed for a representative library, but it also provides us with a variety of ways in which we can focus attention on the differences between various cells or tissues, and thus identify genes that are selectively expressed in different environments or in different tissues.

However, we cannot directly clone the mRNA. We have to produce a complementary DNA (cDNA) copy; hence the designation of such a library as a *cDNA library*. The synthesis of the cDNA is carried out using an enzyme known as *reverse transcriptase*. (Since transcription refers to the production of RNA from a DNA template, the opposite process – RNA-directed DNA synthesis – is known as reverse transcription.) Although this is not a normal process in most cells, some types of viruses, such as leukaemia viruses and HIV, replicate in this fashion; the viral particle contains RNA which is copied into DNA after infection, using a virus-encoded enzyme. Some cellular DNA polymerases also have reverse transcription capability.

7.3.1 Isolation of mRNA

Most of the RNA in a cell is not messenger RNA. The initial RNA preparation will contain substantial amounts of ribosomal RNA and transfer RNA. For effective production of cDNA for construction of a library it is highly desirable

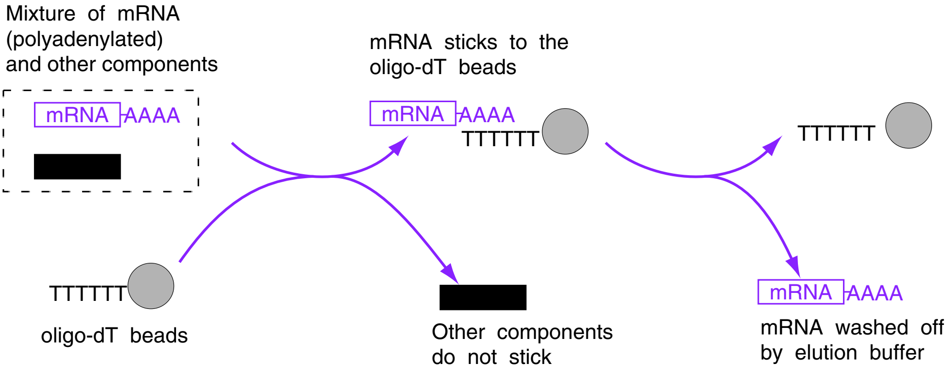

Figure 7.5 Principle of oligo-dT purification of mRNA

to purify the mRNA. This can be done with eukaryotic cells, where we can take advantage of the fact that mRNA carries a tail at the 3′ end – a string of A residues that is added post-transcriptionally. The polyadenylated mRNA will anneal to synthetic oligo(dT) sequences (i.e. short polymers of deoxythymidine, or in other words short stretches of synthetic DNA containing just T residues). Other RNA species, and non-RNA components will not anneal and can be washed off (Figure 7.5). Although some mRNA in bacteria does have polyA tails, these are much shorter and only a small proportion of mRNA is polyadenylated. Therefore, this does not provide a reliable way of isolating bacterial mRNA. The question of the production of bacterial cDNA is discussed below.

When the RNA preparation is passed through a column of a polymer coated with synthetic oligo(dT) fragments, the polyA tail will anneal to the oligo(dT) residues and will be retained on the column while other RNA species will pass through. This is in effect a hybridization process, and as such the hybrids can be made unstable by lowering the salt concentration and raising the temperature, enabling the elution of purified mRNA from the column (Figure 7.6). This will consist of a complex mixture of all the mRNA species present in the cell at the time of extraction. The relative amounts of the different transcripts will vary substantially, which has major implications for the ease of obtaining certain cDNA clones. This is a further clear distinction from a genomic library. Although the following description is presented in terms of a single mRNA, bear in mind that we would in reality be dealing with a complex mixture.

7.3.2 cDNA synthesis

The presence of the polyA tail is also used to advantage in the reverse transcription step (Figure 7.7). Reverse transcriptase, like DNA-directed DNA polymerase, requires a primer for initiation. An oligo(dT) primer will anneal to

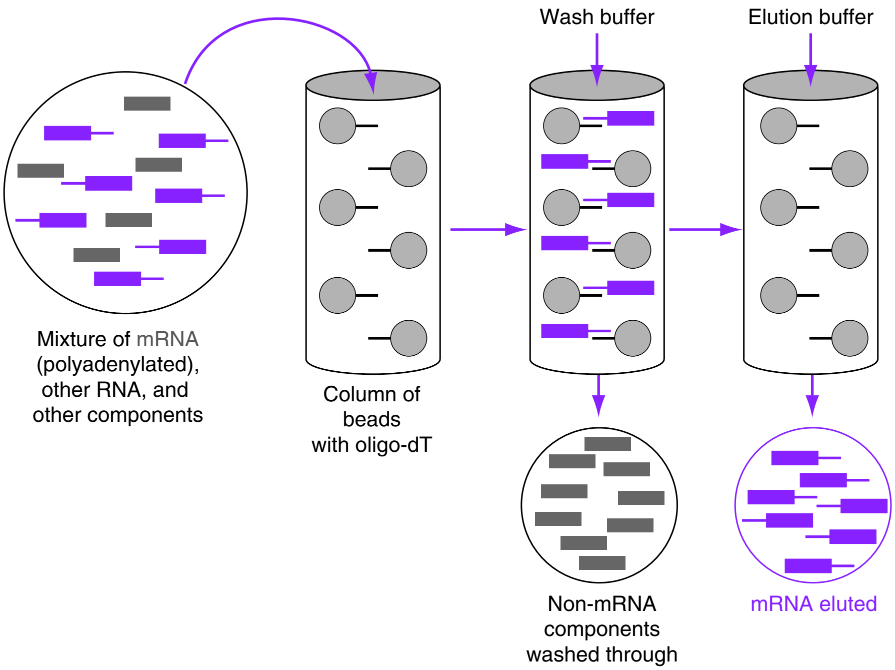

Figure 7.6 Purification of mRNA through oligo-dT column

the polyA tail; reverse transcriptase will then extend this primer, using the mRNA as the template, and will produce a single-stranded cDNA copy.

We now have a double-stranded molecule that is a hybrid between DNA and RNA. In order to obtain a molecule that is stable and can be cloned into a vector, we need to replace the RNA with a DNA strand of the same sequence. First, the RNA is partially degraded by a specific RNAse called RNAse A, which is able to attack double-stranded molecules. This leaves the cDNA strand largely in single-stranded form. Single-stranded nucleic acid molecules tend to form secondary structures, looping back on themselves, because of the hydrophobicity of the bases. The single-stranded cDNA will therefore tend to form a hairpin loop at the 3′ end. This hairpin loop, and the partial remains of the RNA strand, are used by DNA polymerase I as primers for *second strand synthesis*. The product is a double-stranded DNA molecule, with a hairpin loop at one end. That loop is then removed by treatment with S1 nuclease (which will cut single-stranded DNA, including exposed loops). Further treatment with DNA polymerase will ensure that the molecule is fully blunt-ended.

For cloning the cDNA, adapters (see Chapter 5) are added, by blunt-end ligation, to make the cDNA molecules compatible with the chosen vector (Figure 7.8). After size-fractionation, eliminating excess adapters and small, abortive cDNA fragments, the library is inserted into the vector in a second

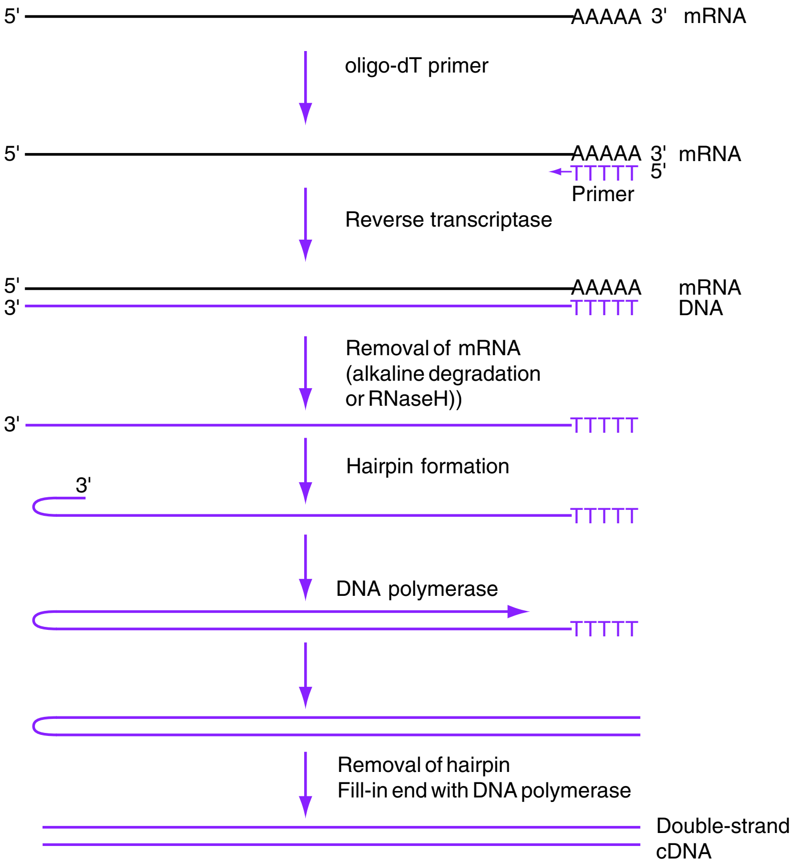

Figure 7.7 Synthesis of cDNA from mRNA

ligation. Because transcripts are not usually longer than a few kb, larger vectors are not considered for cDNA libraries – the choice is essentially between a plasmid vector, or a phage lambda insertion vector such as gt10 or gt11. (The relative advantages and disadvantages of these vectors were discussed in Chapter 6.)

One limitation of the basic procedure described above is that you may not get full-length cDNA. Constraints such as elements of secondary structure in the mRNA may interfere with reverse transcription, so that the enzyme rarely, if ever, reaches the end of the mRNA. As a result, the regions at the 5′ end of the mRNA may be under-represented in the cDNA library. This can be partially addressed by using random primers rather than oligo-dT primers. These random primers will initiate first strand cDNA synthesis at intermediate points, and hence the enzyme will be more likely to reach the 5′ end of the mRNA (see Figure 7.9). Obviously, you will not then get any clones containing

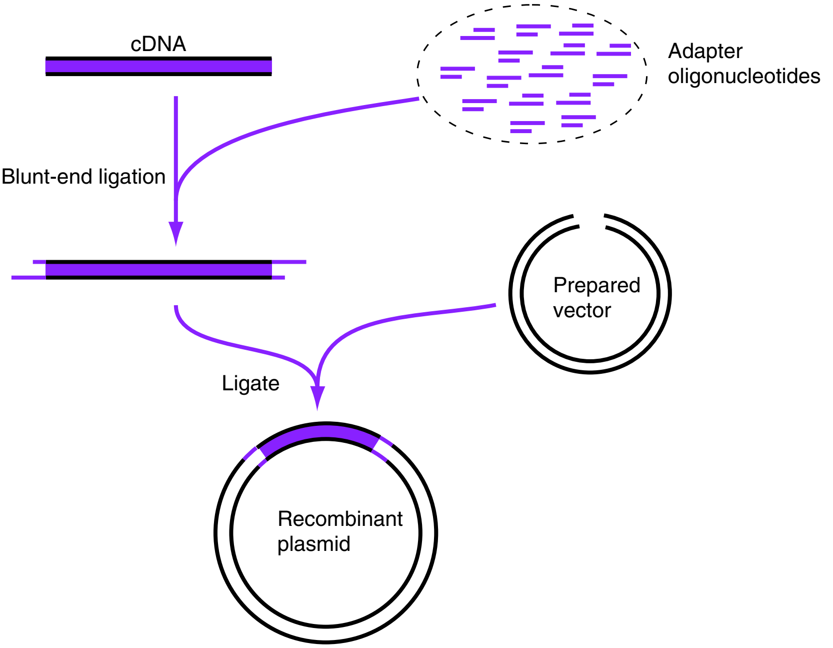

Figure 7.8 Cloning cDNA

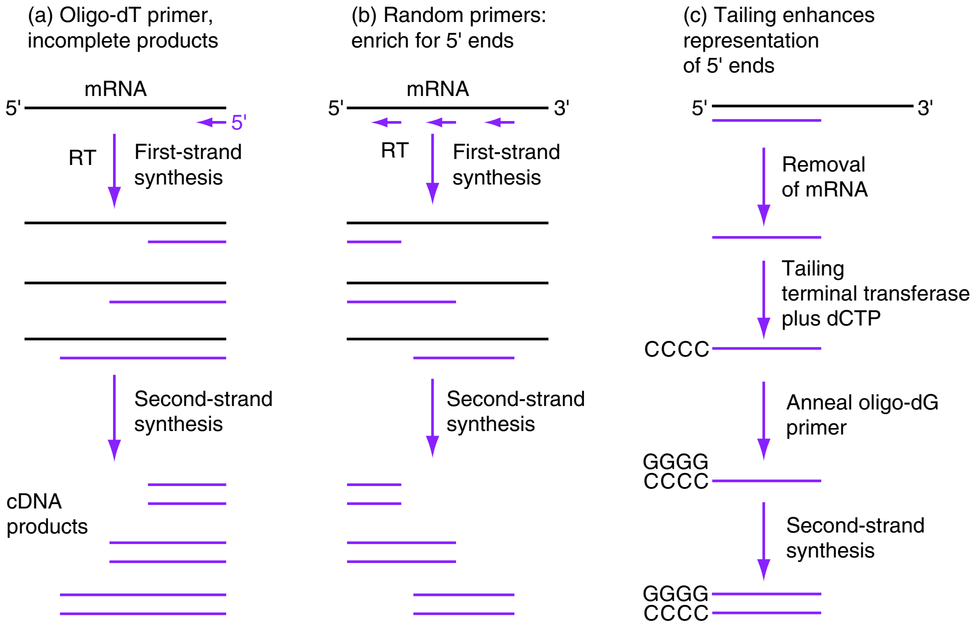

Figure 7.9 cDNA synthesis: enhancing representation of 5′ mRNA ends

full-length cDNA, but these clones containing the 5′ end can be compared to other clones carrying the 3′ portion, making it possible to devise strategies for obtaining full-length molecules. The use of random primers rather than oligo-dT primers also overcomes the problem that some RNA molecules (e.g. bacterial mRNA and genomic RNA from viruses) are not polyadenylated.

The requirement for hairpin formation to prime second strand synthesis can also reduce the representation of the 5′ ends. One strategy to counter this is to use terminal transferase to add a tail to the 3′ end of the first cDNA strand (see Figure 7.9). As described in Chapter 5, terminal transferase, if provided with, say, dCTP, will add a string of C residues to the 3′ ends of DNA molecules. This enables the use of an oligo-dG primer to initiate second strand synthesis, without requiring hairpin formation.

Many variations of these strategies have been devised to obtain full-length cDNA. The most powerful strategy, known as *rapid amplification of cDNA ends* (*RACE*), exploits the amplification power of the polymerase chain reaction; this is discussed in Chapter 9.

7.3.3 Bacterial cDNA

The arguments in favour of cDNA, rather than genomic, libraries carry much less force with bacterial targets. The smaller size of bacterial genomes, and the (virtual) absence of introns, means that a genomic library is usually quite adequate – and a lot easier to construct. There are additional technical difficulties in producing cDNA with bacteria. Not only is the mRNA not consistently polyadenylated, but it is also remarkably unstable – many bacterial mRNA species have a half-life (*in vivo*) of only a minute or two. Furthermore, the organization of bacterial genes into polycistronic operons (groups of genes that are transcribed into a single long mRNA) means that a bacterial mRNA can be as much as 10–20 kb in length. Not only is it difficult to isolate this mRNA intact, but it would be very difficult to produce a full-length cDNA copy from it.

As a consequence, bacterial cDNA libraries are rarely produced. However, for some purposes, such as the analysis of gene expression (see Chapter 13), cloning of bacterial cDNA can play an important role, for example in identifying those transcripts that are relatively abundant in the bacterial cells under selected conditions.

7.4 Random, Arrayed and Ordered Libraries

So far we have envisaged a gene library as a single tube containing a mixture of a large number of clones. When you want to screen the library, you plate it out to give plates with a large number of bacterial colonies or phage plaques. For many purposes, this is perfectly adequate. However, there are circumstances when you do not want to treat the library as a random collection of a large number of clones. For example, if you have a very complex screening procedure, you may be able to test only a portion of the library at a time. This might mean taking a small aliquot of the library and testing say 100 clones. If you then take another aliquot, and again test 100 clones, then some of those clones may be the same ones that you have already tested. (Remember that your library has been amplified. so each independent clone is present in many copies.)

One way around this is to produce an *arrayed* or *gridded* library. If we go back to the original transformation step (or infection with the packaged phage particles), then instead of simply pooling all the clones and storing them in one tube, we can pick them individually and store them separately (Figure 7.10). This can be done using individual wells in microtitre trays, or sometimes on filters. If you are doing this manually, then you are limited in the size of library that you can handle (depending on how patient you are). For a bacterial library with, say, a few thousand clones, this is possible. However, there are now machines available that will identify colonies on a plate, pick them individually and transfer them to individual wells in a microtitre tray. Once you have an arrayed library in a microtitre tray, it is then relatively easy to subculture the clones to trays with fresh culture medium, creating multiple and identical copies of your library. You can then work your way through the library, testing each clone individually, knowing that every well contains a different clone. Alternatively, the library can be replicated to agar plates, or directly to membranes, which can then be screened to identify specific clones, as described in Chapter 8.

Replicating such a library onto a membrane is one way of producing another form of array, consisting of spots of DNA rather than viable clones. For a

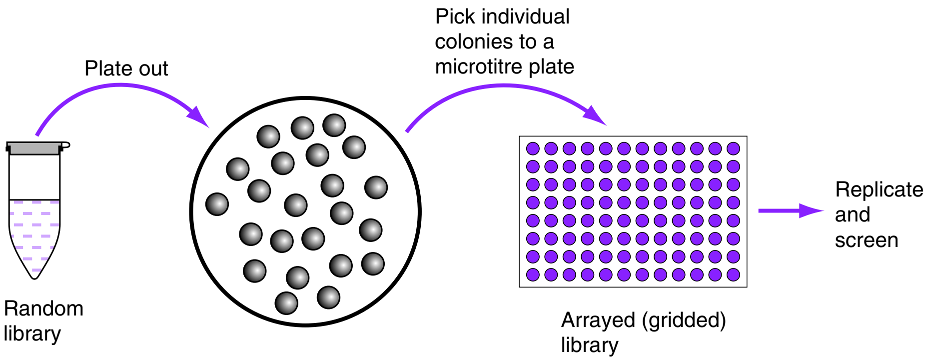

Figure 7.10 Production of an arrayed, or gridded, library

larger library, for example a human gene library, you would want to screen the library at a much higher density. That is, you would want to put the spots on the filter much closer together than would be obtained by merely replicating from the microtitre tray. This requires very precise positioning of the spots on the filter, which is achieved by using another robot.

The availability of genome sequence data makes it possible to produce DNA arrays without constructing a gene library, using PCR products or synthetic oligonucleotides. These either use nylon membranes (*macroarrays*) or glass slides (*microarrays*), and are especially useful for analysing variations in genome content and genome-wide analyses of transcription. The use of such arrays is considered in Chapters 12 and 13.

It is not necessary to be able to produce your own arrays, or to own your own robots. Arrays representing genomic and cDNA libraries from a considerable range of organisms are readily available from public and commercial resource centres, as well as facilities for producing such arrays from your own libraries. The most comprehensive collection is housed by the German Human Genome Project (http://www.rzpd.de). After obtaining the array and screening it, the investigator can order the positive clones from the resource centre.

An arrayed library still consists of a random set of clones. Without screening it, we have no information as to the nature of the insert in each clone, or the

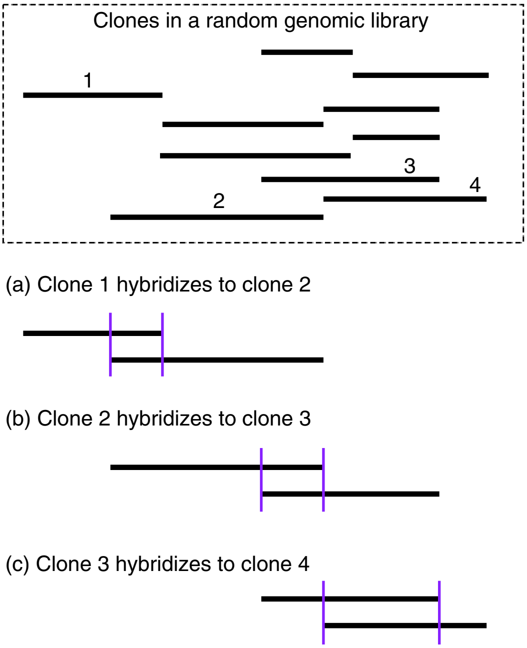

Figure 7.11 Production of an ordered library

relationship between clones. A further development of the concept is to establish which clones overlap so that we can produce a set of clones that can be arranged in order, so as to cover the whole genome. This can be done by hybridization between sets of clones, so that if the insert in clone 1 overlaps with that in clone 2 then we know that they are adjacent and overlapping in the genome (Figure 7.11). Because of the inherent redundancy in a random library, the number of clones required in an *ordered* library is much less; some of the clones in the random library will not be needed. The actual number of clones needed will depend on the degree of overlap between the chosen clones, as well as the insert size.

An ordered library can be a valuable resource. Ordered libraries have contributed substantially to some of the genome sequencing projects – notably the public human genome sequencing consortium used an ordered library of BAC clones. However, the work required to establish an ordered library is substantial, even for a relatively small genome such as that of a bacterium, and even using relatively large inserts such as those in a cosmid library. More recent developments in sequencing technology, and in the computer techniques for assembling sequence data from large numbers of small fragments (see Chapter 10), provide alternatives to the production of ordered libraries as strategies for genome sequencing.

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8 Finding the Right Clone

8.1 Screening Libraries with Gene Probes

In the previous chapter, we described how to create a gene library. Whether it is a genomic library or a cDNA library, it is a large collection of random clones, and we still have to find a way of identifying which clone(s) carry the gene that we are interested in. (We can ignore the more specialized topic of ordered libraries.) This means that we need ways of rapidly *screening* very large numbers of clones. This is most commonly done using a nucleic acid *probe* (DNA or RNA), which will *hybridize* to the DNA sequence you are looking for in a specific clone. The principle involved is that the library (in the form of bacterial colonies or phage plaques) is replicated onto a filter, which is then treated to release the DNA and bind it to the filter, which then carries a pattern of DNA spots that replicates the position of the colonies or plaques on the original plate. The filter is then hybridized with the probe, which has first been labelled so that it can be easily detected. This allows you to detect which DNA spots hybridize to the probe, and recover the corresponding clones from the original plate.

In order to appreciate the power of this technique it is necessary to consider more closely the question of hybridization. Later in this chapter we will consider an alternative strategy, which involves using antibodies to screen an expression library.

8.1.1 Hybridization

Hybridization is based on the difference in stability between the covalent bonds in the nucleic acid backbone of each strand, and the much weaker hydrogen bonds that bind the two strands in the double helix together by base pairing. Thanks to this arrangement, the two strands can be safely separated – both in the cell and in the test tube – under conditions that are much too mild to pose any threat to the covalent bonds in the backbone. This is referred to as *denaturation* of DNA, and unlike the denaturation of most proteins it is reversible. Because of the complementarity of the base pairs, the strands will easily join together again and *renature*. In the test tube, DNA is readily denatured by heating, and the denaturation process is therefore often referred to as melting even when it is accomplished enzymatically (e.g. by DNA polymerase) or chemically (e.g. by NaOH). The separation of the strands during renaturation causes a radical change in the physical properties of DNA, such as optical density (Figure 8.1). During melting of DNA, the optical density changes dramatically during a short temperature interval, and then stabilizes after the strands have separated entirely. The midpoint of this temperature interval is denoted the *melting temperature* (T_m). Under physiological conditions, the T_m is usually 85–95°C (depending on the base composition of the DNA). In the laboratory, we can adjust other factors, such as the salt concentration (see below) to bring the melting temperature down to a more convenient range.

The reason that the T_m varies according to the base composition of the DNA is that guanine–cytosine base pairs are joined together by three hydrogen bonds, whereas adenosine–thymidine base pairs have only two. If we take reasonably large DNA molecules, such as might be obtained by isolating total DNA from an organism, we can make an estimate of its base composition by measuring the T_m. Alternatively, if we know the base composition, we can calculate the T_m. For shorter sequences, such as the 20–30 base synthetic oligonucleotides that are commonly used as *primers*, other factors have to be taken into account. The strength of the association between two bases (expressed as ΔG, the energy released on formation of a base pair) depends also on the adjacent bases, because hydrophobic interactions between adjacent bases (*stacking*) also affects the stability of the pairing. Some examples are shown in Figure 8.2, where it can be seen that the free energy of base pairing (kcal/mol) for the CG/GC doublet is not the same as for the GC/CG doublet. The negative values indicate that energy is *released* on formation of a base-paired structure. More energy released means greater stability. For short oligonucleotides, calculation of the T_m therefore has to take account of the context of each base in the sequence. (Computer programs are readily available to do this for

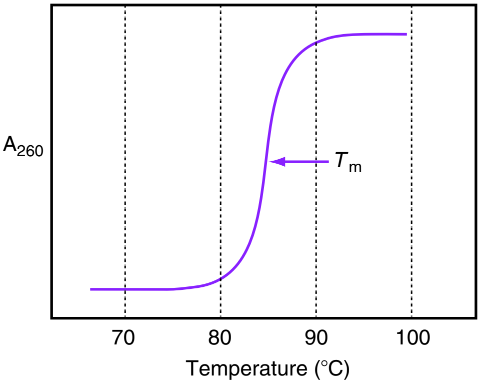

Figure 8.1 Melting (denaturation) of DNA

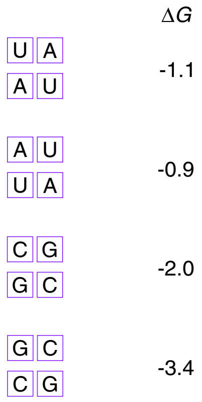

Figure 8.2 Free energy of base pairing

you.) This is not likely to be such an important factor in determining the T_m of a longer stretch of DNA because there will be a tendency for these differences to average out.

Although the normal base pairs (A–T and G–C) are the only forms that are fully compatible with the canonical Watson–Crick double helix, pairing of other bases can occur, especially in situations where a regular double helix is less important (such as the folding of single-stranded nucleic acids into secondary structures – see below).

In addition to the hydrogen bonds, the double-stranded DNA structure is maintained by hydrophobic interactions between the bases on opposite strands. (This is in addition to the hydrophobic stacking of adjacent bases on the same strand, as referred to above.) The hydrophobic nature of the bases means that a single-stranded structure, in which the bases are exposed to the aqueous environment, is unstable; pairing of the bases enables them to be removed from interaction with the surrounding water. In contrast to the hydrogen bonding, hydrophobic interactions are relatively non-specific, i.e. nucleic acid strands will tend to stick together even in the absence of specific base-pairing, although the specific interactions enables a stronger association. The specificity of the interaction can therefore be increased by the use of chemicals (such as formamide) that reduce the hydrophobic interactions.

What happens if there is only a single strand, as is the case normally with RNA, and sometimes with DNA? In that case, removal of bases from the surrounding water is accomplished by the formation of secondary structures in which the nucleic acid folds up on itself to form localized double-stranded regions, including structures referred to as hairpins or stem-loop structures. At room temperature, in the absence of denaturing agents, a single-stranded nucleic acid will normally consist of a complex set of such localized secondary structure elements.

A further factor to be taken into account is the negative charge on the phosphate groups in the nucleic acid backbone. This works in the opposite direction to the hydrogen bonds and hydrophobic interactions; the strong negative charge on the DNA strands causes electrostatic repulsion that tends to repel the two strands. In the presence of salt, this effect is counteracted by the presence of a cloud of counterions surrounding the molecule, neutralising the negative charge on the phosphate groups. However, if you reduce the salt concentration, any weak interactions between the strands will be disrupted by electrostatic repulsion – hence the use of low salt conditions to increase the specificity of hybridization (see below).

If two similar, but different, double-stranded DNA fragments are mixed, melted, and then left to renature, some of them will reform hybrids with their perfectly complementary halves, but others will have formed hybrids (Figure 8.3). This would happen, for example, if you were to mix the cDNA molecules encoding the human red- and green-sensitive photopigments, or the actin genes from mouse and rat. Any base pairs that do not match will cause imperfections in the resulting hybrid double helix. A higher number of mismatches will lead to a less stable hybrid – in other words one that would have a lower melting temperature.

This is very important when single strands of nucleic acids are hybridized in the laboratory. The investigator can choose conditions that would be more or less forgiving of partial mismatches, depending on if they are wanted or not. This is referred to as varying the *stringency* of the hybridization. The most important and obvious way to do this is by altering the temperature. Secondly, hybrids are more stable at higher salt concentrations. At low salt concentrations, the negative charges of the phosphate groups in the backbones cause

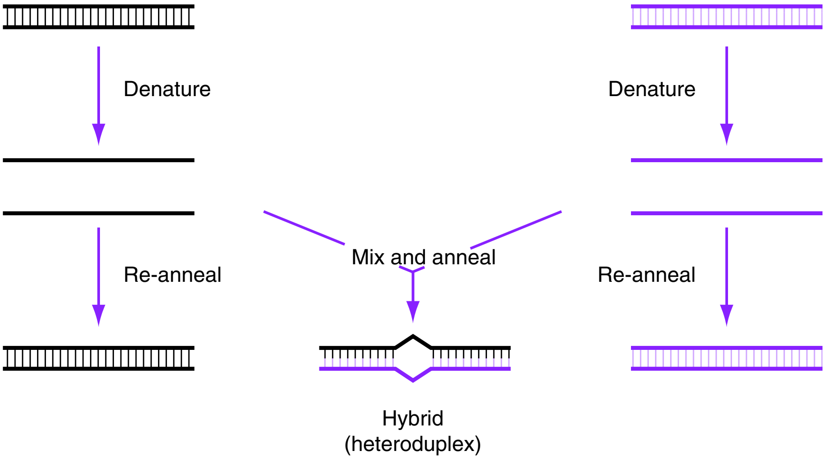

Figure 8.3 Formation of hybrid DNA between similar but non-identical DNA molecules

an electrostatic repulsion between the two strands. At higher salt concentrations, the presence of positive counterions will relieve this repulsion. In the laboratory, salt concentration is usually regulated as multiples of SSC (Standard Saline Citrate; 1 × SSC is defined as 0.15 M sodium chloride and 0.015 M sodium citrate). Sometimes, formamide is added to the hybridization solution. Formamide lowers the melting temperature and is therefore used in situations where hybridization temperatures need to be kept low, such as when carrying out *in situ* hybridization (see Chapter 13).

The basic types of hybridization that are used in the laboratory are filter hybridization, solution hybridization, and *in situ* hybridization. In all three of them, one nucleic acid fragment, the probe, is labelled in order to detect and locate a complementary DNA strand – the target.

8.1.2 Labelling probes

A fundamental feature of nucleic acid hybridization is that the probe is labelled in a way which will make it possible to detect it – and thereby (indirectly) the target it has bound to – after the hybridization. The classic labelling method is to incorporate a radioactive isotope into the probe molecule. Isotopes that are used for this include ^{32}P, ^{33}P, ^{35}S, and ^{3}H. A more energetic isotope, such as ^{32}P, gives a stronger signal, but less good resolution. This makes it useful for experiments where sub-millimetre resolution is irrelevant, such as Southern blot hybridization (see below). At the other extreme, ^{3}H gives a weaker signal and thus requires a much longer exposure time to be detectable. On the other hand, it provides excellent resolution, and can be used in experiments such as *in situ* hybridization (Chapter 13), where it is important to be able to assign the probe labelling not only to specific cells, but even to specific regions of chromosomes.

The classical way to detect radiolabelled probes is to place the probe-target hybrid on an X-ray film. Radioactive particles will expose the region of the film with which they are in contact just like X-rays or visible light would. A more modern method for detecting binding of radioisotopes is phosphoimaging. This method requires specialized and expensive apparatus but is, on the other hand, faster and the phosphoimaging plates can be reused, in contrast to X-ray films.

Many investigators have abandoned radioactive labelling methods in favour of non-radioactive ones. These have advantages over radioactive ones in terms of worker safety, detection speed, and cost. They are also unaffected by the continuous decay of radioisotopes, and are therefore more stable. There are a variety of non-radioactive labels that can be used. Nucleotides substituted with biotin or digoxigenin can be incorporated into the probe, and then detected with specific antibodies (or, in the case of biotin, using avidin, which bind very strongly and specifically to biotin). The antibody (or avidin) that is used is itself labelled with an enzyme, such as horseradish peroxidase (HRP) or alkaline phosphatase, so it can be detected using a chromogenic substrate (i.e. a substrate that yields a coloured product when reacted with the enzyme) or a chemiluminescent substrate (where the initial reaction product is unstable and light is emitted as it breaks down). In the latter case, the emitted light will darken X-ray film just like a radioisotope would. As with radioactive methods, specialized equipment can be used instead of X-ray film.

Alternatively, the probe can be labelled directly with HRP, or a fluorescent label can be incorporated, which allows direct detection of the labelled probe. Such probes are especially useful in the technique known as *fluorescent* in situ *hybridization* or *FISH* (Chapter 16).

8.1.3 Steps in a hybridization experiment

Nucleic acid probes have a tendency to bind non-specifically to other materials on the filter, or even to the filter itself. To minimize this non-specific probe binding, the hybridization solution contains various blocking agents, which may include detergents, bovine serum albumin, and non-homologous DNA. It is often advantageous to pretreat the filter with the hybridization solution without added probe (*prehybridization*).

If the probe is double-stranded DNA, it will need to be heat-denatured by boiling prior to hybridization in order to make it accessible to the target. It is then added to the target in the hybridization solution.

After hybridization, which typically takes place in a controlled-temperature chamber overnight, non-specifically bound probe is removed by washing. This is the step where the investigator can most conveniently decide how tolerant the experiment should be of partially mismatched probe-target hybrids, by choosing an appropriate combination of temperature and salt concentration. If, for example, the intention is to hybridize a cDNA probe with genomic DNA from the same species, then high stringency conditions (i.e. high temperature and low salt concentration) should be chosen (Figure 8.4). This will ensure that the probe will remain bound to the membrane only where it has annealed to the correct complementary sequences. Where the probe has annealed to DNA that is partially similar (for example members of the same gene family), the annealing will be disrupted by these conditions, and the probe will be washed off the filter. If, on the other hand, the investigator wishes to detect, say, a fish gene with a probe made from human DNA (a heterologous probe), then low stringency (i.e. low temperature and high salt concentration) should be chosen in order to protect the expected partially mismatched hybrids.

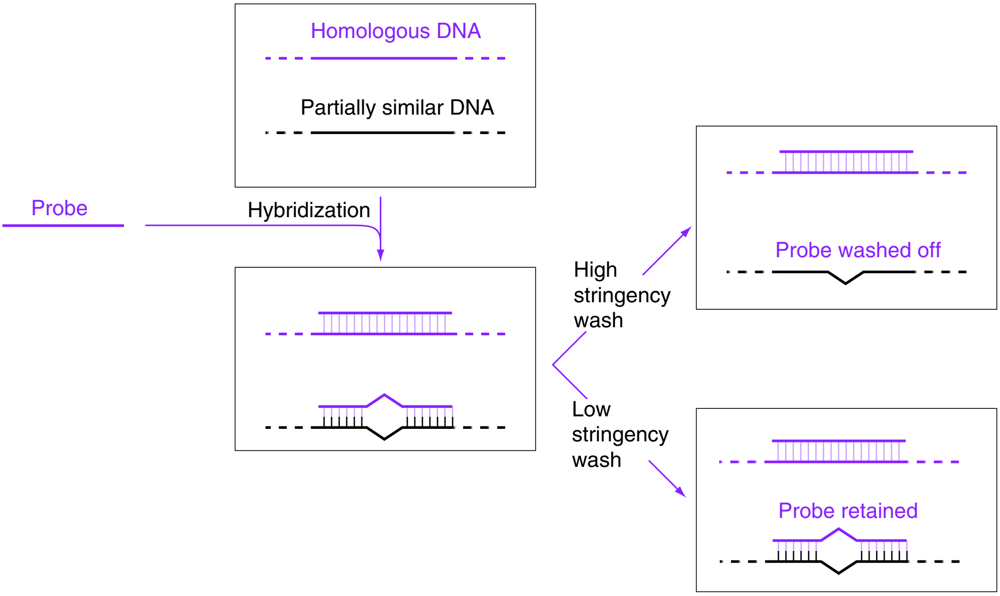

Figure 8.4 High and low stringency washing

The hybridization of the filter with an appropriate probe is the first example of *filter hybridization* that we will come across in this book. In filter hybridization, as the name implies, the target is immobilized in a filter and the probe is free in the hybridization solution until it binds to the target. Other important application of filter hybridization include *Southern blots* (see later in this chapter) and *Northern blots* (Chapter 13). In both cases, the target nucleic acids are first size-separated in an electrophoresis gel before being transferred onto the membrane. The difference between them is that in Southern blots the target nucleic acids are fragments of DNA while Northern blots are used for the identification of RNA. The subsequent hybridization of these filters follows the same principles as outlined above.

8.1.4 Screening procedure

We can now return to the question of screening a gene library, and how you use hybridization with a gene probe to identify a specific clone. Remember that at this stage your gene library is in the form of a number of colonies (or phage plaques) on an agar plate (or a number of such plates). The first step is to produce a replica *filter* of each plate, using a procedure known as a *colony lift* (or a *plaque lift*). A nitrocellulose or nylon membrane is placed on top of the plate, and the orientation is marked by piercing the membrane and the plate with a needle dipped in ink. During the minute or so that the membrane is left on the plate, part of each bacterial colony, or some of the phages from each plaque, will bind to the membrane. The membrane is then soaked in a sodium hydroxide solution, which releases the DNA from the cells or phage, and denatures it. After neutralization with a buffer solution, the single-stranded DNA molecules are fixed to the membrane by heat or UV irradiation. The membrane filter is then hybridized with the appropriate probe. Following hybridization, the filter is washed, under the chosen stringency conditions, before detection of the probe, using procedures appropriate for the nature of the label.

If you are successful, you will now have an X-ray film with one or more black spots on it that marks the position of the clones that you want on the original plate. You then return to that plate, align it with the X-ray film image, pick that colony and subculture it. This is the clone that you want. This procedure is summarized in Figure 8.5.

In practice, especially if you are screening a library at high density, you are unlikely to be able to pick an individual clone without also collecting some of the neighbouring ones. You will then have to use this mixture of clones for rescreening at a lower density. Furthermore, some of the clones will be false positives (for various reasons), and so it will be necessary to submit them to further testing to verify their identity. We will come back to these concepts later on.

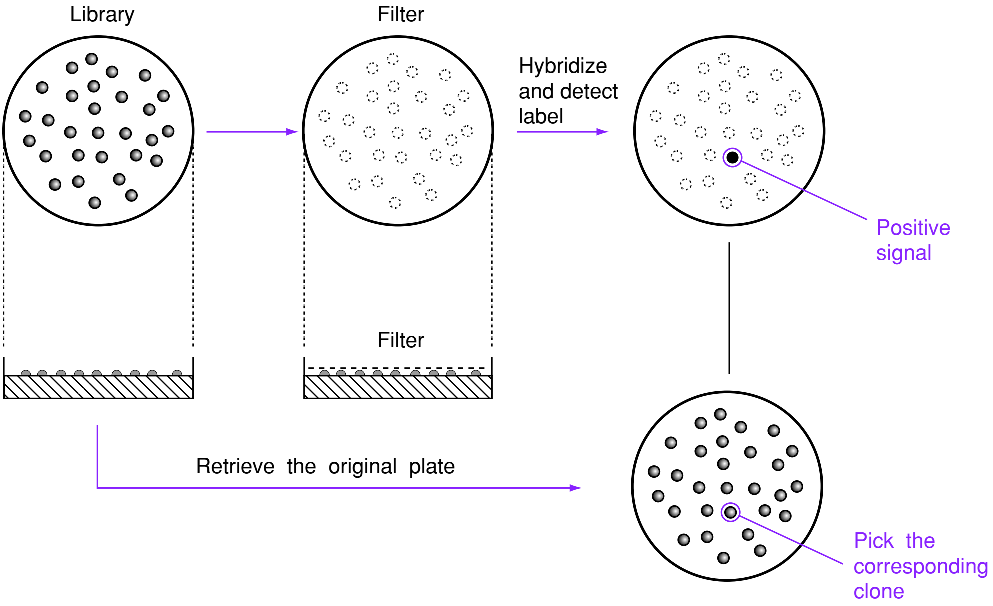

Figure 8.5 Screening a gene library by hybridization with a gene probe

8.1.5 Probe selection and generation

Screening a gene library with a nucleic acid probe implies that you have access to a suitable DNA (or RNA) fragment. Yet the whole purpose of screening a gene library is to isolate a clone carrying a novel piece of DNA. There are a variety of ways around this circular argument. First, there are cases where you actually *are* screening with a probe emanating from the same gene and the same species; this is called *homologous probing*. In this chapter we will describe this, the use of *heterologous probes* and probes generated by *back translation* (as well as the use of antibodies to screen *expression libraries*). Chapter 9 will describe another approach, which is the use of PCR-based techniques for generating suitable probes, and differential and subtractive hybridization methods are described in Chapter 13.

Homologous probes

Why would you use a probe coming from the same gene in the same species? This is not as strange an idea as it may seem. First, you may already have a clone, but an incomplete one. You may not have the complete cDNA sequence, or you may want to walk further down a chromosome, for example in the search for further promoter elements. You may also have access to a cDNA clone, but desire to know the genomic sequence that encodes it. Finally, your business may be to study genetic variation between individuals or strains, whether polymorphisms or mutations causing disease. However, for at least some of these purposes it would be much easier to use PCR (see Chapter 9) to amplify that bit of DNA from the genome of other individuals than to screen a gene library with the homologous probe.

Heterologous probes

If the gene we are trying to clone is not completely unknown, but a corresponding one has already been cloned and characterized from another source, then we can use that characterized clone as a probe. So in isolating the human insulin gene, for example, it was possible to use the previously characterized rat insulin gene to probe a human gene library.

This takes advantage of the fact that genes with the same function are often very similar in different organisms (we will see examples of this in Chapter 11.) As we would expect, the similarity tends to be greatest for the most closely related species (you might not like to think it but we are closely related to rats in this sense!). The extent of the relationship varies from one gene to another;

some genes are very highly conserved while others are more variable. Generally, the closer the relationship between the two species, the more reliable the screening is likely to be.

If we move in the other direction, towards less closely related species, then the similarity is likely to decline to a level at which we can no longer detect hybridization using high stringency conditions. This does not mean that the probe is useless. It is quite common to screen gene libraries with heterologous probes that require lower stringency (e.g. lower temperature or higher ionic strength) in order to hybridize to the target clone. However, there is a price to be paid. As you lower the stringency in order to allow your heterologous probe to hybridize, so you also allow the probe to hybridize to other genes which are similar to the gene you are trying to clone. In other words, you are more likely to get false positive signals. A certain proportion of false positives can be tolerated (indeed you are likely to encounter some false positives, however good your probe is); these have to be eliminated by further screening and checking (see below). However, if the ratio of false to genuine positives becomes too high, then the re-screening becomes prohibitively time-consuming. (The precise point at which this ratio becomes unacceptable will depend on how badly you want the clone and whether you can think of any other way of getting it.)

Back translation

An alternative strategy becomes possible if you are able to obtain in pure form the protein that is encoded by the gene of interest. It is then possible to determine part of the amino acid sequence of the protein (usually starting from the N-terminal region), and to use that information to infer the likely sequence of the gene itself. Since this process (which does not occur in nature, only in your mind – or your computer) is the reverse of normal translation it is referred to as *back translation* or *reverse translation*. Once you have done that, you can then synthesize an appropriate nucleic acid probe.

In addition, this approach can be used even when no protein has been purified or sequenced. A back translation can be made from a predicted region of a protein you believe to exist, either by analogy with a different species or using a specific known domain.

However, you have to remember the redundancy of the genetic code. If you know the DNA or RNA sequence you can predict accurately the amino acid sequence of the protein (subject to a few peripheral assumptions). The fact that several different codons end up coding for the same amino acid does not affect the inference in the real translation direction. However, in the reverse direction it is a different matter. If you know there is, say, a leucine residue in the protein at a specific position, then you have considerable uncertainty over what the DNA sequence actually is. It could be any one of six codons (see Box 8.1).

Box 8.1 Genetic code: possible codons for each amino acid

Amino acid	Possible codons						Number of codons
Alanine	GCU	GCC	GCA	GCG			4
Arginine	CGU	CGC	CGA	CGG	AGA	AGG	6
Asparagine	AAU	AAC					2
Aspartate	GAU	GAC					2
Cysteine	UGU	UGC					2
Glutamate	GAA	GAG					2
Glutamine	CAA	CAG					2
Glycine	GGU	GGC	GGA	GGG			4
Histidine	CAU	CAC					2
Isoleucine	AUU	AUC	AUA				3
Leucine	UUA	UUG	CUU	CUC	CUA	CUG	6
Lysine	AAA	AAG					2
Methionine	AUG						1
Phenylalanine	UUU	UUC					2
Proline	CCU	CCC	CCA	CCG			4
Serine	UCU	UCC	UCA	UCG	AGU	AGC	6
Threonine	ACU	ACC	ACA	ACG			4
Tryptophan	UGG						1
Tyrosine	UAU	UAC					2
Valine	GUU	GUC	GUA	GUG			4
Stop	UAA	UAG	UGA				3

You can easily accommodate ambiguity in the sequence by programming the DNA synthesizer to include a mixture of bases at that position. But every such ambiguity reduces the specificity of the probe. If you allow too much ambiguity you will have a probe that will react with an unacceptable number of non-specific clones in the library. One way of reducing the ambiguity of the probe is to take account of the codon usage of the organism. If this shows that there is a very marked preference for one codon over another synonymous codon, then you can use that information in selecting the codon to be included in your probe. This clearly involves taking a chance, and in many organisms this preference is not marked enough to be very helpful. Instead, you can reduce the required ambiguity by careful selection of the region of the amino acid sequence to be included, so as to avoid amino acids such as leucine, arginine or serine (with six possible codons) in favour of those amino acids with unique codons (methionine, tryptophan) or with only two possible codons (e.g. tyrosine, histidine). Oligonucleotides (oligomers) based on reverse-translation deduction are often called *guessmers*.

To decrease the number of false positives, you can make duplicate lifts from your library and produce two guessmer probes from different regions. The chances of a clone giving a false positive signal in both of these is much smaller than with one. This approach can also be used for the design of PCR primers for the identification of unknown genes (see Chapter 9).

The process of protein sequencing used to be a difficult one, requiring the purification of substantial quantities of pure protein, and often failing to yield a usable amino acid sequence (for technical reasons). Improvements in sequencing technology have made it a more viable option, and it is now possible to obtain sufficient sequence from a spot eluted from a 2D gel; this is widely used in surveys of protein expression, especially in conjunction with genome sequence data (see the section on *Proteomics*, in Chapter 13).

8.2 Screening Expression Libraries with Antibodies

Libraries made with an expression vector such as lambda gt11 (see Chapter 6) can be screened with nucleic acid probes as described above. However, such a library also allows an alternative method of screening, using antibodies (see Box 8.2). For this, the library is cultured at conditions which permit the expression of fusion proteins. This means growing at 42°C, to inactivate the temperature-sensitive repressor, and in the presence of isopropyl galactoside (IPTG), in order to induce expression from the *lacZ* promoter. The plates are then overlaid with a nitrocellulose or nylon filter in the same way as with nucleic acid probe screening, except the filters are not treated with sodium hydroxide. Instead, they are incubated with a diluted antibody to the protein in question. This antibody could have been produced by purifying the protein in question by biochemical methods and injecting it into an animal such as a rabbit together with an adjuvant to boost the immune response. Alternatively, a synthetic peptide could have been produced corresponding to a known or assumed part of the protein, and used similarly for immunization. It is also possible to use

Box 8.2 Antibodies

Antibodies are made by animals in response to antigens – usually proteins or peptides, for our purposes. If a protein is injected into an animal, the normal response is to produce a wide range of antibodies (immunoglobulins), which recognize different parts of the protein. The region recognized by an antibody is known as an *epitope*. Some antibodies will bind to simple *linear* epitopes; these are short sequences of amino acids that can be recognized by a specific antibody, irrespective of conformation. Many such antibodies will bind to a short synthetic peptide, and if you use such a peptide to raise antibodies, you will *only* get antibodies that recognize linear epitopes. On the other hand, some antibodies raised using a protein antigen will bind to epitopes that are formed by folding the amino acid chain; these are known as *conformational* epitopes. Antibodies that are specific for a conformational epitope will only react with the protein in its native conformation, not with denatured protein. This is an important distinction. Many procedures in molecular biology employ denatured protein, so it is important to know that the antibody can react with a linear epitope. A further consideration is that the protein used to immunize the animal may have been modified post-translationally – in particular it may be glycosylated. Some of the antibodies produced will recognize a glycosylated epitope, and these will be of no use for screening gene libraries.

With antiserum from immunizing an animal, these considerations may not be crucial. It will contain a mixture of antibodies, and some of these will recognize linear, non-glycosylated epitopes. A disadvantage is that the antibodies will vary in specificity – some will react with proteins other than the one you want. The antiserum may also contain antibodies to other proteins to which the animal has been exposed. In particular rabbits often have antibodies to *E. coli* antigens, which is a problem if you want to detect expression of a recombinant protein in *E. coli*. It is therefore necessary to test the specificity of such an antiserum – and sometimes to purify it by absorbing out the cross-reacting antibodies.

An alternative would be to isolate and culture individual antibody-producing cells, so obtaining a single antibody species rather than the mixture you get in an antiserum. Although antibody-producing cells cannot be maintained in culture, they can be fused with other cells to form a *hybridoma* – a hybrid cell that can be grown as a cell line and continue to produce a single antibody. This is a *monoclonal antibody*, i.e. an antibody made by a single clone of antibody-producing cells. Although monoclonal antibodies have important applications where specificity is needed (e.g. in diagnosis), they are not *inherently* specific. Their specificity arises through testing a lot of clones to select those that make antibodies that *are* specific. In molecular biology, this specificity may be useful, but also can be a disadvantage. If the monoclonal antibody recognizes a glycosylated or conformational epitope, it will be useless for many of our applications.

The other advantage of monoclonal antibodies is their constancy. If you use antiserum from an animal, then once you have used up your supply you have to immunize another animal. The antiserum will be different – not only in the antibody titre, but also in its specificity and in the strength of binding to your antigen. (Larger animals are better in this respect – a goat or a sheep can supply antibodies for years – but they are expensive.) However, a monoclonal antibody is produced from a permanent cell line. As long as the hybridoma is maintained, you can obtain supplies of identical antibody.

For detecting a target antigen, you need to label the antibody. In molecular biology, this label is usually an enzyme, such as horseradish peroxidase. You can then detect the enzyme using chromogenic or chemiluminescent substrates. However, this would mean having to attach this label to every antibody you used. It is more convenient to use a second antibody. For example, if the antibody comes from a rabbit, then you can use a second antibody that reacts with rabbit immunoglobulin (e.g. mouse anti-rabbit IgG is an antibody raised in a mouse that reacts with rabbit antibodies). A wide range of labelled second antibodies can be bought off the shelf.

lambda gt11 libraries to identify antigens that have not been characterized, for example by using antisera from experimentally infected animals, or from human subjects recovering from an infectious disease. This has proved to be a very powerful way of identifying those antigens that are especially important in the natural course of infection, or for protection against infection.

After incubating the filter with the antibody, excess antibody is washed off, and the filter incubated with a labelled *secondary antibody* that will bind to the first one, such as for example anti-rabbit immunoglobulin, allowing detection of the clones that reacted with the primary antibody (see Box 8.2).

Note the limitations of using antibody screening. In contrast to the use of nucleic acid probes, where expression is not required, screening a gene library with antibodies obviously needs expression of the cloned gene – hence the common use of a vector such as lambda gt11. Furthermore, the protein that is expressed in *E. coli* may not fold into its correct, natural conformation – especially if you are using a vector like lambda gt11 that generates a fusion protein. You therefore need an antibody that binds to a *linear epitope* (see Box 8.2). In addition, it must recognize a non-glycosylated epitope, since the protein you are trying to detect will not be correctly glycosylated in *E. coli*.

An antiserum derived from immunizing an animal with the purified protein will contain a variety of different antibodies, some of which will recognize linear, non-modified epitopes. The problem with conformational or modified (e.g. glycosylated) epitopes is most likely to occur with monoclonal antibodies (see Box 8.2), where it is necessary to confirm the nature of the recognized epitope before using it for screening libraries. Similar considerations arise when using antibodies for Western blotting (see Chapter 13).

8.3 Rescreening

As mentioned above, several thousands of recombinants need to be plated in each culture dish in order to keep the total number of plates reasonably low. This means that you are unlikely to be able to pick your positive recombinants pure and uncontaminated by their neighbours the first time around. Instead, you would lift a small agar plug containing the positive and neighbouring recombinants and elute them into a buffer. The eluted mixture would then be replated onto a new plate for rescreening with the same probe or antibody (Figure 8.6). This serves two purposes. First, it is almost inevitable that some of the clones are false positives, caused by non-specific binding of probe to the membrane. Indeed the likelihood of this is sufficiently high that it is common practice to use two duplicate filters for hybridization; only those clones that give a positive signal on both filters are regarded as worth following up. Second, for those that are true positives, the secondary screening will allow you to pick a recombinant that is free from contaminating neighbours.

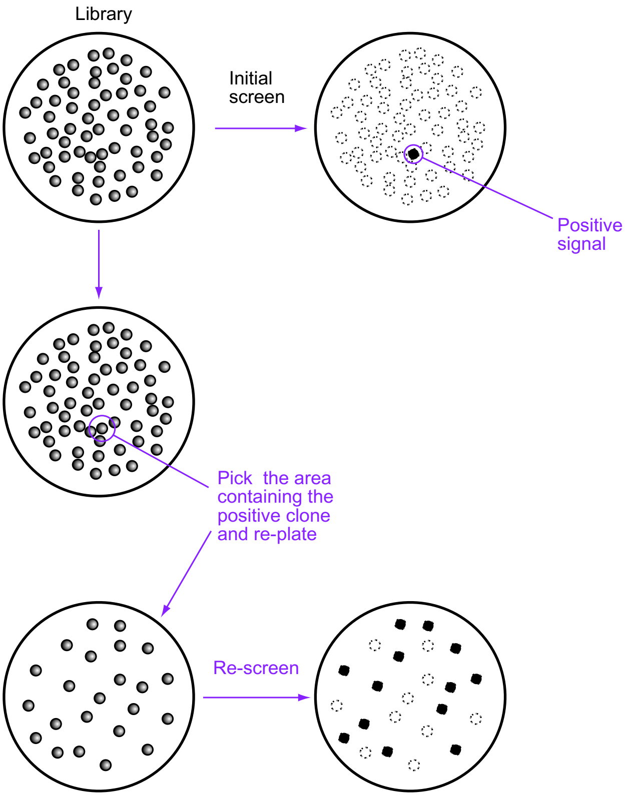

Figure 8.6 Re-screening a clone from a gene library

8.4 Subcloning

In subcloning, the recombinant insert is transferred to another vector, almost invariably a plasmid, which is more amenable for growth, purification, and analysis. Clearly, if the original vector is a plasmid, subcloning may be unnecessary. However, the plasmid may not be amenable for the downstream experiments that you have in mind, so you may want to reclone the insert, after verification, into an expression vector(see Chapters 6 and 15). For recombinant clones with somewhat larger inserts, such as lambda or cosmid clones, it may be possible to divide the insert up into a set of defined restriction fragments and clone each of those fragments, or to identify which fragment carries the DNA that hybridizes to your probe and just clone that fragment into a plasmid vector.

If the insert is even larger than that, such as those found in yeast and bacterial artificial chromosomes (YACs and BACs), this approach is likely to be too time-consuming. Instead you would probably want to fragment the insert from the selected clone into a number of smaller fragments and produce a mini-library of those fragments which would then be re-screened in the same way as the original library (although much more easily).

The first step in subcloning is to grow up enough material. This means either growing up a sufficient number of *E. coli* cells carrying the plasmid or the artificial chromosome, or infecting an *E. coli* culture with a phage clone. The purified recombinant vector DNA is then digested with the appropriate restriction enzyme. In some older vectors, where the insertion site is a single restriction enzyme recognition site, the insert will be flanked by sites recognized by that enzyme. The choice is then obvious. However, most commonly used vectors now have multiple cloning sites, allowing you a lot more flexibility. In particular, if it is important to be able to excise the insert in one piece, then you will need to be able to use an enzyme that does not cut the insert itself. For this purpose, you would be likely to choose an enzyme that cuts DNA infrequently such as *Not*I, which you can predict is unlikely to have a recognition site within the insert. On the other hand, if you want to subclone a smaller piece of the insert, you might choose a medium-frequency cutter such as *Eco*RI. After digestion, you would probably separate half of the reaction mixture by agarose gel electrophoresis (Figure 8.7). This can then be blotted (see the description of Southern blotting below) and probed with the same probe you used to screen the library. With the correct DNA fragment thus identified, you can run out another gel just like the other one, cut out the DNA fragment, purify it from the agarose, and ligate it to your plasmid vector.

Artificial chromosome vectors (BAC, PAC) have inserts measuring hundreds of kilobasepairs, making it impractical to digest and separate them. An

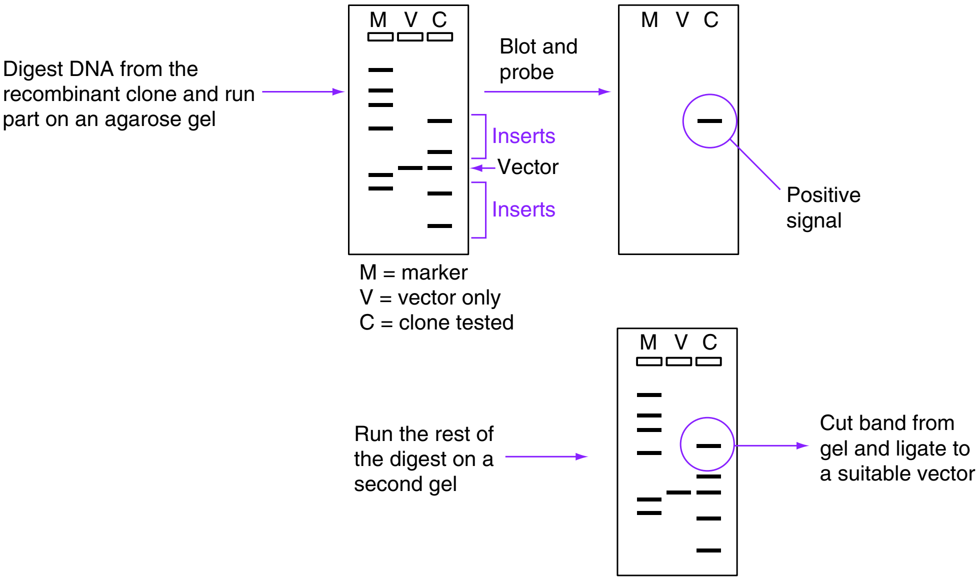

Figure 8.7 Sub-cloning a fragment from a recombinant clone

alternative approach for these is *shotgun subcloning*. This method is based on random physical shearing of the recombinant vector. The resulting fragments are cloned into plasmids, transformed into bacteria, and plated. A replica membrane is then lifted from this plate, and probed with the same probe. In this way, subclones containing the desired sequence can be identified.

8.5 Characterization of plasmid clones

Whether your clone is produced directly or by subcloning from a larger fragment from a gene library, at some stage you are certain to want to characterize the plasmid, and the fragment that it contains. The basic techniques for doing this were described in Chapter 4, so we only need a recapitulation of the major features here. The most basic procedure is to run total DNA extracts or crude plasmid preparations on an agarose gel and look for a change in the size of the plasmid compared to the original vector. If the insert is a reasonable size compared with the vector, you will be able to distinguish recombinant plasmids (carrying the insert) from the parental vector. However, you need to be aware that it is not easy to determine the size of the insert reliably in this way. Conventional size markers are linear DNA fragments, while the intact plasmid will be a supercoiled circular structure, and will therefore run differently on the gel.

8.5.1 Restriction digests and agarose gel electrophoresis

More accurate and more reliable estimates of the size of the insert require some degree of plasmid purification so that the plasmid can be digested with a restriction endonuclease. Simple plasmid purification protocols usually depend on the supercoiled nature of the plasmid: denaturation and rapid renaturation will result in the plasmid maintaining its natural conformation, while the linear DNA fragments that are formed by random degradation of the chromosomal DNA will form an insoluble aggregate. Further purification of the plasmid DNA is obtained by phenol extraction (to remove proteins) and ethanol precipitation (to remove low molecular weight contaminants) and any remaining phenol, as well as concentrating the plasmid DNA. There are many varieties of the procedure, including commercially available kits, which improve the yield and purity of the resulting material as well as avoiding the need for phenol extraction.

The purified plasmid can then be digested with an appropriate restriction enzyme to generate linear fragments that can be accurately sized on an agarose gel. A common procedure is to use the same enzyme that was involved in the cloning step. For example, if you have inserted an *Eco*RI fragment into a unique *Eco*RI site on the cloning vector, then digestion with *Eco*RI will yield two fragments: one corresponding to the linearized vector, and the second being the inserted fragment. Using a standard marker containing a set of linear DNA fragments of known size enable the gel to be calibrated so that the size of the insert can be determined (within the limits of accuracy of the system).

Further restriction digestion can be used to verify the construct. If you know the location of other restriction sites within your cloned fragment, you can confirm the nature of the insert by additional digests. The use of a site that is asymmetrically placed within the insert will enable you to check the orientation of the insert. Figure 8.8 shows an example. In this case, we have inserted a 4.5 kb *Eco*RI fragment into the *Eco*RI site (E) of a 2.5 kb vector plasmid. The *Eco*RI digest confirms that both plasmids A and B have a 4.5 kb insert, but the insert could be either way round. Fortunately, there is a *Hin*dIII site asymmetrically placed within the insert, and another in the vector, adjacent to the *Eco*RI site. So a *Hin*dIII digest will settle the matter. The gel confirms that plasmid A yields two *Hin*dIII fragments, of 5.5 kb and 1.5 kb, while plasmid B has the insert in the other orientation, yielding fragments of 3 kb and 4 kb. Since this is simple to do, it is possible to investigate a number of colonies to find one in which the insert is the right way round. Ultimately, however, you will want to use sequence determination to make absolutely sure.

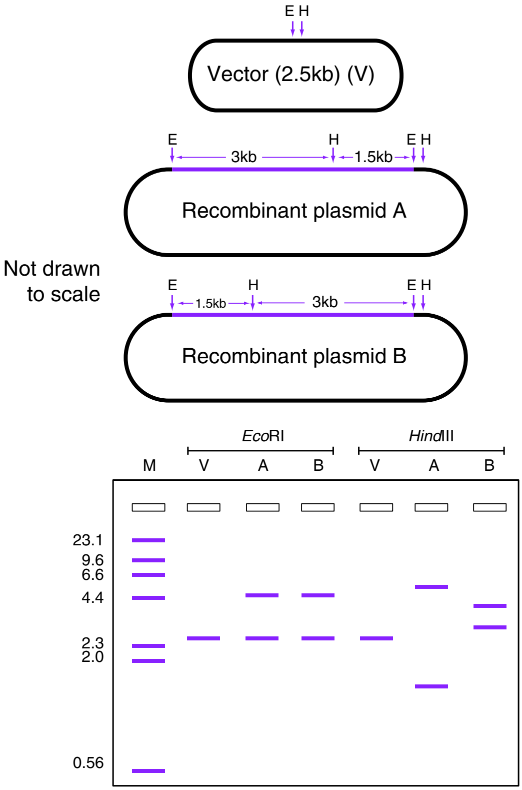

Figure 8.8 Orientation of insert fragment

8.5.2 *Southern blots*

Further confirmation of the nature of your insert comes, once again, from hybridization. You cannot hybridize a probe to DNA fragments while they are in an agarose gel, you need to transfer them to a filter first. The technique for doing this is one that we have already alluded to: Southern blotting, named after E.M. Southern who developed it. The basis of the method is illustrated in Figure 8.9. A membrane (nitrocellulose or, now more commonly, nylon-based) is placed on the gel and a stack of dry paper towels on top of the filter. Buffer is drawn up through the gel and filter by capillary action, and carries the DNA fragments with it. They are trapped on the membrane, which thus acquires a pattern of DNA bands that corresponds to the position of those fragments in the agarose gel. The arrangement shown in the figure looks very crude, and there are much more elegant pieces of equipment available; nevertheless, many molecular biologists prefer to use their own home-made apparatus. Following transfer of the DNA to the filter, the

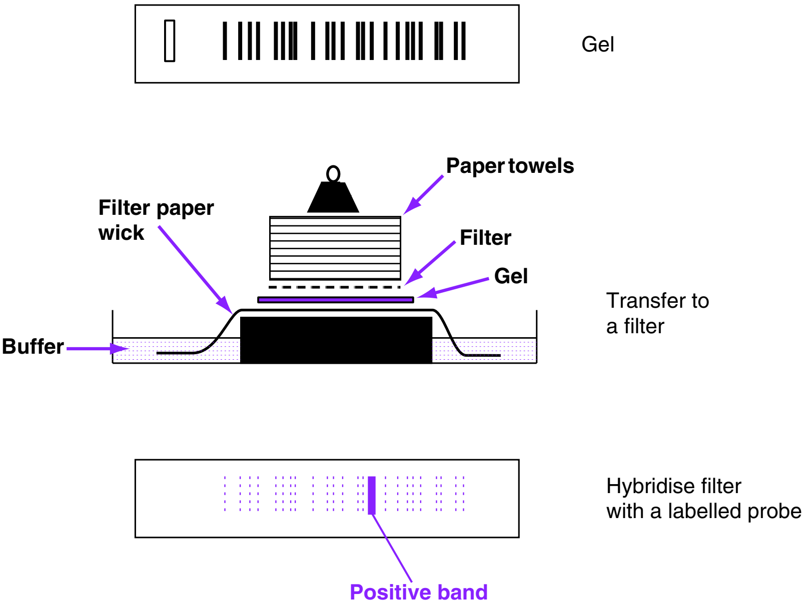

Figure 8.9 Southern blotting

relevant DNA fragment can be identified by hybridization in the ways already described.

Southern blotting is a very useful way of verifying that the band you can see on a gel is indeed the insert that you have been seeking. However, that is only the beginning of the applications of Southern blotting. We will come across it again later on, particularly in Chapters 12 and 16 where it is invaluable for detecting specific gene fragments in a digest of total chromosomal DNA (which just looks like a smear if stained with ethidium bromide), and for comparing the banding patterns obtained with specific probes in DNA from different strains or different individuals.

8.5.3 PCR and sequence analysis

Usually the sequence of the vector is known. It is therefore simple to design a pair of primers that will hybridize to a region at either side of the cloning site, and use these primers to PCR amplify a fragment containing the inserted DNA (see Chapter 9). In this case there is no need to purify the plasmid DNA from each recombinant clone. You can simply resuspend a portion of the colony in water and boil it. PCR amplification will then produce a product of a characteristic size, if you have cloned the right piece of DNA. You can screen a substantial number of potential recombinants in this way. Once you have found one that does produce the correct size of product in the PCR, you can determine the sequence of the PCR product to confirm the nature of the insert.

If you are more confident about the nature of your recombinant product – for example if you are simply re-cloning a known fragment rather than recovering a recombinant from a library, you can skip the PCR step and simply carry out sequencing on purified plasmid DNA from the chosen colony.

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9

Polymerase Chain Reaction (PCR)

On 10 December 1993, Kary B Mullis received the Nobel Prize in Chemistry from King Carl XVI Gustaf of Sweden for his invention of the polymerase chain reaction (PCR) method. Mullis had published and patented his invention only eight years earlier, in 1985. The same year, the Physiology and Medicine prize was awarded to Richard J Roberts and Phillip A Sharp, for their independent discovery of split genes. These laureates had waited twice as long, 16 years, since their fundamental discovery, made in 1977. This will give you some idea of the immense impact PCR has had. Although it is (as we shall see) quite a simple method with obvious limitations, the applications of this method have revolutionized both basic and applied biology. This has been dramatically illustrated in forensic science, where numerous old open cases have been solved, and in clinical applications, where it has suddenly become possible to make diagnoses in hours that previously took weeks.

The key factor in transforming the initial method into one that could have such an impact was the introduction of a thermostable DNA polymerase called *Taq* polymerase. It was originally isolated from *Thermophilus aquaticus*, a thermophilic archaebacterium that thrives in hot springs at temperatures close to the boiling point of water. As a result, all enzymes in this organism have evolved to withstand high temperatures where all proteins from most other organisms would denature immediately and irreversibly. Apart from this distinct feature, *Taq* polymerase (not to be confused with *Taq*I, a restriction enzyme from the same species) is a normal DNA polymerase. It will synthesize a new DNA strand complementary to a single-stranded DNA template. Like all other DNA polymerases, it requires a *primer*, a more or less short strand of complementary DNA to start its synthesis from. In fact, as we will see, it is not a particularly good DNA polymerase. Although it has high processivity (which means it adds successive nucleotides without dissociating from the DNA), it lacks proofreading activity, so it is unable to correct erroneously incorporated nucleotide bases.

PCR uses *Taq* polymerase (or other thermostable DNA polymerases that are now available) for the exponential amplification of a DNA fragment from a longer template, which could be as long as a whole chromosome. The amplified fragment is defined by two short synthetic oligonucleotides that are complementary to the opposing DNA strands of the template that is being amplified. This introduces another limitation to the method. You must know the sequence for at least part of the DNA molecule you wish to amplify – or at least you must be able to make an educated guess.

So how do we go from a small amount of, say, total human genomic DNA to a large amount of one short region that has been exponentially amplified to the extent that it entirely dominates the reaction mixture? This will be clear if you go through the first few cycles in a PCR amplification.

9.1 The PCR Reaction

In this example, we will be starting with genomic DNA purified from human leukocytes. The preparation will contain more or less sheared chromosome segments. Only a small amount is required even from such a complex template – in fact, as little as a single cell has been used as starting material.

We will also need two primers. These can be synthesized to your specifications from a specialist supplier at a very low cost within a day or two. In this example, we will replicate the work of Mullis and his colleagues and use primers flanking a region for the human β-globin gene that is mutated in sickle cell anaemia. A very considerable excess of primer molecules is added to the reaction. (This of course refers to an excess in molar, or molecular, terms; since the primer is very much smaller than the template, the amount of primer *by weight* is quite low – see the discussion in Chapter 5.)

The binding, *annealing*, of the primer to the template is a typical DNA:DNA hybridization reaction, and follows similar principles to the hybridization of probes as described in Chapter 8. First, the double-stranded template needs to be *denatured*. The temperature used for this in PCR, 94°C, does not do much damage to the *Taq* polymerase molecule during the minute or so that the PCR reaction is heated to this temperature.

The temperature is then lowered to the optimal annealing temperature, where the two primers can bind to the opposing DNA strands (Figure 9.1). This is the only temperature in a PCR cycle that can be varied widely. Thus it can be optimized for maximum binding of the primer to the correct template, and minimum binding to other sequences. If the annealing temperature is too low, the primers will bind at other positions on the template, resulting in false products or no product at all. If the annealing temperature is too high, the primers may fail to bind at the correct site. The temperature needed will depend on the sequence and length of the primers. Since G–C pairing is stronger than A–T, the more Gs and Cs there are in the primer, the stronger it will bind (and

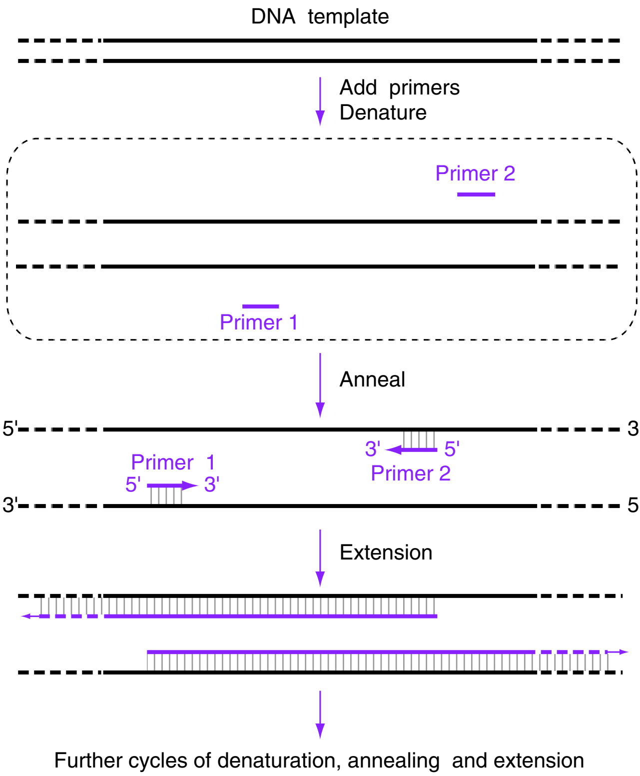

Figure 9.1 Polymerase chain reaction: first cycle

therefore higher annealing temperatures can be used). (See Chapter 8 for a discussion of other factors that influence the optimal annealing temperature.) Although computer programs are available to predict the optimal annealing temperature for a primer, in practice a certain amount of trial and error is needed. Normally, the annealing temperature is chosen somewhere between 40 and 60°C, although for templates with a high GC content annealing temperatures as high as 72°C (the normal extension temperature, see below) may be used. Note also that, as with hybridization, we may want to use conditions that will allow priming from sites that are only partially matched to the primers, and for this purpose we would use a lower annealing temperature. Since the primers are small, and at relatively high molar concentrations, annealing is rapid, taking only a minute or less.

The temperature is then raised to approximately 72°C, which is normally the optimum *extension* temperature for a PCR reaction. The *Taq* polymerase will now produce complementary DNA strands starting from the primers. The extension proceeds at approximately 1000 bases per minute.

Because the template in this case is many times larger than that, the polymerization will proceed until it is interrupted. This happens when the temperature is yet again raised to 94° in order to start the next *cycle* in the PCR reaction, which is normally identical in temperature and duration to the previous ones. As we finish the first PCR cycle, we note that we have two double-stranded DNA molecules for each one that we started with. Each one contains one strand of the original template, and one novel strand which is defined at one end, specifically, by the oligonucleotide primer and at the other end, non-specifically, by the time we allowed for extension.

The advantage of *Taq* DNA polymerase over, say, *E coli* DNA polymerase will now become apparent. The *E coli* polymerase could have done the extension, although it operates at 37°C which means that you would not be able to increase stringency and would risk non-specific hybridization. Above all, however, you would lose all enzyme activity in the denaturation step, and would have to add fresh enzyme. (In the initial manifestation of PCR, before the introduction of *Taq*, this is precisely what had to be done.) *Taq* polymerase, by contrast, survives the denaturation step unscathed.

This second denaturation step creates four single-stranded template molecules. At the following annealing step, one molecule of the complementary primer will bind to each of these single strands. As the temperature is again raised to 72°C, *Taq* polymerase will begin to extend the primers (Figure 9.2). Two of the four extensions, where the primer has again bound to the original chromosomal template, are identical to the first extension in that they terminate only when the temperature is raised. Note, however, that this does not apply to the two strands that are produced with the new strands as templates. These templates end abruptly where the opposite primers had bound.

If you envisage the next cycle, then again the two original template strands will give rise to a long product, limited only by the duration of the extension reaction. Priming on all the other strands will yield a product that is defined and delimited by the primers at both ends. Each subsequent cycle will produce two new long strands, but the number of new short strands made will increase exponentially, so that eventually the reaction mixture will be completely dominated by the newly formed short DNA strands with one primer at each end. It follows from this that the ends of the new DNA are actually defined by the primers, unlike the intervening regions which are entirely defined by the original template (apart from any mistakes in the amplification).

This means that if the sequence of the primer is in any way different from the template, this will not be apparent in the final product, where the ends will be the sequences of the primers not that of the original template. This in turn means that modifications can be introduced through the primers. One particularly useful application of this principle is in the addition of restriction enzyme

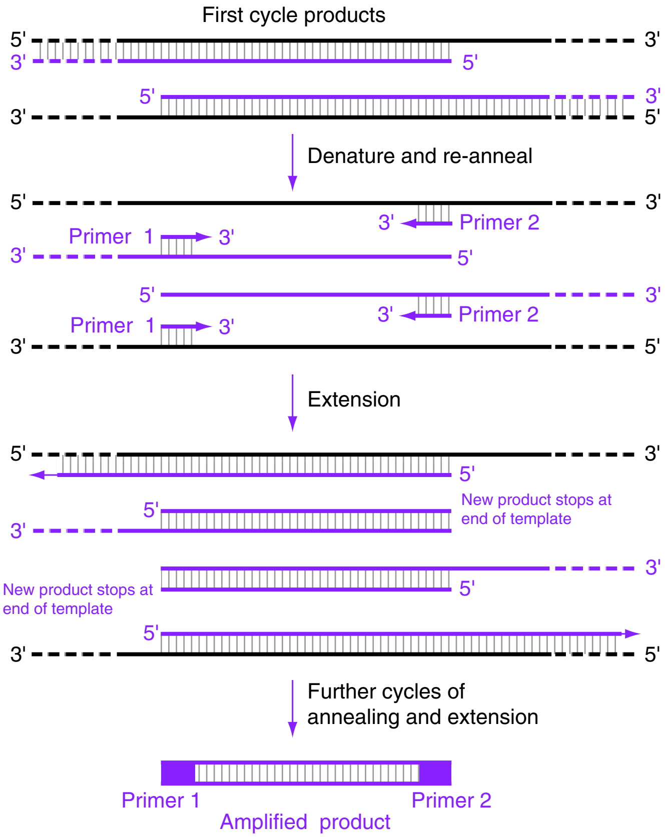

Figure 9.2 Polymerase chain reaction: second cycle

recognition sites to the ends of a PCR product, to make it easier to clone. In order to do this (Figure 9.3), the 5′ ends of the primer sequences are modified to incorporate the appropriate recognition site. In the first PCR cycle, the 5′ ends of the primer will not pair with the template, but that does not prevent the annealing or extension steps. (It is essential for the 3′ end of the primer to be a perfect match, but not the 5′ end). In subsequent rounds, the part of the primer carrying the restriction site (which is now incorporated into the product) will be accurately replicated, so the primers are now a perfect match. The end product is a DNA fragment carrying the restriction site near the ends, so it can be cut with the restriction enzyme and ligated with an appropriate vector.

The same principle can be employed to introduce other modifications into the product, including base changes. Attaching fluorescent dyes, or other labels, to one or both primers provides a good way of obtaining a labelled product.

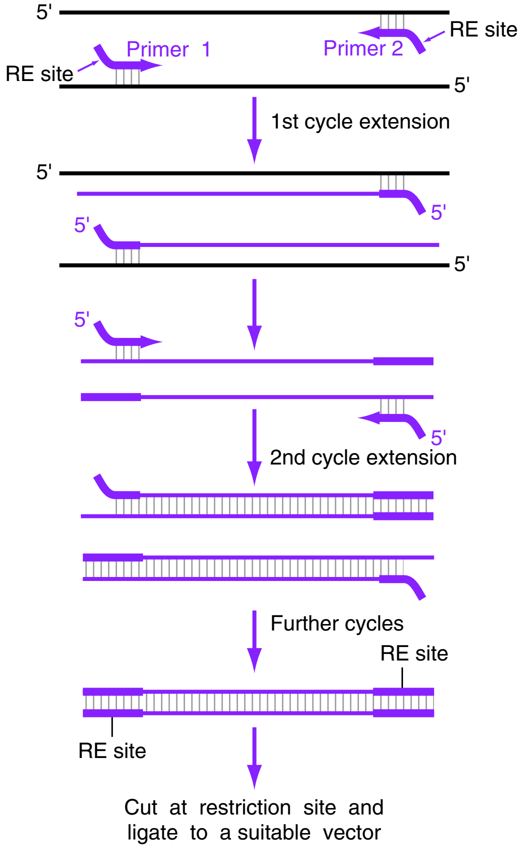

Figure 9.3 Adding restriction sites to a PCR product

9.2 PCR in Practice

During the initial stages of its development, PCR was performed by manually transferring tubes between thermostat-controlled water baths. It was obvious that this was not desirable for the reproducibility of the reaction (nor for the mental well-being of the operator). Therefore, the launching of PCR as a revolutionary new technology was associated with the development of the programmable thermocycler. These instruments are based on metal heating blocks with holes for the PCR tubes. These blocks are designed to switch between the programmed series of temperature steps with great speed and precision by a combination of heating and cooling systems. The use of small (0.2–0.5 m), thin-walled, tubes helps to ensure a rapid change of temperature. Alternatively, for larger numbers of samples, microtitre trays are used.

Because the PCR reaction is performed in a small volume (typically 25–50 μl), and because of the high temperatures involved, it can be easily imagined that the water would quickly evaporate and end up on the inside of the lid rather than on the bottom of the tube. There are two ways of preventing this: the first one is to place a drop of mineral oil over the reaction; an alternative, and less messy, approach is to heat the lids of the tubes to prevent condensation.

9.2.1 Optimization of the PCR reaction

The importance of the annealing temperature in PCR has already been discussed. If the temperature is too high, binding of the primers to the target will not be stable enough for amplification to take place. If it is too low, the system will become too tolerant of partial primer-target mismatches, and will therefore be non-specific.

The most important PCR parameter by far is the design of the primers. The primers must be chosen to define a target of appropriate length. As far as possible, complementarity to other targets in the template mix should be avoided. To a degree, this can be pre-empted by database searches of putative sequences, but often it is, at least in part, a matter of trial and error. The primers should also not anneal to themselves or to one another, and should be unable to form stable secondary structures. Computer programs are available to check that the chosen sequences are suitable in these respects.

Just as conserved gene regions can be used to create ‘guessmers’ for library screening, they can also be used to create degenerate pools of PCR primers. In this way, if two regions within a gene are known or can be predicted by an educated guess, it is often possible to amplify a novel gene by this method. This can be used for the cloning of a known gene from another species, or for discovering a novel gene that is related to one that is previously known (see later in this chapter). It is of course important to remember that the flanking sequences of the product are determined by the primer and not by the template.

Another important factor is the concentration of magnesium ions, a necessary cofactor for the enzyme. A higher magnesium concentration gives a higher yield, but also a lower specificity.

9.2.2 Analysis of PCR products

The normal way of analysing the products of a PCR reaction is to run out the samples in an agarose electrophoresis gel. This allows the user to ascertain that only one band is obtained in each reaction, which is usually the objective. By comparing the size of the amplified band to a molecular weight standard, it is also possible to ascertain that the molecular weight is the same as the predicted one (which is usually known). The assumption is that if the band is the predicted size, then it probably corresponds to the predicted fragment – an assumption which occasionally leads you down the wrong track. If the result is important, it is worth checking the identity of the band (see below).

On the other hand, you may get a band of a different size from that predicted. Frequently a specific amplification product (of the correct size) is found together with a strong band of low molecular weight. These low molecular weight bands are called primer dimers, and are caused by binding of the primers to each other. They can usually be ignored, although the tendency of primers to form dimers can cause problems with a PCR, due (amongst other things) to competition between primer–primer and primer–template binding. Another effect may be an amplification that appears to be specific (as shown by the absence of a band of that size in control reactions), but gives a product of the wrong size. This may indicate that one, or both, of the primers is binding at a different position, producing an artefactual product. This may also be manifested as a specific band together with a more or less complex mixture of non-specific ones. In these cases, greater specificity may be obtained by adjusting one or more of the PCR parameters (see the previous section).

Alternatively, nested PCR may be used to increase the specificity (and/or the sensitivity) of the PCR. In nested PCR (Figure 9.4), a small aliquot of the original reaction is transferred to a second, 'nested' PCR reaction. In the nested

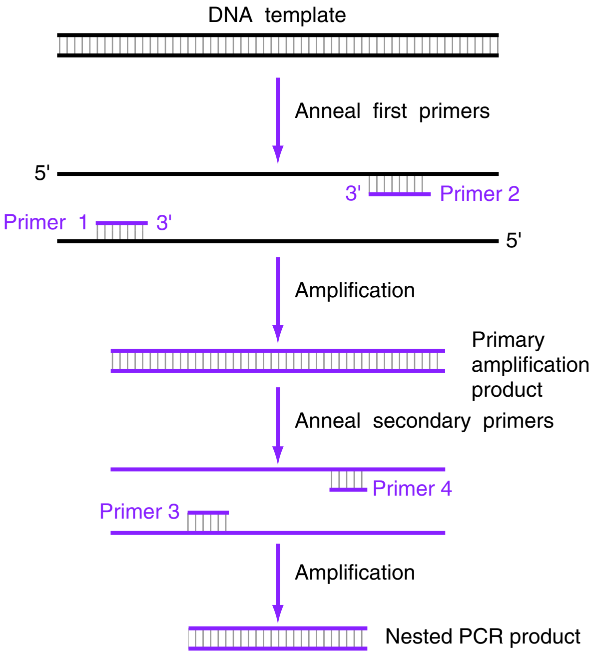

Figure 9.4 Nested PCR

PCR reaction, one or both primers are replaced with a second set of primers which will bind specifically within the desired amplification product. In this way, the undesirable ones which have typically been amplified because of a coincidental sequence similarity in the region of the original primers, will normally disappear.

In order to provide another level of certainly about the identity of a PCR product, in particular if the exact molecular weight is not known, the gel may be blotted and hybridized (see Chapter 8) with a probe complementary to the expected product. Alternatively, because of the remarkable recent progress in sequencing technology, many people find it faster, easier, and cheaper to perform direct sequencing on the PCR product (see Chapter 10).

9.3 Cloning PCR Products

Although PCR is commonly used merely to detect the presence of a specific sequence in different templates, it is also often employed for the amplification of such sequences as a convenient way of obtaining a specific product for cloning. This is especially important when the starting material is very scarce, as in such cases the conventional routes (for example constructing and screening a gene library) are impossible.

You might expect, from the description so far, that the PCR products would be blunt-ended, and could therefore be cloned by normal blunt-ended cloning (see Chapter 5). In practice, this is often not very successful. Not only is blunt-ended cloning comparatively inefficient, compared with cloning sticky-ended fragments, but also, as described below, *Taq* polymerase often tends to add a non-specific adenosine residue to the $3'$ ends of the product. The product is therefore not blunt-ended, and cloning with a blunt-ended vector will fail. Even the use of linkers (see Chapter 5) will be unsuccessful in this situation.

One way round this has already been alluded to. This is to use a modified primer that contains an additional restriction enzyme recognition site in its $5'$ end. The resulting products can be cut and ligated to a plasmid that has been cut with the same enzyme, with great efficiency (Figure 9.5). This is particularly useful when cloning larger PCR fragments.

An alternative, commonly used, method is so-called TA cloning, which takes advantage of the propensity of *Taq* polymerase to non-specifically add an adenosine to the $3'$ ends of each new DNA fragment. This is exploited by using a linearized vector plasmid which has been engineered to contain a thymidine overhang at each end. This produces short, but still sticky ends which will anneal, allowing quite efficient ligation (see Figure 9.5, and also Chapter 5). Note, however, that not all polymerases add adenosine residues to the product; the ‘proof-reading’ polymerases produce genuinely blunt-ended PCR products.

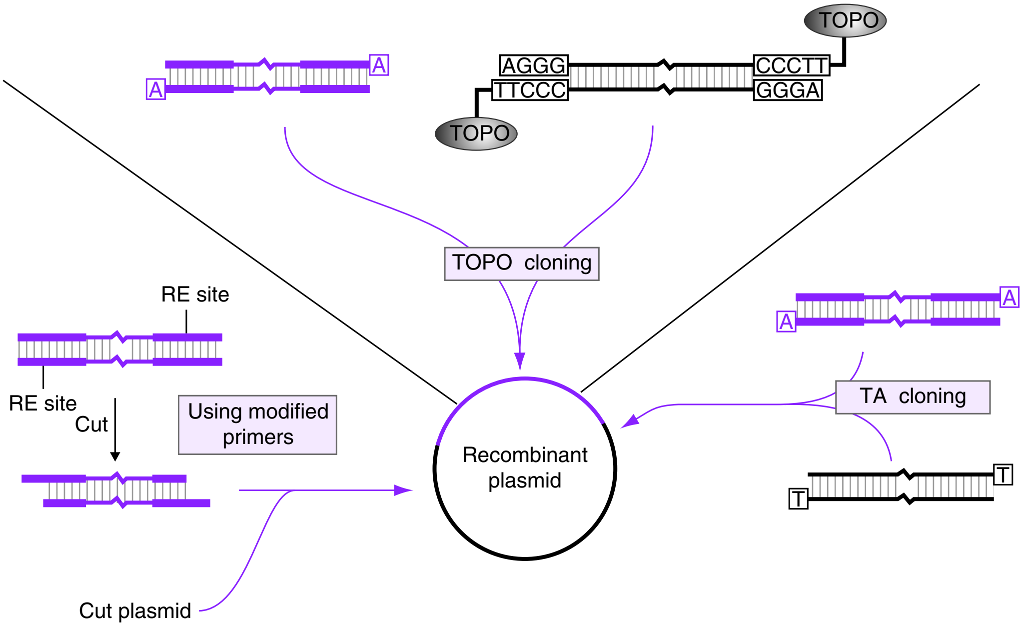

Figure 9.5 Cloning PCR fragments

A further improvement to PCR cloning technology is the use of *Vaccinia* virus DNA topoisomerase I (TOPO) (see also Chapter 5). *In vivo*, topoisomerases are involved in the supercoiling/relaxation of circular DNA. They will cleave DNA at specific sites, leaving a sticky end. The energy released by breaking the phosphodiester bond is stored in a covalent bond between the enzyme and one of the cleaved strands. The enzyme trapped in the sticky-end will then rapidly and efficiently release its stored energy into the formation of a new phosphodiester band as soon as the sticky end encounters its complementary partner. Thus, TOPO has both endonuclease and ligase activity. Commercially available TOPO vectors offer sticky-end overhangs, ranging from TA cloning to more complex sequences bound to the TOPO enzyme. This makes for rapid and ligase-free cloning.

9.4 Long-range PCR

We have already discussed the fact that *Taq* polymerase lacks proofreading activity and is therefore unable to correct its own errors. These errors occur approximately once per 9000 nucleotides, on average. These mistakes will then be perpetuated in all new molecules that have been created by the amplification of these ones. If direct sequencing is used, this is not really a problem, because

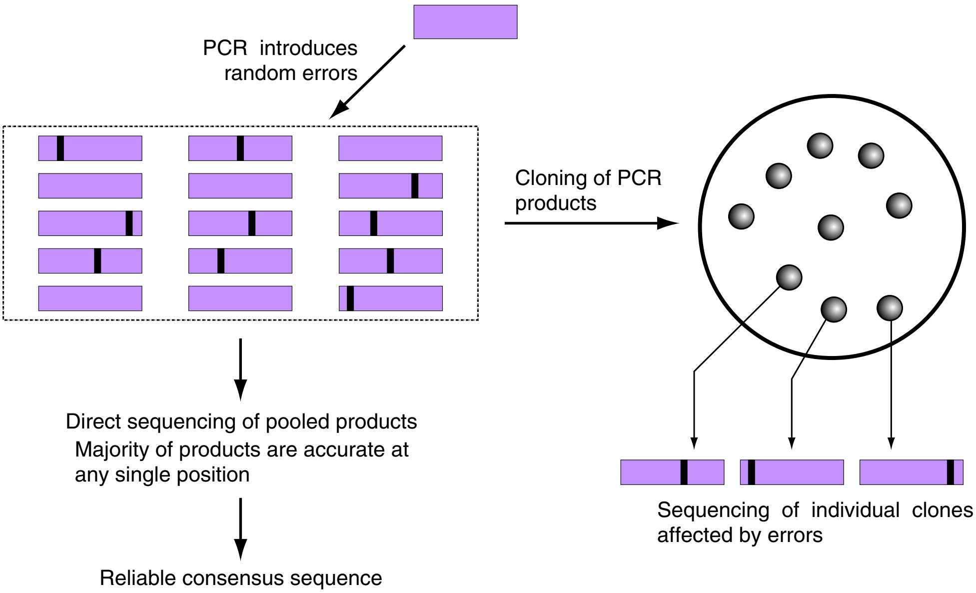

Figure 9.6 Detection of PCR errors

the defects will be randomly distributed and the vast majority of molecules will be correct in any given position. However, if the PCR products are cloned it will potentially be a problem, because any error in one molecule will be perpetuated in all the offspring of that one clone (Figure 9.6). For this reason, investigators normally sequence at least three PCR clones in order to ensure that they are identical.

Another consequence of the relative sloppiness of *Taq* polymerase is the fact that *Taq* polymerase can only efficiently amplify fragments of a few thousand base pairs. Both these problems can be solved by the introduction of thermostable DNA polymerases from other thermophilic organisms, such as *Pfu* and *Pwo* DNA polymerases. Unlike *Taq* polymerase, these enzymes do have proofreading activity. They are not necessarily as efficient, however. This has been ingeniously overcome by the introduction of proprietary mixtures of *Taq* and proofreading DNA polymerases. By combining proofreading and high yield, these mixtures allow the efficient amplification of DNA fragments as large as 50 kb.

9.5 Reverse-transcription PCR

In Chapter 7 we described the use of reverse transcriptase to obtain a cDNA copy of mRNA, and the construction of cDNA libraries. Combining reverse transcription (RT) with PCR, a procedure known as RT–PCR, extends the application of PCR into the analysis of gene expression, either qualitatively or quantitatively, as well as greatly facilitating the construction of cDNA libraries or the cloning of specific cDNAs. The use of RT–PCR for the analysis of gene expression is described in Chapter 13.

One problem with RT–PCR is that the initial mRNA preparation is likely to be contaminated with DNA. The PCR step can then result in amplification of the contaminating DNA even if no mRNA was present (and hence no cDNA produced by reverse transcription). When working with eukaryotic material, this can often be overcome by designing primers using sequences from adjacent exons. In this way, amplification of any genomic DNA will either be prevented altogether (because the presence of the intervening intron makes the sequence too large to be amplified under the chosen conditions), or readily distinguished from amplification of cDNA (because of the different size of the product). Otherwise, it is necessary to remove all traces of DNA from the mRNA, usually by treatment with DNase. This in turn creates further problems. The DNase has to be removed before the reverse transcription step. It is also important to avoid any contamination of the DNase with RNase, which would destroy any mRNA Ribonuclease is a very stable enzyme, not destroyed by heat, so it used to be very difficult to remove it without also destroying the DNase. Fortunately there are now commercial preparations of DNase that are free of ribonuclease, and these are now sufficiently reliable to be used for this purpose. However, it is essential that you use an RNase-free DNase rather than the cheaper grades.

9.6 Rapid Amplification of cDNA Ends (RACE)

The procedure described in Chapter 7 for generating cDNA suffers from the problem that the products are often incomplete, i.e. the ends of the mRNA molecules are under-represented in the clones generated. In Chapter 7 we considered ways of at least partially overcoming this problem, using random priming and tailing with terminal transferase. PCR provides further enhancement of these techniques, by specifically amplifying the ends of mRNA molecules.

The method known as *rapid amplification of cDNA ends* (RACE) is based on the amplification between a gene-specific primer (GSP) within a known area, and a universal primer at either end of the cDNA molecule. Note that the use of a gene-specific primer is only possible if you already know, or can in some way infer, the sequence of at least a small part of the gene.

The most straightforward type is 3′ RACE (Figure 9.7), which allows amplification of cDNA corresponding to the 3′ end of the mRNA. As in the description of cDNA synthesis in Chapter 7, this method takes advantage of

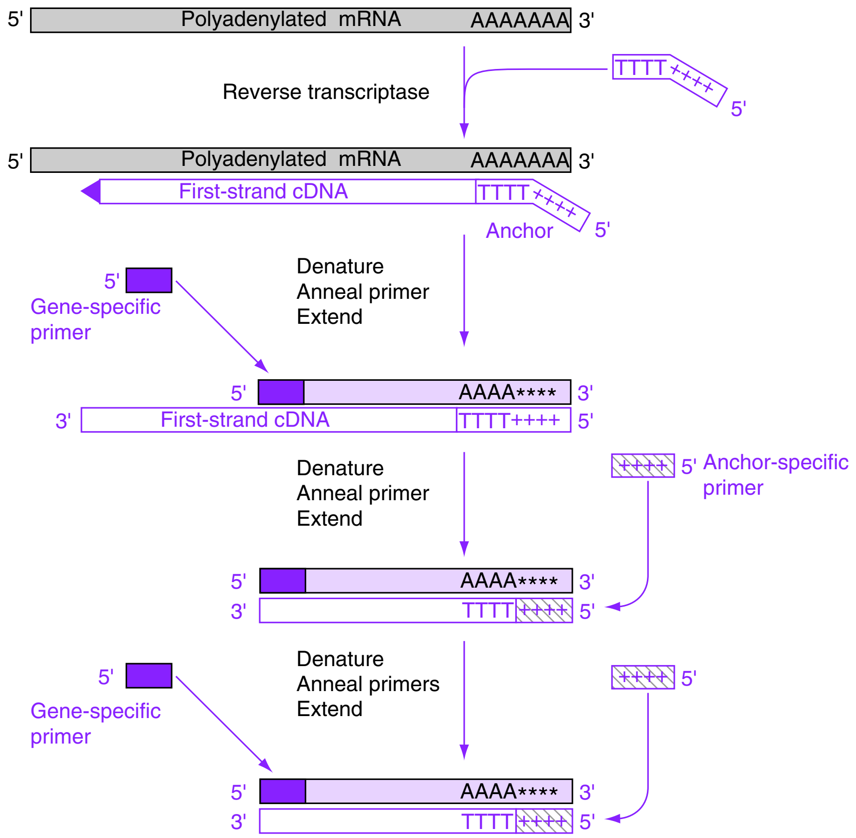

Figure 9.7 Rapid amplification of cDNA ends: 3′ RACE

the fact that eukaryotic mRNA has a poly-A tail. This tail is used as the binding site for a complementary, universal oligo-dT primer, for synthesis of the first cDNA strand. For 3′ RACE, this primer has an additional sequence, known as an *anchor*, which provides a more effective site for subsequent amplification (and often includes a restriction site to assist in subsequent cloning). The second cDNA strand is initiated from a gene-specific primer. The resulting double-stranded DNA can then be amplified by the usual PCR procedures, using the original primers, or replacing the oligo-dT-anchor primer with one just containing the anchor sequence.

Normally, PCR would use two specific primers. In the basic 3′ RACE system, as shown in Figure 9.7 , one primer (the GSP) is specific for the gene concerned, while the other primer (the oligo-dT-anchor primer) will act as primer for any polyadenylated mRNA. The specificity of the system can be enhanced by a nested PCR in which further amplification takes place using a second, nested primer (also specific for the gene in question) in place of the GSP.

In a similar way, 5′ RACE enables amplification of cDNA corresponding to the 5′ end of the mRNA. The key feature of this procedure is the incorporation of a universal tag at the 3′ end of all first strand cDNA molecules. This is accomplished by the enzyme terminal deoxynucleotidyl transferase, usually known – unsurprisingly – as terminal transferase or TdT. This enzyme will non-specifically add deoxynucleotides to the 3′ end of a single DNA strand. By making only one dNTP available, for example deoxycytosine, a 5′ tail analogous to the 3′ poly-A tail can be added.

In the procedure shown in Figure 9.8, the mRNA is initially reverse transcribed using random primers, which are more likely to produce first strand cDNA corresponding to the 5′ ends of the mRNA than using oligo-dT primers. This is a non-specific step, and will produce a mixture of single-stranded cDNA molecules, ideally reflecting all the mRNA present in the original material. The

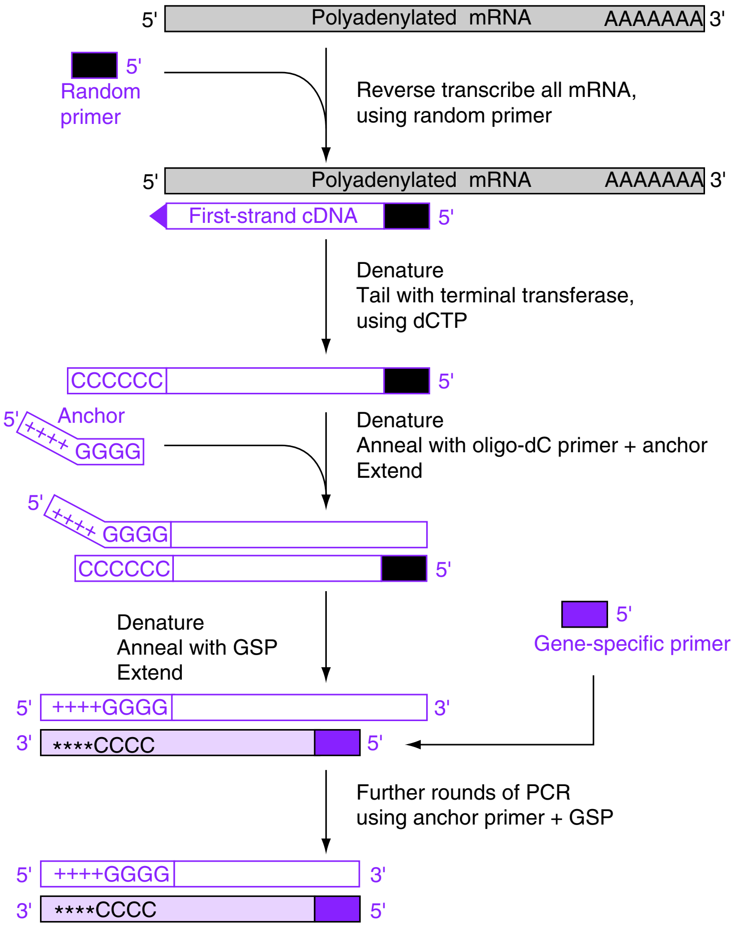

Figure 9.8 Rapid amplification of cDNA ends: 5′ RACE

cDNA pool is then tailed with terminal transferase, to provide a binding site for an oligo-dG primer coupled to an anchor sequence, for second-strand cDNA synthesis. This is then followed by PCR amplification, using the anchor primer and a gene-specific primer. As with 3′ RACE, additional specificity can be gained by using nested PCR, with a second specific primer replacing the first GSP.

Thus, even if a gene is novel, PCR can be used to obtain the entire cDNA sequence without ever using a vector-based library. This has made many laboratories abandon cDNA library screening altogether, although it is currently experiencing a revival thanks to the development of array libraries from national genome facilities.

9.7 Applications of PCR

9.7.1 PCR cloning strategies

PCR can be used in several ways to provide alternatives to the screening of random libraries as described in Chapters 7 and 8. For example, analysis of the known sequences of a set of related genes may show that although the genes are not similar enough to devise reliable hybridization probes, there may be some regions that are relatively highly conserved. It is possible to construct pairs of primers directed at these conserved regions, and use these to amplify the corresponding fragment from the genomic DNA of your target organism (Figure 9.9). Nested PCR is often used to add to the power of this approach. You can then sequence this product, to try to confirm that it is indeed derived from the correct gene, and you can use it as a probe for screening a gene library to isolate clones carrying the complete gene.

This approach needs to be treated carefully. One reason part of a gene may be highly conserved is that it codes for an essential substrate-binding site, so any gene which uses the same substrate is likely to have a similar sequence. For example, many enzymes that use ATP as a substrate have a similar sequence that represents the ATP-binding site. Therefore, other methods have to be used to determine the real function of your novel gene.

On the other hand, if you already know the sequence of the gene, then you can devise a reliable PCR system to amplify the whole gene or part of it, whether from genomic DNA or cDNA, and either clone or sequence the product. Cloning and/or sequencing a gene of which you already know the sequence is much more useful than it sounds. For example, you may want to know whether the sequence varies at all in different strains (see Chapter 12). PCR amplification and sequencing provides a quick route to answering that

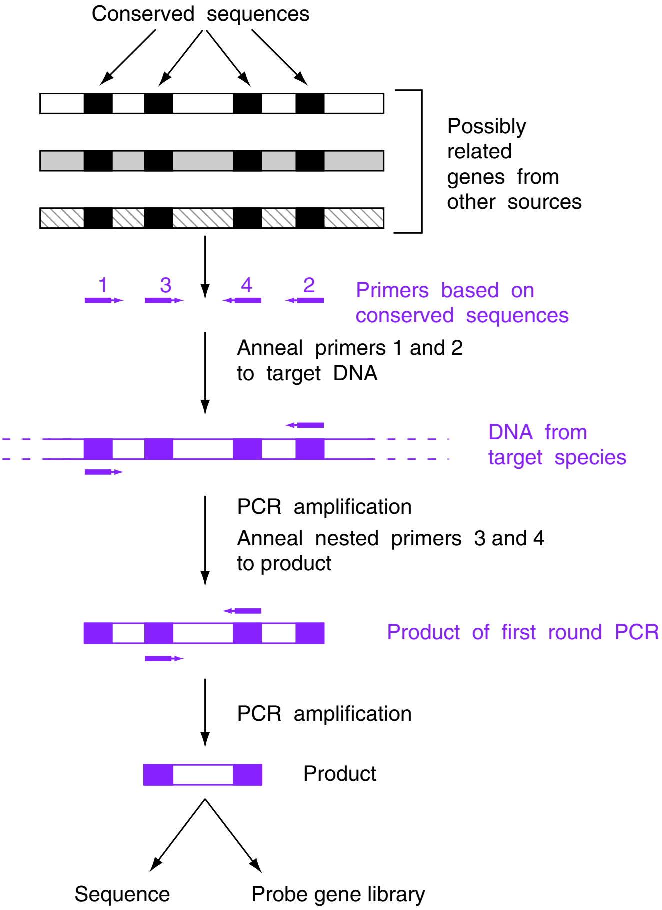

Figure 9.9 Use of conserved sequences to design PCR primers

question. It is also employed in medical genetics in the search for individual sequence differences which may cause disease.

Alternatively, you may have cloned a gene in one vector and you then want to re-clone it in a different plasmid – perhaps moving it into an expression vector, or removing extraneous DNA that was present in the original clone from a random gene library. You can easily devise a pair of primers that will produce exactly the product you want – down to adjusting the reading frame to fit in with your expression vector (see Chapter 13), or adding or removing restriction sites at the end of the fragment to aid subsequent manipulation.

In Chapter 15, we discuss the introduction of changes into the coding sequence of a gene (*site-directed mutagenesis*); PCR plays a major role in this as well. A further topic in Chapter 15 is the use of a procedure known as *assembly PCR* for assembling synthetic genes. There is virtually no end to the versatility of the procedures to which you can adapt PCR as an aid to cloning and re-cloning DNA fragments in a research laboratory.

9.7.2 Analysis of recombinant clones and rare events

The best way of characterizing a recombinant clone, to make sure you have indeed produced the structure that you intended, is to sequence it – or at least to sequence across the new junctions that have been made. For this, you can either directly sequence the appropriate part of the recombinant plasmid, or you can amplify across the inserted fragment and sequence the PCR product.

PCR can also be used to detect specific events that occur *in vivo*, without having to clone the affected region of the genome. For example, if we want to detect the transposition of an insertion sequence into a specific site, or if we want to test if our gene knockouts (see Chapter 17) have really inserted a foreign DNA fragment into that site, we can use PCR. We would use one primer directed at the insert and one at the genomic flanking sequence. We would only get a product if the event we are looking for has indeed occurred. Otherwise the two primers are binding to completely different bits of DNA.

However, this needs a word of caution which applies to some extent in many applications of PCR. The technique is splendid for amplifying DNA where a genuine target exists. When there is no genuine target, there is the possibility of artefacts being created, due to the power of the technique for amplifying extremely rare events, such as the possibility of the polymerase 'jumping' from one DNA molecule to another. This may seem highly unlikely (although there are reports of it happening) – but it only needs to happen once, in the early stages, and the product produced will then be amplified fully effectively, leading to an incorrect conclusion.

A similar effect can occur if the template contains a stable hairpin (or stem-loop) structure. This will cause the polymerase to pause (or even to stop altogether). However, while it is paused, it may encounter the other side of the hairpin and 'jump' across – yielding a product that lacks the hairpin. Although this rarely happens, the product will then amplify much more effectively than the original template, and the final product will be shorter than it should be, leading to the suggestion that your strain contains a deletion at that point.

9.7.3 Diagnostic applications

The polymerase chain reaction has many important practical applications of PCR (i.e. in the real world outside molecular biology laboratories). Amongst these are forensic applications – the detection and identification of specific DNA fragments that can be traced to a particular individual – and uses in medical diagnostics, including the detection of mutations causing human genetic diseases. PCR can be employed for the detection of pathogenic microorganisms, especially those which are difficult to culture, and in particular it plays a central role in the detection of many viruses, such as HIV. In these contexts, the problem of contamination has to be taken very seriously. The ability of PCR to amplify tiny amounts of DNA makes it an extremely sensitive test, but it also makes it extremely sensitive to low levels of contaminating DNA. In a diagnostic situation, extensive measures have to be taken to avoid contamination, notably the use of separate rooms and equipment for the preparation of the clean (pre-PCR amplification) material and the analysis of the post-PCR products (which contain large amounts of material that can act as template for further amplification. A battery of additional precautions must be taken, including the use of negative controls with each batch of samples. We will look at some of these applications further in later chapters.

For some applications, it is desirable (or even necessary) to be able to use PCR not merely to *detect* a specific template, but to determine *how much* of it is present. The best methods for quantitative PCR are collectively known as *real-time PCR*, since you can monitor the accumulation of product during the reaction, rather than merely assaying it at the end. This also has advantages in diagnostic applications, since a result is obtained much more quickly. Real-time PCR is described more fully in Chapter 13.

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10 DNA Sequencing

In 1975, the first complete DNA genome was sequenced – that of the small bacteriophage øX174 (5 kb). Other complete sequences followed gradually, including the SV40 virus (5 kb, 1977), human mitochondrial DNA (16 kb, 1981), bacteriophage lambda (49 kb, 1982) and the Epstein–Barr virus, containing 170 kb of DNA (1984). A new dimension was opened up in 1995 by the first sequence of the genome of an independently living organism, that of the bacterium *Haemophilus influenzae*, which is ten times larger (1800 kb). Only six years later, in February 2001, two competing entities, the publicly funded Human Genome Project and the private company Celera, published the human genome sequence of no less than three billion base pairs. On the way, we have seen the complete genomic sequences of important model organisms such as *E. coli*, the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila*, and the plant *Arabidopsis*. All of these have been received with great excitement and interest in the media, especially of course the human genome sequence – not least because of the rivalry and acrimony between the two consortia involved, and the failed attempts at co-operation. From a scientific point of view, these projects are obviously revolutionary. They are also themselves the result of a technical revolution – the development of sophisticated high-throughput sequencing technology.

Sequencing is the primary way of characterizing a macromolecule, whether it be determining the order of amino acids in a protein or of bases in a nucleic acid. Protein sequencing was a very important tool before genes could be cloned and sequenced. With the advent of recombinant DNA technology, however, it was superseded by the much more efficient method of DNA sequencing, with the sequence of the encoded protein being deduced from the sequence of the gene. Although direct protein sequencing is currently experiencing a renaissance, this chapter is dedicated to the sequencing of DNA.

10.1 Principles of DNA Sequencing

The most fundamental way of analysing the structure of DNA, whether it is a recombinant plasmid, a natural gene, or a whole genome, is to determine the sequence of bases of which it is composed. The advent of automated DNA sequencing machines has made the determination of the sequence of individual fragments very much faster and more reliable, and the dramatic increase in the available computer power has enabled the assembly of very large numbers of fragments (as well as the analysis of very large sequences) – but at the same time the underlying principle of the sequencing method remains the same.

The principle of this procedure, known as dideoxy sequencing or the Sanger method, is illustrated in Figure 10.1. (It should be noted that there is an alternative procedure, known as the Maxam–Gilbert method, which works by selective chemical degradation. This is no longer in common use for routine sequencing, and will not be described here.) To understand the dideoxy procedure it is necessary to remember two fundamental facts about DNA synthesis: first, synthesis of a DNA strand does not start from scratch. It requires a

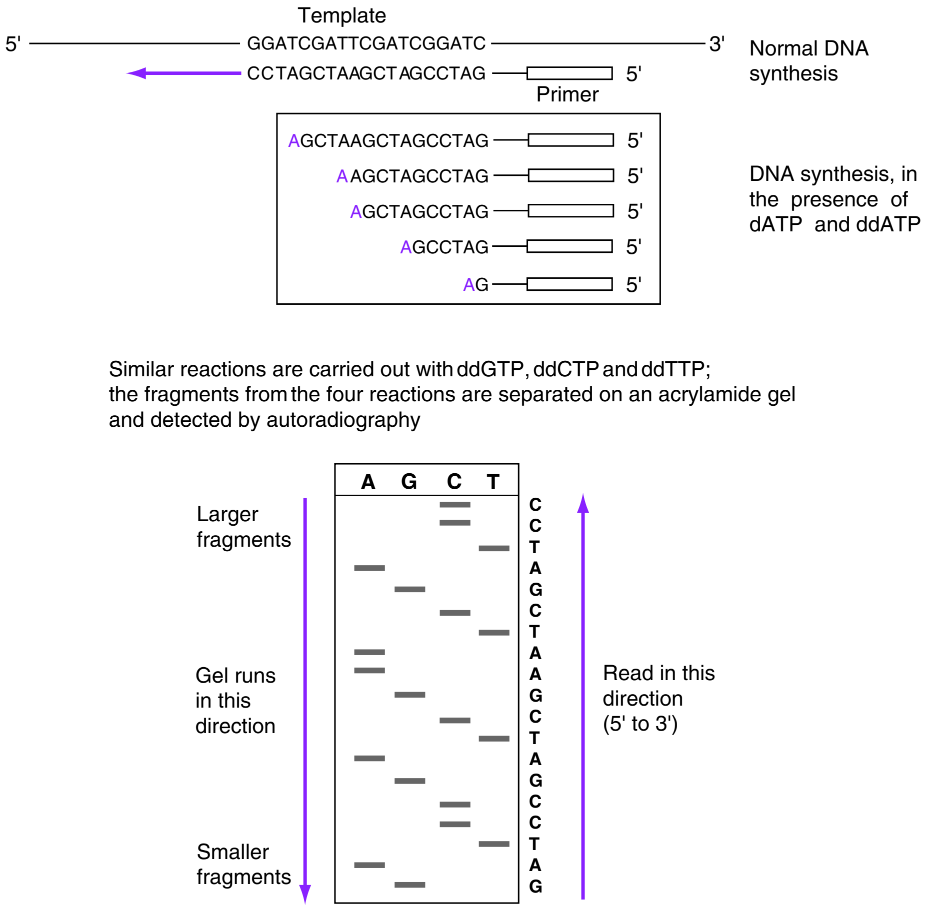

Figure 10.1 Determination of DNA sequence

primer annealed to the template strand. Synthesis of the new strand works by adding bases to the primer that are complementary to the template. So by using a primer that will bind to a specific position on the template we can ensure that all the new DNA that is made starts from the same point. Second, the addition of bases to the growing strand occurs by formation of a covalent phosphodiester bond between the 5′-phosphate on the nucleotide to be added and the 3′-OH group on the existing molecule. The substrate for this reaction is a 5′-dNTP, i.e. a deoxynucleotide with three phosphates at the 5′-position of the deoxyribose sugar; two of these phosphates are eliminated in the reaction.

The sugar part of the natural substrate is more specifically a 2′-deoxyribose, i.e. it does not have a hydroxyl group at the 2′-position (this distinguishes it from the ribose sugars that occur in RNA). However, it does have a 3′-OH group, which is necessary for the formation of the next phosphodiester bond when the next base is incorporated. What happens if we use, instead of the natural substrate, one in which there is no 3′-OH group, i.e. a 2′,3′-dideoxy derivative, a ddNTP (see Figure 10.2)? This can be incorporated into the DNA, by formation of a phosphodiester bond between its 5′-phosphate and the 3′-OH on the previous residue. However, that reaction produces a strand which does not have a 3′-OH at the end, and so no further bases can be added. DNA synthesis will therefore terminate at that point.

So if we replace one of the dNTPs with a dideoxy derivative – for example we use a mixture of dGTP, dCTP and dGTP (the normal substrates) but replace dATP with the 2′,3′-dideoxy derivative which we will label ddATP – then DNA synthesis will proceed only as far as the first A residue and will then stop (Figure 10.3). If we carry out a set of four such reactions, in each case replacing one of the dNTPs with its corresponding ddNTP, we would produce four molecules of different lengths, each proceeding just as far as the first occurrence of the relevant nucleotide in the sequence.

Just determining the first occurrence of each base would not be much use. However, instead of completely replacing, say, dATP with ddATP, we can use

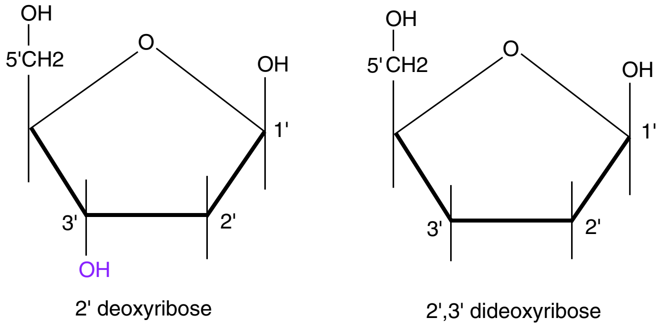

Figure 10.2 2′ deoxyribose and 2′, 3′ dideoxyribose

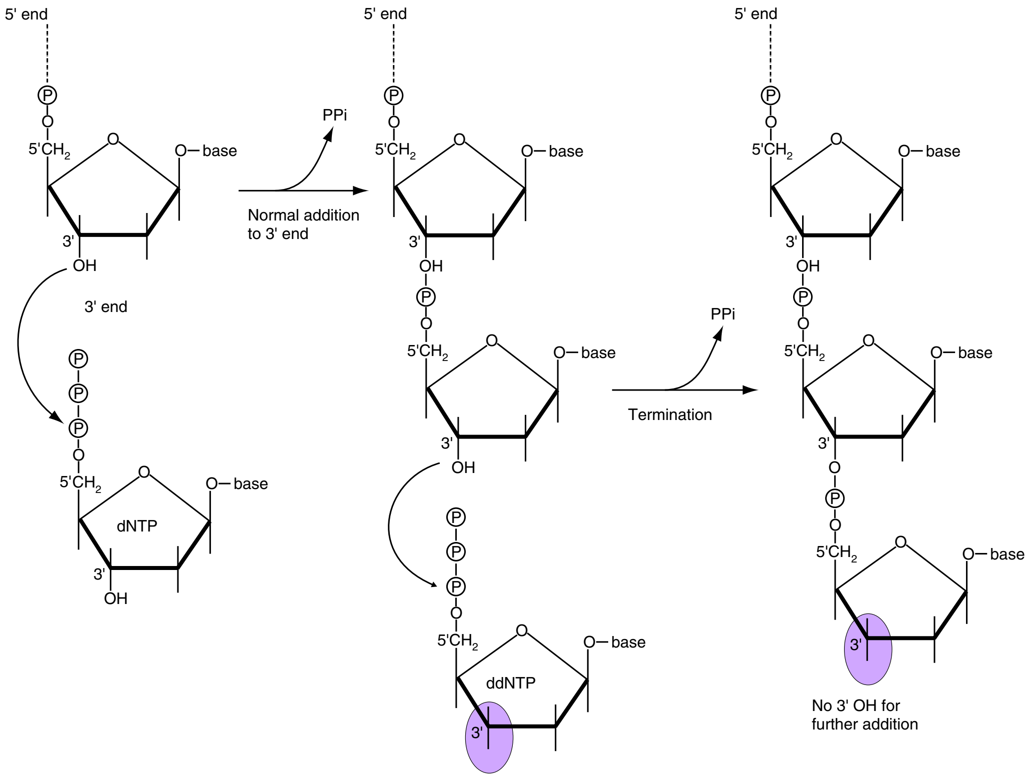

Figure 10.3 Chain termination by ddNTP

a mixture of the two substrates. So at the first T residue in the template, a few molecules of the new strand will have ddA added (and will therefore terminate) but most will have the normal A residue, and the reaction will be able to proceed. At the next T in the template, some more molecules will terminate, and so on. We will thus have a series of molecules of different chain lengths, each ending with ddA (see Figure 10.1). These can be separated by electrophoresis in a polyacrylamide gel, using denaturing conditions to prevent the DNA strands from folding up. We also use a gel that is much thinner and longer than normal acrylamide gels. This increases the resolving power of the gel so that it can resolve differences in length as small as a single base. The molecules are separated on the basis of their size, with the smaller molecules running faster through the gel. We therefore get a series of bands, corresponding to the positions of A residues in the new strand (or T residues in the template). With a set of four reactions, each using a different ddNTP, we get a set of four lanes, from which the sequence can be read as shown in the figure.

For manual DNA sequencing, we would normally carry out the reaction with one of the dNTPs radioactively labelled, so that exposure of the gel to an X-ray film would result in a pattern of black bands on the film. Because the smallest fragments (which are closest to the primer) will migrate faster, they will be at the bottom of the gel; the gel is therefore read from bottom to top.

10.2 Automated Sequencing

Today, virtually all DNA sequencing uses automated sequencers. Although the principle is the same, the method of detection is different. For automated sequencing, either the primer or the ddNTPs are labelled by incorporation of a fluorescent dye. Thus, rather than running the gel for a finite time and reading the result, the machine uses a laser to read the fluorescence of the dye as the bands pass a fixed point. Much longer sequences can be read from each track in this way. A further advantage is that the sequence read by the machine is fed automatically into a computer. This is not only much quicker than reading a gel manually and typing the resulting sequence into a computer, but also avoids the errors that are virtually inescapable with manual data entry. For larger scale genome sequencing projects, the processes of sample preparation, setting up the sequencing reactions, and loading the products into the sequencer, are also automated, using robotic methods.

If the primer is labelled, all the products carry the same dye, and so you still have to use four lanes. However, if the four ddNTPs are each labelled with different dyes, the sequencing reactions can be performed in a single tube and separated in a single lane, thus increasing the capacity of the machine. A further development on this is to replace the polyacrylamide gel, which needs to be cast anew for each run, with a reusable matrix-filled capillary. Apart from the development of supervectors which allow the cloning of very large DNA fragments (see Chapter 6) and the enormous increase in computer power, it is this development of simplified automatic sequencers, where sample preparations and sample loading are performed by robots, that have made possible the sequencing of eukaryotic genomes such as the human one. The strategies for large-scale genome sequencing are considered later in this chapter.

Both manual and automated DNA sequencing methods suffer from problems that can give rise to errors in the sequence. These are often associated with the presence of certain combinations of bases in the DNA – for example runs of identical nucleotides, when it can be difficult to determine exactly how many bases there are in the run, and the presence of secondary structures in the DNA such as hairpin loops, which can interfere either with DNA synthesis (causing premature termination) or with the running of the fragment on the gel. Some of these potential errors can be identified and minimized by altering the reaction conditions, by sequencing a different overlapping fragment covering the problem region, and by determining the sequence of the complementary strand.

A good complete sequence will therefore be derived by reading and assembling several overlapping sequences in each direction.

10.3 Extending the Sequence

Although automated DNA sequencers allow much longer reads than manual methods, the length of sequence that can be obtained from a single run is still limited. As the fragments get larger the degree of separation between the bands is less, and the amount of product in each band is reduced, making the signal weaker. (In addition to the required termination due to incorporation of dideoxynucleotides, synthesis may pause or even stop for other reasons, such as secondary structure in the DNA.) The sequence thus becomes progressively less reliable. Sometimes this is not a problem: if we are using sequencing to verify the structure of a recombinant plasmid that we have made, or if we are looking at the variation of a specific region between different strains, then the sequence of a few hundred bases may be quite sufficient. However, if we want to sequence a whole gene, or especially a whole genome, then we will usually require longer sequence data.

One strategy for extending the length of sequence determined is referred to as *walking* (Figure 10.4). Remember that the sequence depends on DNA synthesis starting from a specific primer. Usually, with an unknown cloned sequence, we would start with a primer directed at the cloning vector close to the point of insertion. Since most sequencing projects use the same cloning vector (either a pUC plasmid or an M13 vector, which have the same sequence flanking the cloning site), we can use the same primers for any clone. These are therefore known as *universal primers*.

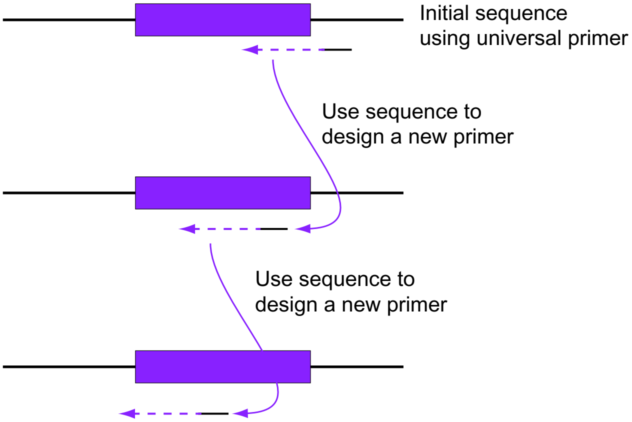

Figure 10.4 Extending a sequence by primer walking

The (forward) primer from one side of the insert will read the sequence in one direction, while the sequence of the complementary strand will be obtained using the (reverse) primer from the other side of the insert. The sequence we obtain would thus start with a small amount of vector sequence (which has to be removed prior to analysis or to deposition in a databank, see below) and would then read through into the unknown insert. We can extend the length of sequence determined by using the first piece of data to design a new primer that would start synthesis further along the insert. That will produce a further length of sequence, which can then be used to design a third primer, and so on. This procedure is quite effective for relatively short sequences but becomes excessively tedious if long stretches of DNA are to be sequenced. It can, however, be a useful strategy for finishing larger sequences (such as whole genome sequences) when other approaches have left short gaps (see below).

10.4 Shotgun Sequencing; Contig Assembly

For sequencing a longer fragment – say a cloned fragment of 10 kb – the best procedure is to split it up into smaller fragments, each of which is a suitable size for sequencing. This is like producing a gene library. The insert from your recombinant vector is fragmented and cloned using a suitable vector. The bacteriophage vector M13 (see Chapter 6) is useful for this purpose, since it will produce single-stranded versions of your fragments which often give cleaner results. However, improvements in the technology mean that good results can also be obtained with double-stranded plasmid templates.

Note that it is *essential* to have overlapping fragments in this library, so mechanical fragmentation is the best procedure (see Chapter 7). You then pick recombinant clones at random from this mini-library and sequence each of them – an approach known as *shotgun* sequencing (see Figure 10.5). At the start, you will have no idea where each bit of sequence comes from in the original fragment – nor even which strand of the original it is derived from. However, once you have a number of such fragments sequenced, you can start to use computer packages to compare each bit of sequence to all the others. The computer will find any overlaps between the fragments, including comparing the complementary strand in case you have two fragments that overlap but are derived from different strands. Where there is an overlap, those fragments will be joined together to form a *contig*. As the project progresses, each contig will grow longer, and will then start to overlap other contigs. So the contigs themselves are joined together until eventually you have one single contig covering the whole of the original piece of DNA, and all the sequenced fragments fit into that single contig (see Figure 10.6).

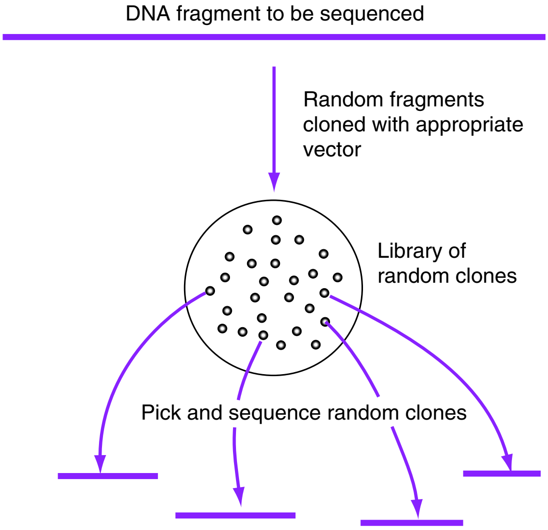

Figure 10.5 Shotgun cloning and sequencing

Of course, while initially each piece of sequence is new information, as you proceed the sequencing becomes less and less productive. Towards the end, nearly all the clones sequenced will be completely contained within one of the existing contigs. Nevertheless, provided all parts of the fragment are equally represented in the library, it is quicker to continue with a shotgun approach rather than try to screen the library for the missing pieces. Sometimes, however, parts of the original fragment are under-represented in the library; they may be difficult to clone for some reason. If this is the case, you will have a gap in your sequence that can be difficult to fill with a shotgun approach. If you have reason to believe that the gap is quite small then you can use primer walking; as described above, to bridge the gap. Alternatively you can use the sequences you have determined to design PCR primers that will enable you to amplify a DNA fragment that spans the gap, and can then be sequenced. Other approaches for bridging gaps in longer sequences, such as genome sequences, are considered below.

For any sequencing project, whatever the size of the DNA to be sequenced, and whatever strategy is used, a diminishing returns effect is very marked. You may get 90 per cent of the sequence accurately determined quite quickly; the next 9 per cent may take as long again; and then the next 0.9 per cent a similar length of time. It is usually necessary to set some limits to this: how complete and how accurate do you need the sequence to be? Will 90 per cent do, or must you have 99 per cent or 99.9 per cent? Without some sort of compromise you can be drawn into a costly exercise trying to determine whether one difficult base in a sequence of a million bases is G or C.

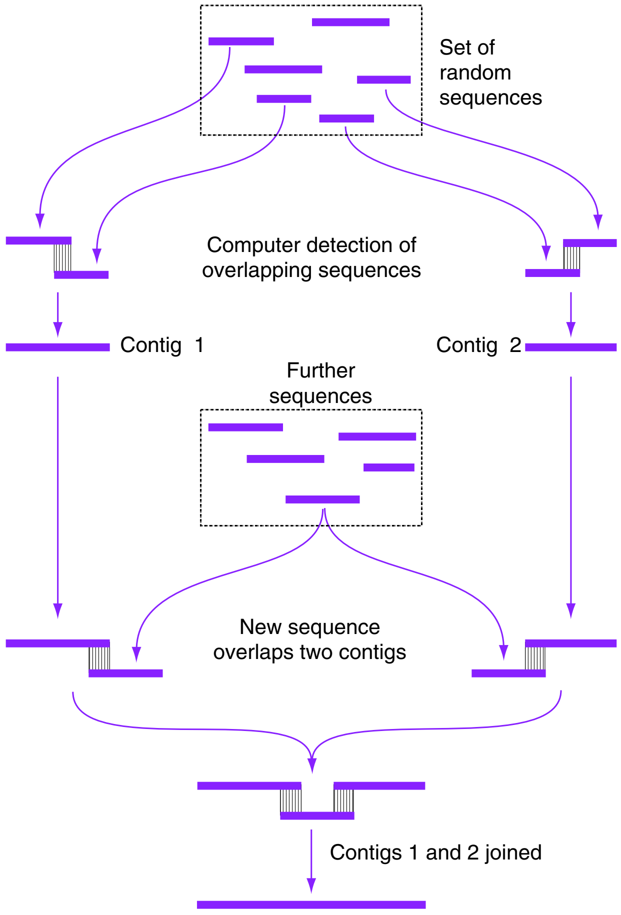

Figure 10.6 Contig formation and joining

10.5 Genome Sequencing

10.5.1 Overview

In 1995, the first sequences of complete genomes (excluding viruses) were published; these were of two bacteria, *Haemophilus influenzae* and *Mycoplasma genitalium*. Although these are fairly small (1.83 Mb and 0.58 Mb, respectively) in comparison with other bacteria, let alone mammalian or plant genomes, they are much larger than the examples we have been considering so far. These have been followed by sequences of the genomes of a number of other bacteria, as well as some eukaryotes, including yeast (*Saccharomyces cerevisiae*) and of course the human genome sequences. Some examples are shown in Box10.1.

Box 10.1 A selection of sequenced genomes

Organism	Size (Mb)
Archaea	
Methanococcus jannaschii	1.66
Archaeoglobus fulgidus	2.18
Bacteria (prokaryotes)	
Mycoplasma genitalium	0.58
Campylobacter jejuni	1.64
Haemophilus influenzae Rd	1.83
Mycobacterium leprae	3.26
Mycobacterium tuberculosis	4.41
Pseudomonas aeruginosa	6.30
Streptomyces coelicolor	8.67
Eukaryotes	
Saccharomyces cerevisiae (yeast)	13
Caenorhabditis elegans (nematode)	97.1
Arabidopsis thaliana (plant)	118
Drosophila melanogaster (fruit fly)	135.6
human (about 70 per cent ‘finished’; remainder in draft form)	3200 (euchromatic, estimated)

The table lists only a small selection of finished genome sequences. For fuller, up-to-date, lists (including projects in progress) see the following web sites.

European Bioinformatics Institute*http://www.ebi.ac.uk/genomes/mot/index.html*
Genome Monitoring Table. Daily updates on progress of genome sequencing

The Institute for Genomic Research
http://www.tigr.org/tdb/mdb/mdbcomplete.html
http://www.tigr.org/tdb/mdb/mdbinprogress.html
Lists microbial genome sequences, complete and in progress, respectively

Genome Sequencing Center, Washington University School of Medicine
http://genome.wustl.edu/gsc/

Human genome sequencing progress:
http://www.ncbi.nlm.nih.gov/genome/seq/
http://www.gdb.org/hugo/ (Human genome organization)

The speed of progress in genome sequencing is such that it is no longer sensible to produce a definitive list; it would be long out of date before this book is published. However, you can see lists of sequenced organisms (and access a lot of information about the sequences) at the Web sites listed. You will find information not only about completed projects but also projects that are still in progress. Some of the genome projects (including the Sanger Centre) have a policy of releasing all sequence data as it becomes available, on a daily basis. This is raw sequence with no annotation (i.e. nothing to tell you what it codes for or any other information), but it can be searched for the occurrence of specific sequences, which is very useful to a research worker.

Once the contigs have been assembled and a stage is reached when a reasonably confident decision can be made as to where all the bits go, then it can be labelled as a *draft sequence*. Note that at this stage there are likely to be gaps in the sequence, although informed predictions can be made as to the position and size of those gaps. There will also be some uncertainties and errors in the sequence. The next stage is the finishing process, which involves filling in the gaps and correcting the more obvious errors and uncertainties. A *finished* sequence will contain no known gaps, and will be accurate to a defined level. The final stage is *annotation*, when protein-coding sequences are identified and predictions made as to the nature of the products. A wide variety of other features are also identified at this stage. (Annotation and further analyses are considered in Chapter 11.)

This description of a genome sequencing project makes it sound like a production line, and indeed the analogy is not inappropriate. In a large project, the various stages – cloning, sequencing, assembly, finishing, annotation – are carried out by separate teams, and the product from one team (clones or sequence data) is passed on to the next stage. However, you should not take the analogy too literally – information flows both ways, and one stage does not have to be completed before the next stage starts, e.g. assembly takes place concurrently with the generation of sequence data.

At the end of the process we have a finished (and annotated) sequence, and we would say that we have determined the complete genome sequence of the target organism We need to consider what we mean when we say this. For the bacterial genomes that have been sequenced, this is literally true – we know (within the chosen limits of accuracy) which base is present at every single position in the genome. However, no eukaryotic genome has been completely sequenced to this level. For these eukaryotic genomes, the definition of 'finished' is set lower, for example to mean more than 95 per cent of the euchromatic regions (which contain most of the genes) sequenced (to the set standard of accuracy). with no gap more than 150 kb. (This definition will vary from one project to another.) The published human genome sequences do not meet even this definition. Overall, at the time when the data were prepared for publication, the coverage was about 85 per cent (or about 90 per cent of euchromatin), and both versions had a large number of gaps in them. The published sequences were therefore labelled as rough drafts. The coverage has increased since the original publication of the human genome sequence data, and at the time of writing about 70 per cent of the euchromatin is considered finished, with the remainder being in draft form. (See the web sites listed in Box 10.1 for an update on these figures.) Furthermore, these are not unique sequences of single individuals, but are a composite of sequences from several sources – unlike the bacterial sequences which are derived from a single strain of that species. These are probably not serious limitations. For example, many of the gaps are due to the occurrence of repetitive DNA (which can be difficult to sequence); this is assumed to be 'junk' DNA. Most, if not all, of the significant coding sequences will be present in the sequence that has been determined – and therefore for practical purposes we 'know' the sequence of the human genome.

10.5.2 Strategies

Various strategies, or combinations of strategies, can be used for determining sequences of DNA as long as a complete genome (or a complete chromosome). One is to extend the concept of shotgun sequencing as described above, that is simply to make a library of small random fragments of the whole genome and sequence them (Figure 10.7). This requires the isolation and sequencing of a

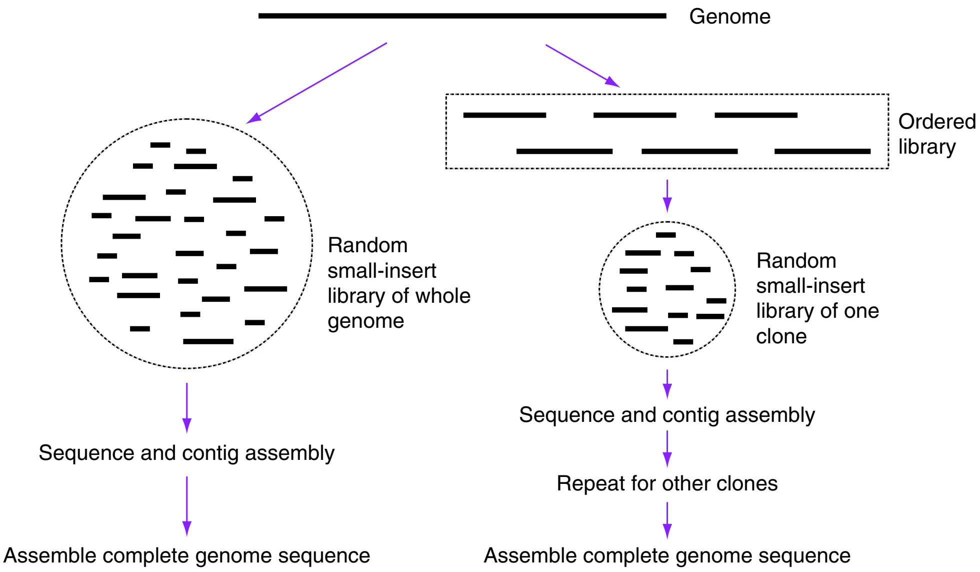

Figure 10.7 Genome sequencing strategies

vast number of clones, which is made possible by the use of robotic methods for all stages of the process – picking the clones, extracting the DNA, setting up the sequencing reactions. Furthermore, the contig assembly of such a large number of individual random sequences requires a very substantial amount of computer power. This is not an impossible strategy; many bacterial genomes have been sequenced in just this way, and this formed a major part of the strategy for one of the human genome sequencing projects. However, it is not the only way of doing it.

The main alternative is to split the problem up into defined units first. For a eukaryotic organism, you might want to separate the chromosomes to start with, and treat each chromosome as a separate project. Then (or instead), you can make a library representing the whole genome or chromosome, using a vector that can carry appropriate sizes of insert (e.g. a cosmid). You would then determine the sequence of the insert in individual cosmids, and subsequently assemble the cosmid sequences into a complete genomic sequence. Some of the sequencing projects have started with random cosmids, while others have attempted to arrange the cosmids into an ordered library first (i.e. one in which the relative position of all of the cosmids has been defined – see Chapter 7).

One advantage of this strategy is that very many laboratories are capable of determining a sequence of the size of an individual cosmid insert, so it is possible to distribute the cosmids to different laboratories for sequencing. On the other hand, the total shotgun approach is highly centralized and only possible in a very few centres. A further advantage of the ‘clone by clone’ approach is that useful information is generated at intermediate stages in the project – the sequence of an individual cosmid is a coherent piece of information – while the shotgun approach consists of large numbers of essentially meaningless sequences of small fragments until sufficiently large contigs have been assembled. On the other hand, the shotgun method does start generating sequence data almost from the beginning, without having to produce cosmid libraries.

10.5.3 Repetitive elements and gaps

Two problems encountered in genome sequencing are worth considering: repetitive elements and gaps. Most genomes contain *repetitive elements*: identical sequences which occur more than once (often many times) in the genome. The amount of repetitive DNA varies substantially between organisms, ranging from a few percent to at least 50 per cent in the human genome. There are various classes of repeat sequences, including *dispersed* (or *interspersed) repeats*, mainly mobile elements such as insertion sequences and transposons, which occur at different sites distributed around the genome, and *tandem repeats* of shorter sequences (even as short as 1–3 nucleotides) which occur many times in succession at one locus. There may also be segmental duplications, where a block of perhaps several hundred kilobases of DNA has been copied to a different region of the genome. Any repetitive element is likely to cause problems in contig assembly. Two fragments of sequence that end with part of a repetitive element may be identified by the computer as overlapping, when in fact they are derived from quite different parts of the genome. This is more of a problem with the total shotgun approach than when sequencing individual cosmids, because the separate cosmids are much less likely to carry more than one copy of an insertion sequence. Tandem repeats cause a rather different problem: the overlapping fragments are genuinely adjacent, but the number of copies of the tandem repeat may be miscounted.

An additional factor has also to be borne in mind: many of these repetitive elements are inherently hypervariable; mobile elements can transpose to different sites, and tandem repeats can vary in their copy number. Care has to be taken that the starting material is homogeneous, and the possibility of alterations during the various cloning steps has to be considered.

In the discussion of smaller-scale sequencing projects, we considered the problem of *gaps* remaining after contig assembly. This is even more important for genome sequencing, and generally arises because of difficulties in cloning certain fragments of DNA. Primer walking as described above is less likely to be useful in this context because many of the gaps will be too big. PCR can be used to amplify a fragment to bridge the gap, but only if the sequences either side can be aligned and if the gap is relatively short. If there are a number of gaps in the sequence, it is likely to be impossible to guess the order of the sequenced fragments.

Both of these problems can be addressed, at least in part, by the use of different types of clones, and in particular by the use of supervectors (especially bacterial artificial chromosomes, BACs) described in Chapter 6, which are capable of accommodating inserts of hundreds or even thousands of kilobases. These large inserts can bridge substantial gaps in the assembled genome. Hybridization can be used to identify a recombinant carrying sequences overlapping with either side of the gap. Gaps may arise from the lethal nature of certain sequences in multi-copy plasmids. The stringently controlled low copy number of vectors such as BACs tends to reduce this problem. If the gap arises from a sequence that is too lethal even for a low copy number plasmid, then lambda vectors can be used, although the insert size is then limited to about 23 kb. Alternatively, if other approaches have eliminated most of the gaps, the remaining gaps can be bridged by using PCR amplification of the relevant region of the genome, thus avoiding the need for any cloning step.

Once the genome sequence has been finished, to meet the criteria of accuracy and completeness laid down in the project specifications, the final, important, step is *annotation* – identifying the coding regions, the predicted products, and other features of the sequence. This is considered in the next chapter. However, despite all the excitement that is generated by the publication of a genome sequence, knowing the sequence is only a means to an end, not an end in itself. The important factor is what you can do with that vast amount of information.

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11 Analysis of Sequence Data

11.1 Analysis and Annotation

In the previous chapter, we described the strategies and techniques for sequencing DNA. Whether you have sequenced an individual gene or a complete genome, there will be an understandable sense of achievement when contig assembly is complete, and you have finally finished the sequence. However, the job is not yet complete. Without some understanding of the significance of the various components of the sequence, that series of A, G, C and T letters is like reading an unknown foreign language, and without spaces or punctuation. You can see the letters but what does it all mean? A complete understanding of the role of every part of the sequence is an ambitious goal, but there are some basic analyses that need to be done routinely before you are ready to publish the sequence, or to deposit it in a sequence databank.

11.1.1 Open reading frames

The analysis starts with the identification of regions of DNA that you can predict to code for expression of proteins. To keep it simple at this stage, we will start with a discussion of sequences that do not have introns (such as bacterial sequences and cDNA) so we do not have to consider the identification of exons and introns. That will come in the next section.

The key to identifying protein-coding sequences is to remember that mRNA has the potential to be translated into protein in any of three *reading frames*; which one is actually used will depend on where the ribosomes start. Since at this stage we may not know in which direction the DNA is transcribed, there are six possible reading frames altogether – three for an mRNA in one direction and three if the mRNA is produced in the other direction. How do we know which of these reading frames is used?

Fortunately, we are provided with a clue. In those reading frames that are not used for translation, there are usually frequent stop codons. The same applies to any regions of the sequence that do not code for proteins. This is not just a fortunate coincidence – it prevents the ribosomes from accidentally producing useless, or even potentially damaging, products. On the other hand, any region of the sequence that does code for a protein must, obviously, be free of stop codons (until the stop codon that signifies the end of the protein). Such a sequence, without any stop codons, is known as an *open reading frame (ORF)*. So we can use a computer to search the sequence for stop codons, and an ORF (without stop codons) can be predicted to code for a protein, in that reading frame. It is then straightforward to translate the DNA sequence into a protein sequence.

This of course depends on a judgment as to how long an open reading frame must be before being labelled as an ORF. Unless we put a minimum size on it, the concept is meaningless – every reading frame between two stop codons is ‘open’ in the sense that it has no stop codons. On the other hand, by excluding ORFs below an arbitrary size, we would risk excluding genuine ORFs that do actually code for small polypeptides. For example, if we were to consider as significant only those ORFs of more than 300 bases (coding for 100 amino acids, which would make a polypeptide of about 10 kDa), then we would miss any polypeptides that are less than 10 kDa. However, if we make the size limit too small, we run the opposite risk, of predicting expression of a small protein when no such protein exists.

There are three extra clues that we can use. The most important of these is the presence of a start codon. We can refine the concept of an ORF to mean the distance between a start codon and the first stop codon in the same reading frame. This is not actually as straightforward as it sounds. Although we regard ATG (using DNA rather than RNA nomenclature) as the ‘normal’ start codon, many organisms sometimes use other start codons as well, such as GTG, TTG or CTG – in some cases, especially in bacteria, these other start codons can be quite frequent. In such cases, we can also look for the presence of a ribosomal binding site adjacent to the start codon.

The second extra clue is codon usage. The genetic code has many examples of different codons that are synonymous, i.e. they code for the same amino acid. Generally, these synonymous codons are not all used to the same extent – there is a preferred codon usage, which in some cases can be very marked. Some codons may be used so rarely that we could use their presence in our queried ORF as an indication that it is not really translated into a polypeptide. However, we do have to be careful about arguments like this, especially as there is a danger of circularity. If our knowledge of the codon usage of the target organism is based on the sequence of a limited number of genes, our predicted codon usage may not be accurate. If we then exclude ORFs that do not conform to this codon usage, we will reinforce our incorrect assignment of codon usage.

The third extra clue that we can use is a comparison of ORFs with predicted proteins from other organisms. (We do not have to rely on the identified ORFs from other sequences; we can ask the computer to compare the product of our suspected ORF with all the possible products that would arise from translation of other complete sequences in all six reading frames.) If the predicted polypeptide from our query sequence is similar to polypeptides that might be made by other organisms, this suggests that it is a real coding sequence. Similarly we could look for known protein motifs in this sequence. The comparison of sequences, and searching for motifs, are further considered later in this chapter.

These factors can be exemplified by the data shown in Figure 11.1. This is actually a portion (about 7 kb) of one of the cosmids used in the sequencing of the *Mycobacterium tuberculosis* genome. It is quite straightforward to locate all the stop codons on all six reading frames (three in each direction). In this case, the sequence (as a simple text file) has been read by a program known as Artemis (freely available from the Sanger Centre) to provide this display; there are many other programs available that will perform such an analysis. In the figure, the top three bands show the position of stop codons in the three reading frames that are read from left to right, while the bottom three bands show the complementary strand (read from right to left, 5′ to 3′). You will see that the distribution of stop codons is far from uniform. If we consider that only the largest ones are likely to encode ‘real’ proteins, and therefore confine ourselves to regions of, say over 450 bases (coding for a sequence of 150 amino acids), there are three or more such sequences in each of the six reading frames. In some places, four of the six reading frames have no stop codons within such a distance, and it is highly unlikely that all of these are actually used to produce proteins. (In some organisms, especially bacteriophages, overlapping genes do exist, but this normally happens to only a very limited extent in larger genomes.) So we then take start codons into account, and this reduces the number considerably, but still leaves more potential ORFs (including some overlapping ones) than are actually thought to exist.

Databank entries contain a substantial amount of *annotation*, in addition to the sequence itself. This annotation shows the location of significant features of

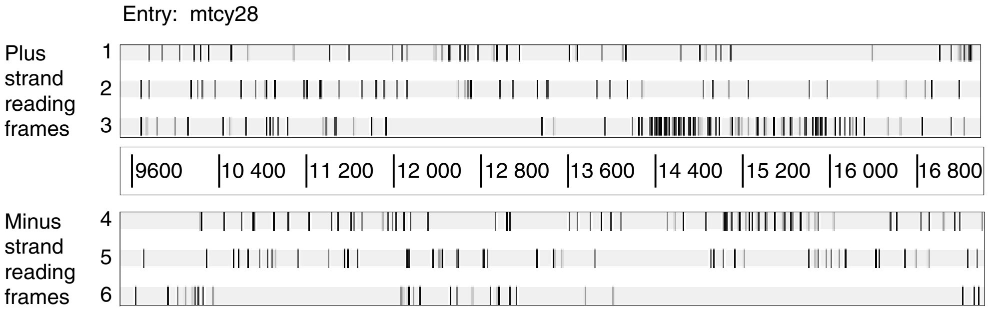
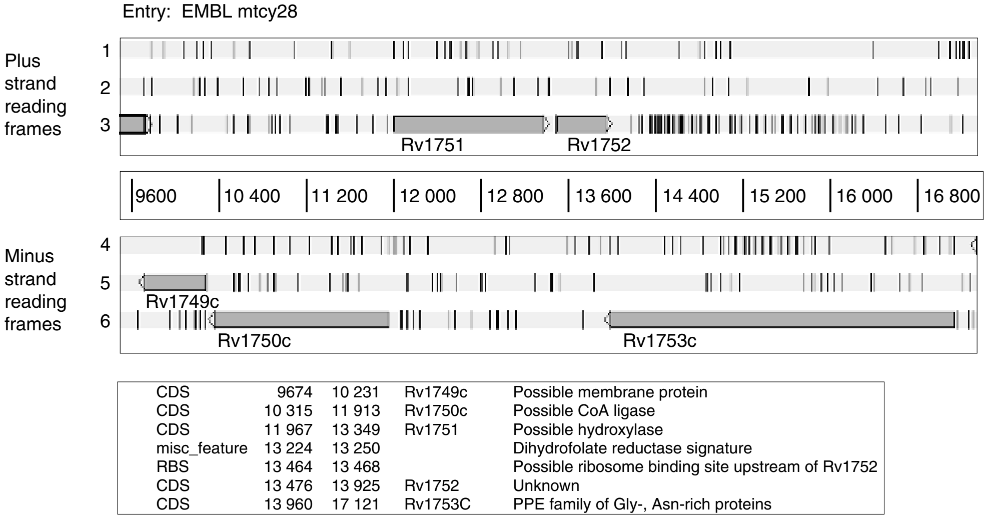


Figure 11.1 Open reading frames – computer mapping of stop codons; edited display from analysis of a DNA sequence using Artemis

the sequence, and, in particular, the assignment of ORFs. Artemis is capable of reading the full EMBL entry and displaying these features. In Figure 11.2, we can see the output from Artemis when it is supplied with this extra information. This shows which of the potential ORFs in this region were considered (by those doing the annotation) to represent 'real' coding sequences, as well as (in some cases) the likely function of the product. It is possible, with a relatively small sequence such as that displayed, to examine each potential ORF individually and in detail. On the other hand, the analysis of a whole genome requires computational methods that are capable of automatically scanning the whole genome and provisionally assigning ORFs on the basis of the factors outlined above. The final annotation still requires manual checking of each potential coding sequence, and its function. We will look further at the annotation of sequences, and the structure of databank entries, later in this chapter.

However it is done, we have to remember that the assignment of ORFs, and the possible function of the corresponding proteins, is only a prediction, and is subject to a degree of uncertainty in the absence of direct evidence as to the existence and properties of the encoded protein. If you extend the argument to analysis of complete genomes, the necessary assumptions about the likely minimum size of a protein means that statistics on the number of predicted proteins have to be treated with care as they may miss an unknown number of small proteins.



CDS	9674	10 231	Rv1749c	Possible membrane protein
CDS	10 315	11 913	Rv1750c	Possible CoA ligase
CDS	11 967	13 349	Rv1751	Possible hydroxylase
misc_feature	13 224	13 250		Dihydrofolate reductase signature
RBS	13 464	13 468		Possible ribosome binding site upstream of Rv1752
CDS	13 476	13 925	Rv1752	Unknown
CDS	13 960	17 121	Rv1753C	PPE family of Gly-, Asn-rich proteins

Figure 11.2 Open reading frames – display of coding sequences; edited display from analysis of a DNA sequence and databank annotations using Artemis

11.1.2 Exon/intron boundaries

In eukaryotes, *introns* are spliced out from the primary transcript as the *exons* are joined into an mRNA molecule (Figure 11.3). This is not a consideration when analysing cDNA sequences. The primary transcript, *heteronuclear RNA* (*hnRNA*) is so short-lived that it is unlikely to be represented in a cDNA library – you can rest comfortably in the assumption that virtually every cDNA clone you pick is derived from processed mRNA. Thus, the search for ORFs in eukaryotic cDNA sequences is similar to prokaryotes, with the very convenient exceptions that each transcription unit is neatly delineated – you know you will normally (if not always) find one ORF in the message, and you know that they are likely to be complete in each clone (if the clone itself is complete).

Genomic DNA sequences are, of course, a different matter. We can only start thinking of predicting the ORF if we can predict the mRNA sequence. Luckily, we can. Human intron/exon boundaries are surrounded by some recurrent motifs (as indeed you would expect, as it stands to reason that the splicing mechanism must be based on sequence recognition). The most obvious and conserved of these is the GU–AG motif. Most introns begin with GU

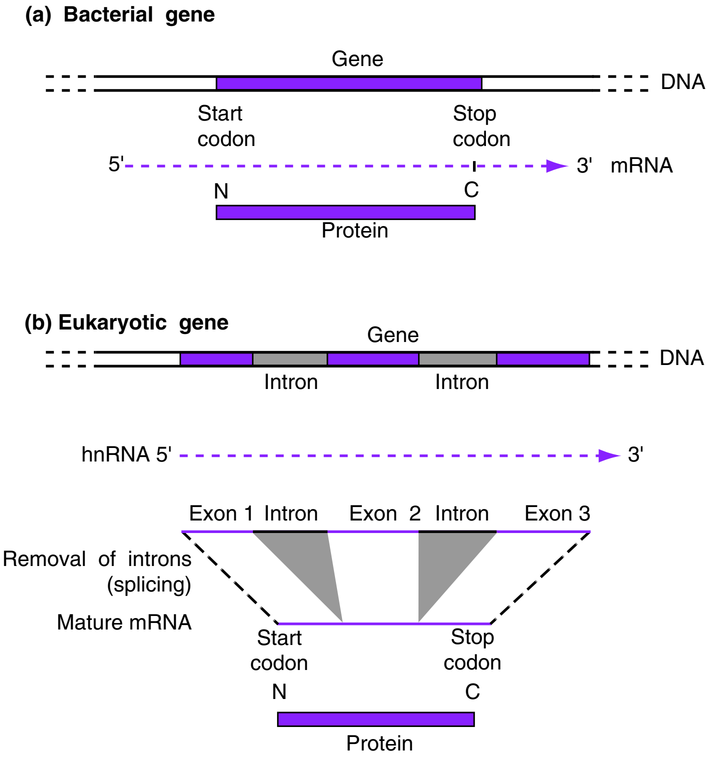


Figure 11.3 Introns and exons

(encoded by GT in the sense strand of the DNA) and finish with AG. Obviously, this is not the whole truth; both of these combinations of two bases will theoretically occur with a frequency of 1:16, so a longer, more variable, sequence is required to define a splice site. There are computer algorithms that will screen any genomic DNA sequences for these and predict any intron/exon boundaries within this sequence. These algorithms are specific for each organism, and are based on knowledge of thousands of intron/exon boundaries determined by comparison of cDNA and genomic sequences.

In this way, the computer in effect excises the predicted introns and thus assembles a *predicted* cDNA sequence. This cannot, however, be done with a certainty that will make it reliable without some corroborating evidence. Fortunately, such evidence is often at hand. The most obvious thing to do is to search GenBank for matching full-length cDNA sequences, from the same species or from a different one. Additional evidence can be obtained from screening databanks of *expressed sequence tags* (ESTs) (see Chapter 13).

11.1.3 Identification of the function of genes and their products

The methods described above will lead to a provisional assignment of ORFs, or coding sequences, in our cloned DNA fragment. The next question is the nature and function of those proteins. If we are dealing with the sequence of a specific clone, we would start with a strong hypothesis as to what the protein actually does. The reliability of that hypothesis will depend on the cloning strategy and other evidence, and it may need confirming by other means. If the hypothesis is reliable enough, we can move straight on to using the DNA sequence to provide information about the protein itself (see below).

However, if we are dealing with an unknown stretch of DNA (and especially if we are analysing a whole genome sequence), we would be starting with no preconceived idea as to what the sequence actually codes for. In this case, we have to resort to comparing each predicted sequence against all the known protein sequences held in the databanks. (The methods for doing this are discussed later on; as we will see then, it is possible to carry out such comparisons with DNA sequences as well as with predicted protein sequences, but proteins usually give better results.) There are a number of possible outcomes to such a search. If we are lucky, our protein may have a high level of similarity to a well-characterized protein from another source (or even better, to a number of such well-characterized proteins). In this case, we are on reasonably safe ground in attaching that label to our protein as well.

There is a snag. Many proteins in the databanks, especially those predicted from gene sequences, have a functional label attached to them not because of an established function, but because of their similarity to another protein with an established function. Unless we look carefully at the evidence for the identity of these proteins, there is a risk of building up a chain of such similarities, becoming less and less reliable. In other words, gene A in one genome is identified as coding for enzyme X. Then gene B, in a second genome, is similar to gene A in the first genome, so it is also labelled as coding for enzyme X. In a third genome we find a gene that is similar to B, so this is also labelled as coding for enzyme X, and so on. We may end up with a gene that is only remotely similar to the originally identified gene A.

We may be less lucky. Our predicted gene product may not have a high degree of similarity to any known protein, so we cannot be certain as to its function. However, it may show some features which are characteristic of certain classes of protein, which would enable us to provisionally label it as, for example, 'probable membrane protein', or 'possible oxidoreductase'. Or, it may show no discernible resemblance to any sequence in the database, in which case it has to be labelled as 'unknown function'. This applies to a substantial proportion of the predicted proteins in a typical genome sequence. We may actually find that although it does have similarity to other proteins (or predicted proteins) in the database, these proteins are already labelled as 'unknown function'; you can see one example (identified as Rv1752) in Figure 11.2).

However good these predictions look, they are still only predictions, which need direct experimental evidence to prove them. This includes not only verification of the biochemical functions of the gene product, but also testing of its role in the physiology of the whole organism – including its potential role in causing a specific disease. If your cloning strategy was devised as a search for a gene with a specific function you will have a good idea where to start. Later on in this chapter, we will discuss what inferences can be made from the predicted protein structure, and elsewhere in this book we consider what deductions can be made from studying the levels and localization of the transcript (Chapter 13), linking genetic and physical maps (Chapter 14), the production and analysis of its protein product (Chapter 15), and the study of what happens if the organism overexpresses or lacks the gene (Chapter 17).

In the course of these sequence comparisons, we may also come across *pseudogenes*. These are DNA sequences which have significant sequence similarity to 'real' genes, i.e. to DNA sequences that are known or believed to code for proteins, but the pseudogenes contain changes in the sequence that make it unlikely to be functional. The simplest type of change is one that puts a stop codon (or several stop codons) within what should be the coding sequence. Some pseudogenes may be transcribed into mRNA (*transcribed pseudogenes*), but these mRNA molecules cannot be translated into functional proteins.

11.1.4 Expression signals

An ORF is of no significance unless that region of the DNA is transcribed (and transcribed in the right direction). So you might consider that our prediction of protein coding sequences could be made more precise by incorporating the detection of transcription start (and stop) sites. Unfortunately, it is not quite that simple. For example, in most bacteria a high proportion of genes are transcribed by RNA polymerase recognizing promoter sites which have two relatively conserved consensus regions: the -35 region (i.e. a region centred at 35 bases before the start of transcription) and a -10 region. The consensus sequences for these two regions are TTGACA and TATAAT respectively (see Chapter 2); but these only represent a consensus. Very few promoters have exactly that sequence at either position, and the distance separating them can vary by a few bases as well. If we build in that degree of flexibility into our search for promoters we will end up with a large number of sites, most of which are not genuine promoters. The binding of RNA polymerase is affected to a lesser extent by the sequence of a larger region, extending over perhaps 70 bases, as well as by other factors such as the supercoiling of that region of the chromosome.

In addition, the specificity of bacterial RNA polymerase can be changed by substitution of a different sigma factor, enabling it to recognize promoters with a markedly different structure. In organisms that are less well characterized than *E. coli*, the structure of these alternative promoters may be unknown, making promoter prediction impossible.

In eukaryotes, as usual the situation is even more complicated. The binding of RNA polymerase II is mediated by a number of canonical elements with similarity of structure, including the TATA-box, GC-box, and CAAT-box. However, the spacing of these elements is not always consistent. Moreover, there is an element of species variation, so the parameters for searching for a transcription start site in a fruit fly are different from those needed to make the corresponding prediction in a human sequence. A number of websites are available for the prediction of transcription start sites in model organisms. These take advantages of neural networks that utilize information from known promoters in the same organism.

Although searching for possible promoters is likely to be of little or no use in predicting coding sequences, the identification of sites from which a gene may be transcribed can be useful information. In other words, rather than searching the whole genome for promoter sequences to define coding regions, we could take specific ORFs and look more carefully at the sequence upstream to see if we can identify a potential promoter, and hence a possible transcription start site. This can then form part of the annotation that is attached to our sequence when we deposit it in the databanks. However, in the light of the above comments, it is very important to distinguish those possible promoters that have been identified merely by prediction (and hence may not be reliable) from those that that have been established by direct experimental evidence (see Chapter 13).

In addition to searching for possible promoters (i.e. sites at which RNA polymerase will bind) we can look for sites at which regulatory proteins can attach to the DNA to repress, or activate, transcription. Many of these proteins have quite well conserved, and characterized, recognition sequences, often known as 'boxes'. For example, in most bacteria iron uptake is regulated by proteins belonging to one of two families, related to either Fur (the Ferric Uptake Regulator protein of *E. coli*) or DtxR (the diphtheria toxin repressor of *Corynebacterium diphtheriae*). These proteins, in the presence of Fe^{2+}, bind to specific DNA sites and repress transcription of the adjacent genes. Identification of a site such as the so-called 'Fur box', to which Fur binds, therefore provides an indication that the associated gene will be repressed in the presence of an adequate supply of iron, which in turn suggests that the function of that gene may be connected with iron uptake. There are a number of other known 'boxes', to which different regulatory proteins will bind, in both prokaryotes and eukaryotes; for example, the E-box (for enhancer) has been associated with an expression pattern following a circadian (24 hour) rhythm. It is possible to screen DNA sequences for each of these boxes, providing evidence not only relating to the regulation of the relevant genes, but also a clue as to their possible function; the methods for searching for such binding sites, and other structural features of a protein, are considered later. As with putative promoter sites, it is essential to distinguish between computer predictions and direct evidence.

11.1.5 Other features of nucleic acid sequences

The analysis of our DNA sequence does not end with the identification of protein binding sites. One of the simplest, and yet very informative, analyses is the base composition of the DNA, i.e. the ratio between G+C and A+T bases. Overall, this ratio is characteristic of a particular species, and tends to be similar between different species within a genus, while varying more widely between less related organisms (sometimes as low as 30 per cent G+C or as high as 70 per cent). Base composition can therefore be used as an aid to establishing the taxonomic relationship between different species.

Within bacterial genomes, the base composition tends to be reasonably uniform from one region to another, but there are notable exceptions. We may for example find, in an organism with an overall base composition of 65 per cent, a group of genes with a much lower G+C content – say 45 per cent.

Usually there is quite a sudden change in base composition at each end of this region, which is known as an *island*. Many of the examples that have been studied concern groups of genes that are connected with bacterial pathogenicity, and are hence referred to as *pathogenicity islands*, but the phenomenon is not restricted to virulence determinants. What is the significance of these islands?

Processes such as DNA replication and transcription, and particularly the regulation of these processes, are to some extent sensitive to the base composition of the DNA. Therefore over an extended evolutionary period, the enzymes involved in these processes, and the composition of the DNA, have evolved together to produce a well-balanced system. Furthermore, the codon usage of the genes is also related to the base composition – so as the codon usage and the specificity of the available tRNAs co-evolves, this will also be reflected in the base composition of the DNA. The inference from this is that these *islands,* with a different base composition, are relatively recent arrivals. They represent DNA that has been acquired by the bacterium by horizontal gene transfer from a different species. This inference has been substantiated in some cases by direct evidence. For example, the genome of *Vibrio cholerae* contains an island in which the genes for the cholera toxin are found. It has been established that this island is in fact phage DNA that has been integrated into the bacterial chromosome. Many other islands have been shown to be integrated bacteriophages, either by direct evidence or by comparison of the sequence with that of known bacteriophages.

Mammalian DNA also contains 'islands' with a different base composition from that of the remainder of the genome, but these have a different significance. The dinucleotide CG (usually written as CpG to emphasize that we are referring to consecutive bases on one strand) occurs much less commonly than would be expected from a random distribution of bases. Yet in some regions, the frequency of this doublet is very much higher, forming regions (CpG-rich islands) up to 2 kb in length, with a much higher G+C content than that of the whole genome. There are many thousands of such regions in the genome. The frequent association of CpG islands with promoter regions means that the identification of such an island can be taken as suggesting a transcriptional initiation region.

Amongst the many other features of DNA that are amenable to computer analysis, we can single out the occurrence of inverted repeat sequences. It is important to be clear about the meaning of an 'inverted repeat'; since DNA strands have a direction to them, and the two strands are in opposite directions, an inverted repeat of say CAT is *not* TAC but ATG (see Figure 11.4). A pair of inverted repeats, in close succession, can anneal together, so that a single strand containing such a sequence will give rise to a *hairpin* structure, or if they are separated by a few bases, a *stem-loop* structure (Figure 11.5). A well-known example of such a structure is the tRNA molecule. Ribosomal RNA also exists

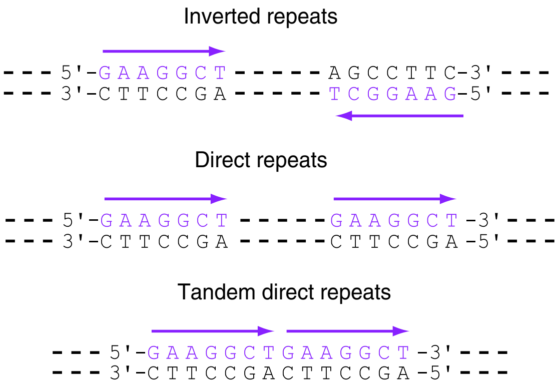


Figure 11.4 Inverted and direct repeats

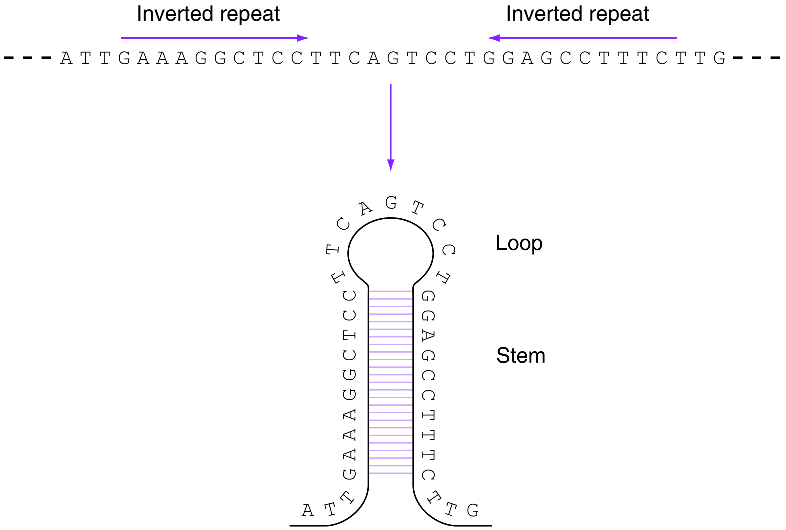


Figure 11.5 Formation of stem-loop structure

in a highly folded conformation, and mRNA transcripts will also form folded structures. Although such structures are most common in single-stranded nucleic acids (mainly RNA), they can also give rise to localized destabilization of double-stranded DNA, most notably when the double helix is unwound during replication. One of the significant aspects of such structures is the role that they play in transcriptional termination; the formation of a stable stem-loop structure in the mRNA favours the dissociation of the nascent mRNA strand from the template DNA, allowing the two DNA strands to reanneal. This causes the RNA polymerase to pause, and ultimately stop transcription.

Regulatory proteins often bind to inverted repeats. Many such proteins are dimeric, so one subunit can bind to one copy of the inverted repeat, and the other subunit to the second copy. The requirement for two binding sites gives a higher degree of specificity to the interaction, as well as establishing a strong interaction.

Repeated sequences are also a frequent cause of variation (see Chapter 12). Inverted repeats are typically found at the ends of mobile genetic elements such as transposons and insertion sequences. In addition, recombination between inverted repeat sequences will lead to inversion of the region between them. In contrast, recombination between two direct repeats will cause deletion of the region between them. These sources of variation are not only an interesting natural phenomenon; they can be a nuisance in gene cloning as the occurrence of repeated sequences within your insert can cause instability of the construct. Tandem direct repeats (that is, direct repeats with no intervening region) also cause variation in another way – by *replication slippage*. When the replication apparatus encounters a tandem direct repeat, it may (very occasionally) jump forwards or backwards, causing a loss or gain of additional copies of the repeated sequence. One example of this is found in Huntington's disease, where a CAG repeat in a gene called huntingtin has a tendency to slip and extend itself. Above a certain length, the encoded poly-glutamate stretch in the resulting protein will cause the disease.

Because the number of copies of a direct repeat at a specific site may therefore vary from one individual to another, it forms the basis of one method of molecular typing (see Chapters 12 and 16).

11.1.6 Protein structure

Having identified an ORF, and used the computer to translate it into a protein sequence, we can then investigate a number of aspects of the structure of that (maybe hypothetical) protein. This is obviously particularly important where we do not already have clues to the function of our gene through its similarity with others that have already been characterized. A full consideration of all the possibilities is beyond the scope of this book, but it is worth a brief and selective overview, especially as an examination of protein structure can provide some leads as to its possible function.

For example, if we look at the amino acid composition of the protein, and especially at the occurrence and distribution of hydrophobic and hydrophilic amino acids, we should be able, just on this evidence, to detect if it is likely to be a membrane protein. Proteins that are embedded in a membrane will normally have substantial stretches of hydrophobic amino acids. Soluble proteins are more likely to be predominantly hydrophilic, although they can contain hydrophobic regions if the protein folds into a pocket that shields those hydrophobic regions from the aqueous environment. If we look more closely at the predicted structure (especially in combination with testing for specific motifs – see below) we may be able to guess whether it is involved in transport across the membrane, or energy generation, or acts as a receptor for signal transduction, or any of the other functions commonly associated with membrane proteins. As an example, Figure 11.6 show a hydrophobicity plot for the human rhodopsin protein. This shows seven regions of marked hydrophobicity, with intervening regions that are more hydrophilic. The hydrophobic regions are embedded in, and span, the membrane, so that at one end of a transmembrane region the protein is exposed to the cytoplasmic environment, while at the other end the protein protrudes into the external environment. This is a commonly occurring theme in membrane proteins, especially in those proteins that function as transporters (that is they ferry material across the membrane) or as signal transducers (i.e. they respond to changes in the external environment and transmit a signal across the membrane to the interior of the cell).

Proteins, of course, do not exist merely as sequences of amino acids, but in their native form adopt higher orders of conformation. Ideally we would like to be able to predict, from the primary sequence of amino acids, which parts will adopt secondary structures such as alpha-helices, and how those elements of secondary structure will fold into the tertiary and higher orders of structure that are characteristic of the native protein. However, the number of possibilities is extremely large, and furthermore the final folding of the protein can be influenced by the folding of the polypeptide chain as it is made. The final structure is not necessarily the most thermodynamically stable one. It is therefore rarely

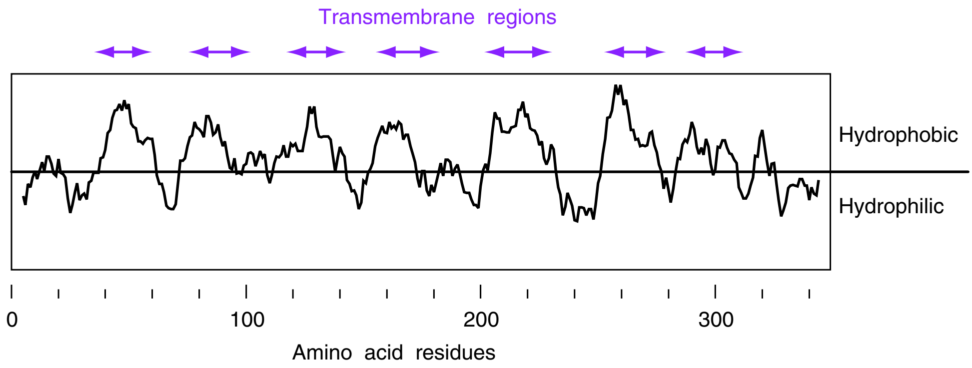


Figure 11.6 Hydrophobicity plot: the illustration shows a Kyte–Doolittle hydropathy plot for human rhodopsin (Swiss-Prot P08100); the hydrophobic regions represent transmembrane structures, with the intervening hydrophilic sequences alternating between cytoplasmic and external structures

possible to predict with any degree of certainty the final folding of a hitherto unknown protein (although it may be possible to identify likely regions of specific secondary structures such as alpha helices). For this, in spite of the huge increase in computer capacity, we are still left with the need to produce crystals of the pure protein for X-ray crystallography.

However, as more and more proteins are characterized at this level, it becomes increasingly likely that the unknown protein we have predicted is a member of a family of proteins, some of which have been characterized structurally. By aligning the sequences, we can therefore make much more reliable predictions as to the likely conformation of our unknown protein.

11.1.7 Protein motifs and domains

Although our protein may not show much overall similarity to any other characterized protein, we may find, if we look more closely, that it contains short sequences of amino acids that are very similar to parts of a number of other proteins. These conserved regions are known as *motifs*. The recognition of motifs provides yet another clue as to the function of our protein, or other aspects of its structure.

Some motifs occur because a wide range of enzymes, with otherwise disparate properties, may use the same substrate. For example, there is a wide range of enzymes that uses ATP as a substrate. In many cases, the region of the enzyme that binds the ATP has a similar structure – although the remainder of the sequence may show no similarity. If we can recognize the presence of a putative ATP-binding site in our protein, then we can infer that it is probably an enzyme that uses ATP as a substrate.

The overall reaction catalysed by some enzymes is actually a series of separate reactions, with different parts of the enzyme responsible for different steps. For example, acetyl-CoA carboxylase (catalysing the first step in fatty acid synthesis) has one component with a covalently attached biotin, a second component with (noncovalent) binding sites for ATP and CO_2 which carboxylates the biotin, and a third component, with an acetyl-CoA binding site, which transfers the carboxyl moiety from the biotin to the acetyl-CoA. In *E. coli* these components are made as separate polypeptides which associate with one another to form the complete holoenzyme. On the other hand, in mammalian cells the enzyme is made as a single polypeptide with different parts of the structure responsible for the different activities. We refer to these elements as *domains*. It is not uncommon to find enzymes that consist of several subunits in one organism but as a single polypeptide with several domains in another source – probably due to a fusion of genes that originally evolved separately (see Chapter 12). Domains often form more or less structurally-independent regions of a protein – in other words each domain folds up into its own secondary structure with little or no structural interaction with the other domains, other than a flexible loop connecting them. You can visualize such a structure, roughly, as a set of balls connected by bits of string.

As well as substrate-binding sites, there are a range of other motifs that indicate sites for structural modification such as lipid or sugar attachment, for secretion or for targeting to specific cellular compartments, or in the case of regulatory proteins, for DNA binding. There are libraries of known motifs available, so it is a simple matter to screen a protein sequence for any of these motifs. One of the longest-established libraries of patterns is PROSITE. Figure 11.7 shows the output from a web-based search for motifs present in protein Rv0194 (a protein from *M. tuberculosis* which is identified as a probable transporter protein; SwissProt accession number O53645), and demonstrates the presence of two ATP-binding motifs, and a signature

```
Edited output:

   [1] PDOC00001 PS00001 ASN_GLYCOSYLATION
   N-glycosylation site

   Number of matches: 2
   1        790–793    NWTY
   2      1079–1082    NLSA
   --------------

   [5] PDOC00007 PS00007 TYR_PHOSPHO_SITE
   Tyrosine kinase phosphorylation site

          1008–1015    REFDVDGY
   --------------

   [7] PDOC00017 PS00017 ATP_GTP_A
   ATP/GTP-binding site motif A (P-loop)

   Number of matches: 2
   1      367–374    GAPGSGKS
   2      976–983    GSTGSGKS

   [8] PDOC00029 PS00029 LEUCINE_ZIPPER
   Leucine zipper pattern

          124–145    LQLVQALLFDVPNVLRHVLTLL

   [9] PDOC00185 PS00211 ABC_TRANSPORTER
   ABC transporters family signature

          471–485    LSGGQRQRIALARAL
```

Figure 11.7 ScanProsite – protein against PROSITE: the query protein was a probable ABC (ATP-binding cassette) transporter protein from *M. tuberculosis* (SWISSPROT 053645); the analysis was done using ScanProsite at *http://ca.expasy.org/tools/*

Trusted matches - domains scoring higher than the gathering threshold

Domain	Start	End	Bits	Evalue	Alignment
ABC_membrane	20	289	116.40	5.3e-3.1	Align
ABC_tran	360	544	179.10	7.2e-50	Align
ABC_membrane	627	898	67.90	2.2e-16	Align
ABC_tran	969	1153	183.50	3.4e-51	Align

Matches to Pfam-B

Domain	Start	End	Evalue	Alignment
Pfam-B_16	317	348	7.3e-12	Align
Pfam-B_16	926	954	1.8e-10	Align

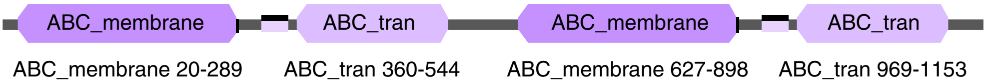


Figure 11.8 Domain families – Pfam database: the query protein was a probable ABC (ATP-binding cassette) transporter protein from *M. tuberculosis* (SWISSPROT 053645), see also Figure 11.7

sequence for the family of proteins known as ABC (ATP-binding cassette) transporters.

A further useful tool is the Pfam database of protein domain families, available at several sites, including the Sanger Centre (http://www.sanger.ac.uk). Rather than identifying specific motifs, this uses a set of multiple sequence alignments for each family. (Technically, these alignments are encoded using a statistical treatment known as Hidden Markov Models; HMMs have a variety of applications in bioinformatics.) Figure 11.8 shows the output from an analysis of the same protein as used in Figure 11.7. The first table shows the matches to the Pfam-A database, which is a high-quality, curated database (i.e. the quality is maintained manually). The second table shows matches to Pfam-B families, which are computer-generated, of lower quality and do not contain annotation. Rather than merely identifying short motifs, this analysis has identified domains that are characteristic of this family of proteins. The motifs identified by Prosite will be found within the domains labelled ABC_tran.

11.2 Databanks

Once we have determined a DNA sequence, it should be made publicly and freely accessible by submitting it to a databank (EMBL, GenBank, or the

Japanese databank DDBJ). This is done electronically via the internet, and the databanks have Web-based procedures that make this submission a simple automatic process (see Appendix A for the Web addresses). In practise, the databanks can be considered as one and the same, as they share their information on a daily basis, and wherever you live you can just choose the one whose interface you prefer. It is only necessary to submit your sequence to (or to search in) one of them. Each sequence in the databank is allocated a unique *accession number*, which should be quoted in publications referring to that sequence, and the use of the accession number makes it easy to retrieve that sequence from the databank. An example of an EMBL databank entry for an individual sequence (the gene for the transcarboxylase subunit of acetylCoA carboxylase, *accA*, from *E. coli*) is shown in Figure 11.9. Genome sequence data is annotated in a similar way, but is often, for convenience, included in the databanks in sections which may correspond to actual clones (cosmids for example). These clones can usually be ordered directly from national and international genome resource centres. The fragments of the genome sequence may also be 'virtual clones' (i.e. arbitrarily divided sections of the genome). Whole chromosomes or whole genomes may also be included as a single sequence, and are useful for some forms of analysis – but downloading a whole chromosome sequence and analysing it may stretch the capability of your computer or your software.

Entries in GenBank look slightly different, but the information contained is the same, and you should have little difficulty in switching from one to the other. Both are computer-readable, provided that the software has been set up to recognize the format.

The notes that we have added to the annotation should mainly be self-explanatory, but some aspects need additional comment. The *identifier* (ID) is a short label that may give some clues as to the nature of the entry. In this case it indicates that the sequence is from *E. coli*, and that it contains (part of) the adjacent *polC* (*dnaE*) gene as well as *accA*. (To keep the figure simple, information about *polC* has been removed from the annotation.) The *accession number* (AC) is important as it is the most convenient way of retrieving a specific sequence from the databank, and the one that is referred to in publications; the accession number is the same in the different databanks (whereas the ID differs). It refers to a particular sequence submission, rather than to a specific gene or locus. There may therefore be several entries, with different accession numbers, relating to the same sequence. Some may be partial, incomplete or unassembled sequences, or even inaccurate ones, that have in effect been superseded by later versions. If you want to look for different versions of a specific gene you may therefore need to search the databank by gene name, or alternatively use a known sequence and search for similarities (e.g. using BLAST, see below).

In addition to the sequence itself, there is scope for a considerable amount of *annotation* that makes the sequence information much more useful. The more

Added notes	**EMBL annotation**	
identifier	ID	ECPOLCACC standard; DNA; PRO; 1539 BP.
accession number	AC	M96394;
	SV	M96394.1
entry date	DT	19-SEP-1992 (Rel. 33, Created)
last modified	DT	04-MAR-2000 (Rel. 63, Last updated, Version 15)
definition	DE	acetyl-CoA carboxylase (accA) gene, complete cds.
keywords	KW	accA gene; acetyl-CoA carboxylase; carboxyltranferase;
species	OS	Escherichia coli
classification	OC	Bacteria; Proteobacteria; gamma subdivision; Enterobacteriaceae;
	OC	Escherichia.
Medline reference	RX	MEDLINE; 92380982.
authors	RA	Li S.J., Cronan J.E. Jr.;
title	RT	"The genes encoding the two carboxyltransferase subunits of
	RT	Escherichia coli acetyl-CoA carboxylase";
reference	RL	J. Biol. Chem. 267(24):16841–16847 (1992).
protein database	DR	SWISS-PROT; P30867; ACCA_ECOLI.
features header	FH	Key Location/Qualifiers
features table	FT	source 1..1539
	FT	/organism="Escherichia coli"
	FT	/sub_strain="W3110"
	FT	/strain="K-12"
– ribosome binding site	FT	RBS 340..343
	FT	/gene="accA"
– coding sequence limits	FT	CDS 349..1308
	FT	/codon_start=1
	FT	/db_xref="SWISS-PROT:P30867"
	FT	/evidence=EXPERIMENTAL
	FT	/transl_table=11
	FT	/gene="accA"
	FT	/product="acetyl-CoA carboxylase"
	FT	/protein_id="AAA70370.1"
– translated sequence*	FT	/translation= "`MSLNFLDFEQPIAELEAKIDSLTAVSRQD...`"
	FT	terminator 1324..1352
	FT	/gene="accA"
sequence information	SQ	Sequence 1539 BP; 371 A; 366 C; 433 G; 369 T; 0 other;
DNA sequence*	`cgtcagcgac aggtcaggtc agcttgatgc tcagcggtgg...`	
	//	

Figure 11.9 Sequence annotation: some elements of the annotation have been omitted; translated and DNA sequence data have been truncated

annotation you can reliably include, the more useful your submitted sequence is. Obviously you will need to include information about the source of the sequenced DNA. In addition, the identification and extent of any open reading frame is a basic requirement (together with the computer prediction of the protein sequence), intron/exon boundaries (if applicable) and any further information on expression signals, motifs, structural elements, as identified above will enhance the value of the submission, as well as identification of the presumed or actual function of the gene. This information goes into the *features table* (lines starting with FT), and it is this information that is read by programs such as Artemis (see above).

In addition to DNA sequences, there are databanks of protein sequences available via the same sources. The principal one is known as SWISS-PROT, and you will see in the figure that a reference is provided that identifies the corresponding entry in the SwissProt database. There are two types of protein sequence information. Some protein sequences are derived from direct protein sequencing, but the majority are derived by computer translation of DNA sequences. Some care is needed when using computer-generated protein sequences, especially as there may be no direct evidence that this protein actually exists. Furthermore, it may be based on misidentified intron/exon boundaries, the identification of the start site may be incorrect, or a variety of other factors (including post-translational modification or cleavage) may result in the protein within the cell being substantially different from the primary translation product. In particular, a base missing or incorrectly inserted will result in a shift of the reading frame, and the protein sequence beyond that point will bear no relationship to the real product.

11.3 Sequence Comparisons

11.3.1 DNA sequences

There is a vast, and rapidly growing, amount of sequence data available in the databanks. How can we find out if our sequence is the same as (or very similar to) some other sequence that has already been determined?

We can start by considering the simplest approach. Suppose we have 1 kb of sequence data (and we will for the moment ignore the possible complication of introns). We can put our sequence (the *query sequence*) alongside each of the entries in the databanks, and count the number of bases that match. (Fortunately we do not have to do this one at a time; the computer software will automatically pull out each sequence entered in the databank and compare them for us in a remarkably short period of time.) However, there will be a lot of fragments of different lengths, and they will not necessarily start at the same place. So we (or rather the computer) must slide our sequence along each of the sequences it is being compared with and see where the best match comes. Clearly, we do not want to know how well it matches with all the sequences in the databanks, but we can instruct the computer to list, say, the best 50 matches.

There is, however, another problem. Our sequence may have one or more gaps in it, compared with the potential match in the databank. This can happen in several ways: we may be trying to compare cDNA and genomic sequences, there may be an error in our sequence, or one or more bases may have been deleted in the DNA of the organism from which we have cloned this fragment. We also have to recognize that the sequence in the databank may be wrong. This is particularly likely in the case of expressed sequence tags (ESTs, see Chapter 13), which are not produced with a high requirement for accuracy.

For example our sequence may read, at one point, GGACT, while at the corresponding point in the databank sequence, which otherwise matches perfectly, the sequence is GG*G*ACT. This is a very small difference, but the consequence would be that part of our sequence would line up perfectly, while the rest would not match at all (or more strictly it would match just as well as any two random bits of DNA). In order to accommodate this problem, we can allow the computer to introduce gaps into one or both sequences.

We cannot allow the computer indefinite licence to introduce as many gaps as it likes, otherwise it would produce perfect matches between any two sequences, just by repeatedly sliding them apart until it found the next base that matched. The algorithm therefore incorporates a *gap penalty*; every time a gap is introduced, into either sequence, there is a reduction in the score. The gap penalty can be set at different levels, including different penalties for different lengths of gaps, although the software usually has a default value so we do not have to think about it.

There are several methods available for comparing DNA sequences, and some of them employ algorithms that essentially work as described above (although the description is highly simplified!). However, this requires a lot of computer power for longer sequences, if carried out literally as described. As the computer slides the two sequences along base by base, at each position it has to calculate a score with all possible combinations of gaps – even for a sequence of say 1kb, there is a very large number of possible combinations.

Other programs therefore employ different algorithms that are designed to speed up the process. One approach is basically to split our sequence (the *query sequence*) into small fragments (*words*), using these words to decide how the two sequences will align best and then computing a score for the optimal alignment, including allowing for gaps. This is more or less the basis for one of the common search programs known as FASTA. We will look at an example of a FASTA search, for protein databases, later on.

One of the most widely used programs is known as BLAST (Basic Local Alignment Search Tool). At a highly simplified level, this works by finding very short matches (segment pairs) and then extending that match outwards until the score falls below a set value. Each matched pair of sequences above a certain length is then stored and reported (as High-scoring Segment Pairs, or HSPs), starting with those with the highest score. Figure 11.10 shows the result of a database search using BLAST, using the *E. coli accA* gene as the *query sequence*. (Note that this is an edited version of the output.) The numbers in the first

Query sequence = *acc*A (960 letters) [see Figure 11.9]
Database: embl; 1 415 498 sequences; 3 814 099 291 total letters.

	Sequences producing High-scoring Segment Pairs			High score	Smallest sum probability $P(N)$	N
1	EM_PRO:AE004296	AE004296	Vibrio cholerae chromosome I, section	2268	5.3e-95	1
2	EM_PRO:AE006064	AE006064	Pasteurella multocida PM70 section 31	2122	2.2e-88	1
3	EM_PRO:HI32724	U32724	Haemophilus influenzae Rd section 39	1996	1.1e-82	1
4	EM_PRO:AE004783	AE004783	Pseudomonas aeruginosa PA01, section	1824	3.4e-79	2
5	EM_PRO:AE002462	AE002462	Neisseria meningitidis serogroup B . . .	1716	9.9e-72	2
6	EM_PRO:AE003873	AE003873	Xylella fastidiosa 9a5c, section 19 . . .	1573	1.5e-63	1
7	EM_VI:SV27645	U27645	Stealth virus 1 clone 3B614.	1228	4.6e-48	1
8	EM_PRO:BSPK	D13095	B. stearothermophilus phosphofructokin	1216	1.5e-47	1
9	EM_PRO:AE005962	AE005962	Caulobacter crescentus section 288 . . .	1164	5.0e-45	1
10	EM_PRO:MSD827	D87827	Magnetospirillum sp. gene for magnet . . .	1101	2.0e-43	1
11	EM_PRO:AE008321	AE008321	Agrobacterium tumefaciens strain C58 . . .	1106	2.2e-42	1
12	EM_PRO:AP003002	AP003002	Mesorhizobium loti DNA, complete . . .	1098	5.8e-42	1
13	EM_PRO:SP59236	U59236	Synechococcus PCC7942 ribosomal pro	1088	1.2e-41	1
14	EM_PL:AB029556	AB029556	Pisum sativum accA mRNA for acety . . .	1037	9.2e-40	1
15	EM_PRO:AP001517	AP001517	Bacillus halodurans genomic DNA . . .	1030	6.9e-39	1
16	EM_PRO:AP003134	AP003134	Staphylococcus aureus subsp. aureus . . .	988	5.4e-37	1
17	EM_PRO:AE001970	AE001970	Deinococcus radiodurans R1 section . . .	972	2.6e-36	1
18	EM_PL:AF165159	AF165159	Glycine max carboxyl transferase . . .	958	4.2e-36	1
19	EM_PL:AF056969	AF056969	Arabidopsis thaliana carboxyltransferase	945	2.0e-35	1
20	EM_OR:CHPPPU388	U38804	Porphyra purpurea chloroplast, comple . . .	920	6.4e-34	1
21	EM_PRO:AE006603	AE006603	Streptococcus pyogenes M1 GAS strain . . .	904	2.9e-33	1
22	EM_PRO:CJ11168X2	AL139075	Campylobacter jejuni NCTC11168 . . .	902	4.2e-33	1
23	EM_PRO:AE006311	AE006311	Lactococcus lactis subsp.lactis IL 1403 . . .	845	1.4e-30	1
24	EM_PRO:AE007354	AE007354	Streptococcus pneumoniae section . . .	845	1.4e-30	1
25	EM_PRO:AE001484	AE001484	Helicobacter pylori strain J99 . . .	839	2.5e-30	1
26	EM_PRO:AE002322	AE002322	Chlamydia muridarum, section 53 . . .	788	5.3e-28	1
27	EM_PRO:AE001624	AE001624	Chlamydia pneumoniae section 40 . . .	785	7.3e-28	1
28	EM_OR:CHASPSAC	Z33874	Antithamnion spec. chloroplast psaC . . .	716	5.7e-25	1
29	EM_PRO:STPOL3A	M26046	S.typhimurium DNA polymerase III . . .	496	7.0e-13	1
30	EM_PRO:ECLPXA	M19334	Escherichia coli lipid A biosynthesi . . .	300	0.0026	1
31	EM_PRO:AY034597	AY034597	Bacillus sphaericus putative ace . . .	237	0.88	1

Notes *E. coli accA* sequences, multiple entries from the same species, and data from patents have been deleted. Numbers in the left-hand column have been added for cross- reference to subsequent figures.
$P(N)$ indicates the probability of the match occurring by chance; N = the number of matching regions within a given sequence

Figure 11.10 Results of a database search using BLASTN (DNA search): (a) list of matching sequences with scores: (b) (overleaf)

column have been added by us, to make it possible to compare this figure with subsequent ones. The next column shows the ID of the sequence that matches, including in this example the Division of the EMBL database. EM_PRO indicates it is a bacterial (prokaryotic) sequence, and it is not surprising that most of the entries are bacterial. However, there are some plant and chloroplast genes (EM_PL, EM_OR) as well as one surprising viral sequence (EM_VI). The next columns show the accession numbers and a truncated definition.

```
>EM_PRO:AE004296 AE004296 Vibrio cholerae chromosome I, section 204 of 251 of the
complete chromosome.
     Length = 10 625

Minus Strand HSPs:

Score = 2268 (346.3 bits), Expect = 5.3e-95, P = 5.3e-95
Identities = 692/963 (71%), Positives = 692/963 (71%), Strand = Minus / Plus


Query:   954 GTAACCGTAGCTCATCAGGCGCTGATAACGACGATTTTTTAAATCTTCAGTGCTTAACA- 896
             |||||||||| ||||||  || ||||| || || |     || | ||| ||| |  |
Sbjct:  2836 GTAACCGTAGTTCATCAAACGTTGATAGCGGCGCTCCAGCAAGTTTTC-GTGATCCAGTG 2894

Query:   895 CGTCGAGATCGGCCAGATC-CGCCAGCAGT-TGCGCTTTCAACGATGC-CGCCA-TCGCT 840
             | || ||||||||||  |  ||| || ||  || ||||| | | || | |  || ||  |
Sbjct:  2895 CATCCAGATCGGCCAACTGACGC-AGTAGCGTG-GCTTTTA-C-ATTCTCTGCAGTCTGT 2950

Query:   839 TCCGGGTTACGGTGAGCACCACCCAGTGGTTCCGGGATGATGGAGTCGATCAGTTTCAGT 780
             |   | | ||| || ||||| |||||||| ||    |||||   ||| ||||  | |||
Sbjct:  2951 TTGTGATCACGATGCGCACCGCCCAGTGGCTCTTCAATGATTTCGTCAATCAACTCCAGC 3010

Query:   779 TCTTTCAGACGCGGAGCAATGATACCCATCGCTTCAGCCGCCAGCGGCGCTTTGTCGGCG 720
             ||||||| ||| |||||||| | |||||||||||| |||||  | |||||||| || |
Sbjct:  3011 TCTTTCAAACGTGGAGCAATCAGACCCATCGCTTCTGCCGCTTGTGGCGCTTTATCAGAA 3070

Query:   719 CTCTT-CCACAGAATGGACGCACAACCTTCCGGCGAGATAACGGAATAGGTGCTGTATTG 661
              ||   ||||||||| |||||||||||||| || || || || || |||||   ||||||
Sbjct:  3071 -TCACGCCACAGAATCGACGCACAACCTTCTGGTGAAATCACTGAGTAGGTTGAGTATTG 3129

Query:   660 CAGCATATTCACTTTATCGCCCACGCCAATCGCCAGCGCACCGCCAGAACCACCTTCACC 601
             |||||| ||||| |  || || || |||||||||| |||||| ||||| |||||||| ||
Sbjct:  3130 CAGCATGTTCACATAGTCACCGACACCAATCGCCAACGCACCACCAGAGCCACCTTCGCC 3189

Query:   600 GATAACCGTACAAACTACCGGTACGCCGAGGCGAGACATTTCACGCAGGTTGCGTGCAAT 541
              |  ||  |||| |  || || ||    ||||  |||||  |    |||||    || ||
Sbjct:  3190 CACCACGTTACATATGACTGGCACCTTCAGGCCTGACATCACTTTAAGGTTTTTGGCGAT 3249

Query:   540 GGCTTCAGACTGACCACGCTCTTCTGCGCCCACGCCAGGATAAGCCCCCGGGGTGTCGAT 481
              ||||| |||||||| |||||||| || ||||| || || || || |||| ||| || ||
Sbjct:  3250 CGCTTCCGACTGACCGCGCTCTTCAGCACCCACACCTGGGTATGCGCCCGCGGTATCAAT 3309
```

Notes The match shown has been truncated.
Minus Strand HSPs (High-scoring Pairs) indicates that the reported scores were obtained with the reverse complement of the query sequence
Expect = 5.3e-95, P = 5.3e-95. This shows the probability of the score(s) occurring by chance. This is a very low value, suggesting that this match is highly significant
Strand = Minus / Plus. This indicates that the match shown contains the minus strand (reverse complement) of the query sequence, and the plus strand of the *V. cholerae* sequence

Figure 11.10 (b) example of a reported match, from the search result shown in (a)

The most significant column is that headed P(N), which provides an estimate of the probability of each match occurring by chance; the entries are arranged in descending order, so that the most significant matches are at the top. It is important not to read too much into this; it does not necessarily indicate true phylogenetic relationships, and as we will see later on the order may change, and different matches may be found, if the search is done using a different program (e.g. FASTA instead of BLAST) or using different values for parameters such as the gap penalty.

The BLAST search will also return the pairwise alignment of the query sequence with each of the matches; one example is shown in Figure 11.10(b), although this is not necessarily the optimal alignment (see the section on CLUSTAL below). Note that in this case the computer has used the reverse complement of the query sequence, as indicated by the label ‘Strand = Minus/ Plus’. You will see that although this is the highest scoring match shown in Figure 11.10(a), there are substantial differences between the two sequences (71 per cent identity). We will look later on at the match between these two sequences at the protein level.

11.3.2 Protein sequence comparisons

DNA sequence comparisons are inherently noisy. If you just take two random DNA sequences and put them side by side, there is a 25 per cent chance of a match at any specific position. For example suppose sequence X has a T at position 47; sequence Y, as a random sequence, will have an equal chance of having A, C, G or T at position 47 (assuming also an equal proportion of all four bases), so the chance of it matching is 1 in 4. Sometimes you have to use DNA sequence comparisons, for example if you are working with a non-coding sequence, but generally for searching databanks with an unknown sequence you will get much cleaner results with a protein sequence. (Note that all the predicted coding sequences in the DNA databanks are translated and incorporated in a protein sequence bank such as SwissProt.) A further advantage of searching at the protein level is that related proteins are generally more conserved than are the genes encoding for them.

The initial basis for protein sequence comparisons is just the same as described above for nucleotide sequence comparisons, and the algorithms used are fundamentally the same. The major difference arises from the fact that you have 20 amino acids instead of four bases. This doesn’t have to change anything – you can treat the 20 amino acids as all different from one another, and require a perfect match (*identity*) at any position. However, the computer usually offers you a more sophisticated scoring system, in which some pairs of amino acids are regarded as more different than others. This scoring system is based on a matrix of all the possible amino acid pairings, ranging from some pairs which score almost the same as a perfect match through to other pairs which are regarded as completely different. All of the scores in the matrix are used to calculate the overall score for the match, although the alignment presented will only mark matches above a selected cut-off score.

There are several such matrices to choose from, although here again the package you use will probably have a default in case you do not want to make a choice; but even if you accept the default, you should have some understanding of the basis of the matrix that is used. It is a common misconception that the alignment is calculated solely on the basis of the similarity between the amino acids; for example, a biochemist might group valine, leucine and isoleucine together as they have large nonpolar (hydrophobic) side chains, phenylalanine, tyrosine and tryptophan (aromatic), glutamate with aspartate (acidic, negatively charged), lysine and arginine (basic, positively charged), and so on. A change from one amino acid to another within the same group (say lysine to arginine) would be expected to have less effect on the structure and function of the protein than a change between groups (say lysine to phenylalanine), and so you would expect this similarity to be reflected in the scoring system.

That is indeed partly true; but in fact commonly used matrices such as the Dayhoff Point Accepted Mutation (PAM) matrices are derived empirically from comparisons of related proteins from different sources, and the score is derived from the frequency with which specific changes in amino acid sequence occur. This partly reflects the similarity between the amino acids, but is also influenced by the nature of the genetic code. Where a pair of amino acids can be interchanged by only a single base change in the DNA, this will occur more frequently than a change that requires two or three base changes. There are a series of PAM matrices with different values; one example (the PAM250 matrix) is shown in Figure 11.11. Values of zero indicate neutral changes,

C	12																			
S	0	2																		
T	–2	1	3																	
P	–3	1	0	6																
A	–2	1	1	1	2															
G	–3	1	0	–1	1	5														
N	–4	1	0	–1	0	0	2													
D	–5	0	0	–1	0	1	2	4												
E	–5	0	0	–1	0	0	1	3	4											
Q	–5	–1	–1	0	0	–1	1	2	2	4										
H	–3	–1	–1	0	–1	–2	2	1	1	3	6									
R	–4	0	–1	0	–2	–3	0	–1	–1	1	2	6								
K	–5	0	0	–1	–1	–2	1	0	0	1	0	3	5							
M	–5	–2	–1	–2	–1	–3	–2	–3	–2	–1	–2	0	0	6						
I	–2	–1	0	–2	–1	–3	–2	–2	–2	–2	–2	–2	–2	2	5					
L	–6	–3	–2	–3	–2	–4	–3	–4	–3	–2	–2	–3	–3	4	2	6				
V	–2	–1	0	–1	0	–1	–2	–2	–2	–2	–2	–2	–2	2	4	2	4			
F	–4	–3	–3	–5	–4	–5	–4	–6	–5	–5	–2	–4	–5	0	1	2	–1	9		
Y	0	–3	–3	–5	–3	–5	–2	–4	–4	–4	0	–4	–4	–2	–1	–1	–2	7	10	
W	–8	–2	–5	–6	–6	–7	–4	–7	–7	–5	–3	2	–3	–4	–5	–2	–6	0	0	17
	C	S	T	P	A	G	N	D	E	Q	H	R	K	M	I	L	V	F	Y	W

Figure 11.11 Protein weight matrix: for comparison of amino acid sequences, whether for database searching or for multiple alignments, matrices are used to score every possible pair of amino acids; this example shows the Dayhoff PAM 250 matrix

while increasing positive or negative values indicate increasingly acceptable or unacceptable mutations, respectively. Note that in some cases, a mismatch can give a positive score – and in particular a mismatch between tyrosine (Y) and phenylalanine (F) actually gives a higher score than a perfect match of most other amino acids. If you are not familiar with the one-letter amino acid notation, see Box 11.1.

Box 11-1 Amino acid notations

	Three letter notation	One letter notation
alanine	Ala	A
arginine	Arg	R
asparagine	Asn	N
aspartate	Asp	D
cysteine	Cys	C
glutamate	Glu	E
glutamine	Gln	Q
glycine	Gly	G
histidine	His	H
isoleucine	Ile	I
leucine	Leu	L
lysine	Lys	K
methionine	Met	M
phenylalanine	Phe	F
proline	Pro	P
serine	Ser	S
threonine	Thr	T
tryptophan	Trp	W
tyrosine	Tyr	Y
valine	Val	V

The PAM matrices were derived from studies of proteins that are very similar. However, database searches are commonly used for the identification of more distantly related sequences, and under these conditions the PAM matrices are not necessarily ideal. An alternative family of matrices known as BLOSUM ('blossom') – BLOcks SUbstitution Matrix – can be used; these are derived in a somewhat different way. The same considerations apply; it is just the values that are different. Some of the subsequent comparisons use the BLOSUM62 matrix.

Figure 11.12 shows the output from a BLAST search of the SwissProt database, using, as the query sequence, the translated product of the *E. coli accA* gene that we employed for the nucleic acid search above. The general structure of the table is the same (although we have only shown the ID and not the accession numbers, to simplify it). As before, we have added our own reference numbers in the first column; numbers up to 31 indicate that the protein is derived from the corresponding sequence in Figure 11.10. Higher numbers show sequences that did not figure in the nucleic acid comparison. (We should discount the asterisked ones, where the difference is solely due to an update of the database.) Apart from these, the top six sequences are in the same order as before; thereafter the order is far from identical, and as we go further down the table we find a number of sequences where a match was found at the protein level that did not register in the DNA search. In some cases, the reason is clear: the organisms differ substantially in the base composition of their DNA. This accounts, for example, for the presence of mycobacterial and Streptomyces sequences in this figure but not in the DNA comparison. The protein sequences are quite well conserved, but the difference in the base composition of the DNA (about 65 per cent G+C, compared to 50 per cent for *E. coli*) means that the DNA sequences are less similar.

You should not be too impressed by the fact that most of these matches are labelled as acetyl/propionyl-CoA carboxylase: many of them are from genome sequence data, and the only reason they have this label is precisely because of their similarity to proteins such as the *E. coli* AccA. In this example, it is probably correct, but the potential for a circular argument has to be remembered at all times.

As before, the program will also return a pairwise alignment of the sequences. If you compare the protein alignment shown in part (b) with the alignment of the corresponding DNA sequences in Figure 11.10, you will see that the protein comparison produces a much clearer similarity. For optimal alignment, and for alignment of multiple sequences, CLUSTAL (see below) should be used.

Note that all the cautionary comments discussed in relation to nucleic acid searches also apply to protein sequence comparisons: the list of matches is not definitive, the order shown is not necessarily a true reflection of the order of similarity, and you may get a different set of matches if you use a different

(a) Query = AccA (319 letters) [see Figures 11.9 and (a) 11.10]
Database: swall 729 700 sequences; 231 810 429 total letters.

		Sequences producing High-scoring Segment Pairs:		High Score	Smallest sum Probability $P(N)$	$\overline{N}$
32*	AAL19196	Salmonella typhimurium	ACETYL-COA CARBOXYLASE	1588	7.7e-163	1
33*	CAD08690	Salmonella typhi	ACETYL-COA CARBOXYLASE	1588	7.7e-163	1
34*	CAC89902	Yersinia pestis	ACETYL-COA CARBOXYLASE	1473	1.2e-150	1
1	**Q9KPW8**	**Vibrio cholerae**	**ACETYL-COA CARBOXYLASE**	**1259**	**5.6e-128**	**1**
2	Q9CNX9	Pasteurella multocida.	ACETYL-COA CARBOXYLASE	1249	6.5e-127	1
3	**ACCA_HAEIN**	**Haemophilus influenzae**	**ACETYL-COA CARBOXYLASE**	**1234**	**2.5e-125**	**1**
4	Q9HXZ2	Pseudomonas aeruginosa	ACETYL-COA CARBOXYLASE	1135	7.8e-115	1
5	Q9JRV8	Neisseria meningitidis	ACETYL-COA CARBOXYLASE,	1047	1.6e-105	1
6	Q9PGU5	Xylella fastidiosa	ACETYL-COA CARBOXYLASE	1038	1.5e-104	1
16	Q9K842	Bacillus halodurans	ACETYL-COA CARBOXYLASE	912	3.3e-91	1
13	ACCA_SYNP7	Synechococcus sp..	ACETYL-COA CARBOXYLASE	875	2.8e-87	1
8	Q05914	Bacillus stearothermophilus	HYPOTHETICAL PROTEIN IN P	848	2.0e-84	1
10	P71532	Magnetospirillum sp.	MAGNETIC PARTICLE SURFACE	837	2.9e-83	1
17	**BAB57862**	**Staphylococcus aureus**	**ACETYL-COA CARBOXYLASE**	**832**	**1.0e-82**	**1**
35*	CAC99650	Listeria monocytogenes	ACETYL-COA CARBOXYLASE	826	4.3e-82	1
36	ACCA_BACSU	Bacillus subtilis.	ACETYL-COA CARBOXYLASE	824	7.0e-82	1
11	AAK89767	Agrobacterium tumefaciens.	AGR_L_2394P.	824	7.0e-82	1
9	Q9A448	Caulobacter crescentus	ACETYL-COA CARBOXYLASE	815	6.3e-81	1
21	ACCA_PORPU	Porphyra purpurea.	ACETYL-COA CARBOXYLASE	785	9.5e-78	1
12	Q98FX7	Mesorhizobium loti	ACETYL-COA CARBOXYLASE	768	6.0e-76	1
23	Q9P162	Campylobacter jejuni	ACETYL-COA CARBOXYLASE	748	7.9e-74	1
18	Q9RV16	Deinococcus radiodurans	ACETYL-COA CARBOXYLASE	741	4.4e-73	1
26	ACCA_HELPJ	Helicobacter pylori J99	ACETYL-COA CARBOXYLASE	726	1.7e-71	1
19	**Q9LLQ9**	**Glycine max (Soybean).**	**CARBOXYL TRANSFERASE**	**726**	**1.7e-71**	**1**
29	ACCA_ANTSP	Antithamnion sp.	ACETYL-COA CARBOXYLASE	723	3.5e-71	1
28	Q9Z8C9	Chlamydia pneumoniae	ACCOA CARBOXYLASE/TRANS	723	3.5e-71	1
27	Q9PKC9	Chlamydia muridarum	ACETYL-COA CARBOXYLASE	720	7.4e-71	1
20	Q9LD43	Arabidopsis thaliana (cress).	CARBOXYLTRANSFERASE	710	8.5e-70	1
14	**Q41008**	**Pisum sativum (Garden pea)**	**CHLOROPLAST MEMBRANE P**	**706**	**2.2e-69**	**1**
37	Q97DB2	Clostridium acetobutylicum	ACETYL-COA CARBOXYLASE	686	3.0e-67	1
22	Q99YE1	Streptococcus pyogenes	PUTATIVE ACETYL-COA CARB	653	9.3e-64	1
25	Q9FBB7	Streptococcus pneumoniae	ACETYL-COA CARBOXYLASE	638	3.6e-62	1
24	Q9CHF1	Lactococcus lactis.	ACETYL-COA CARBOXYLASE	604	1.4e-58	1
38	Q9LCG6	Lactobacillus plantarum	CARBOXYLTRANSFERASE	270	3.6e-23	1
39	Q9GQQ8	Giardia lamblia	PUTATIVE ACETYL-COA CARB	265	3.5e-21	1
41	Q9L0A1	Streptomyces coelicolor.	PUTATIVE ACETYL COA CARB	242	1.3e-19	1
42	ACCD_MYCTU	Mycobacterium tuberculosis.	PUTATIVE ACETYL-COA CARB	227	7.9e-17	1
31	AAK57728	Bacillus sphaericus	PUTATIVE ACETYL-COA CARB	172	2.2e-12	1
43	O28066	Archaeoglobus fulgidus	METHYLMALONYL-COA DECA	178	1.3e-10	1
44	BAB65588	Sulfolobus tokodaii	HYPOTHETICAL METHYLMAL	138	4.6e-06	1
45	AAL03498	Rickettsia conorii	PROPIONYL-COA CARBOXYLA	137	5.8e-06	1
46	PCCB_MYCLE	Mycobacterium leprae	PROBABLE PROPIONYL-COA C	134	1.4e-05	1
47	PCCB_SACER	Saccharopolyspora erythraea.	PROPIONYL-COA CARBOXYLA	132	2.3e-05	1
48	**PCCB_HUMAN**	**Homo sapiens (Human).**	**PROPIONYL-COA CARBOXYLA**	**130**	**3.8e-05**	**1**
49	**PCCB_PIG**	**Sus scrofa (Pig)**	**PROPIONYL-COA CARBOXYLA**	**130**	**3.8e-05**	**1**
50	O05766	Mycobacterium smegmatis.	PUTATIVE PROPIONYL-COA	121	4.6e-05	1
51	Q9A3J0	Caulobacter crescentus	CARBAMOYL-PHOSPHATE SYN	130	9.4e-05	1

Notes This is an edited list. Some entries have been deleted, and species names have been added in for explanation. Numbers in the left-hand column have been added for cross-referencing to other figures. Asterisks indicate that the corresponding entries are not in Figure 11.10 but are present in the updated database. Entries in bold are those used for alignment in Figure 11.14. See Figure 11.10 for further notes.

Figure 11.12 Results of a database search using BLASTP (protein search): (a) list of matching sequence with scores; (b) (overleaf)

```
(b) >SWALL:Q9KPW8 ACETYL-COA CARBOXYLASE, CARBOXYL TRANSFERASE
ALPHA SUBUNIT (Vibrio cholerae)
Length = 319

Score = 1259 (448.2 bits), Expect = 5.6e-128, P = 5.6e-128
Identities = 241/318 (75%), Positives = 276/318 (86%)

Query:  1 MSLNFLDFEQPIAELEAKIDSLTAVSRQDEKLDINIDEEVHRLREKSVELTRKIFADLGA  60
          MSLNFLDFE+PI ELE KI +L  VSR      +++D+E+ +L +KS+EL +KIF+DLGA
Sbjct:  1 MSLNFLDFEKPIVELETKIQALRDVSRHSTSASVDLDKELEQLEKKSLELKKKIFSDLGA  60

Query: 61 WQIAQLARHPQRPYTLDYVRLAFDEFDELAGDRAYADDKAIVGGIARLDGRPVMIIGHQK  120
          WQ+AQLARHPQRPYTLDY++  F EFDELAGDRAYADDKAIVGGIARL+GR VM+IGHQK
Sbjct: 61 WQVAQLARHPQRPYTLDYLKHIFTEFDELAGDRAYADDKAIVGGIARLEGRSVMVIGHQK  120

Query: 21 GRETKEKIRRNFGMPAPEGYRKALRLMQMAERFKMPIITFIDTPGAYPGVGAEERGQSEA  180
          GRET+EK++RNFGMP PEGYRKALRLM+MAERF MPIITFIDT GAYPGVGAEERGQSEA
Sbjct: 21 GRETREKVKRNFGMPKPEGYRKALRLMEMAERFNMPIITFIDTAGAYPGVGAEERGQSEA  180

Query: 81 IARNLREMSRLGVPVVCTVIGEGGSGGALAIGVGDKVNMLQYSTYSVISPEGCASILWKS  240
          IA+NL+ MS L VPV+C V+GEGGSGGALAIGVGD VNMLQYSTYSVISPEGCASILW +
Sbjct: 81 IAKNLKVMSGLKVPVICNVVGEGGSGGALAIGVGDYVNMLQYSTYSVISPEGCASILWRD  240

Query: 41 ADKAPLAAEAMGIIAPRLKELKLIDSIIPEPLGGAHRNPEAMAASLKAQLLADLADLDVL  300
          +DKAP AAEAMG+IAPRLKEL+LID II EPLGGAHR+  + A ++KA LL  LADLD L
Sbjct: 41 SDKAPQAAEAMGLIAPRLKELELIDEIIEEPLGGAHRDHKQTAENVKATLLRQLADLDAL  300

Query: 01 STEDLKNRRYQRLMSYGY 318
            E+L  RRYQRLM+YGY
Sbjct: 01 DHENLLERRYQRLMNYGY 318
```

Notes This shows the match between the two proteins corresponding to the DNA sequences compared in Figure 11.10 (b). See also Table CLUSTAL for alignment of these proteins with others selected from part (a) of this table. The line between the two sequences shows identical amino acids, and (marked with +) those positions scored as positive similarities according to the matrix used (in this case BLOSUM62).

Figure 11.12 (b) example of a reported match

program, or set the variables differently. In Figure 11.13 we show the output using the same query sequence, but this time using FASTA instead of BLASTP. The differences are not dramatic; this is a reasonably well-conserved protein and so there are a large number of good matches, which will show up however you do the analysis. As you move further down the table, however, the extent of the differences increases – so much so that there are a number of sequences in this list that cannot be considered as a significant match, although they showed a respectable score in the BLAST comparison. Conversely, some proteins that appear in the BLASTP search were not detected as a match by FASTA.

AccA: 319 aa vs SWISS-PROT All library; 231810429 residues in 729700 sequences FASTA (3.39 May 2001) function [optimized, BL62 matrix (11:−4)] ktup: 2 join: 37, opt: 25, gap-pen: −8/ −1, width: 16

```
     The best scores are:                                         opt   bits  E(729387)
32   AAL19196      ACETYL-COA CARBOXYLASE               (319)    1588   367     2e-100
33   CAD08690      ACETYL-COA CARBOXY                   (319)    1588   367     2e-100
34   CAC89902      ACETYL-COA CARBOXY                   (319)    1473   342    1.2e-92
 1   Q9KPW8        ACETYL-COA CARBOXYLASE               (319)    1259   294    3.2e-78
 2   Q9CNX9        ACCA.                                (317)    1251   292    1.1e-77
 3   ACCA_HAEIN    ACETYL-COA CARBOXY                   (315)    1238   289    8.3e-77
 4   Q9HXZ2        ACETYL-COA CARBOXYLASE               (316)    1138   266    4.6e-70
 5   Q9JRV8        ACETYL-COA CARBOXYLASE               (319)    1050   247    4.1e-64
 6   Q9PGU5        ACETYL-COA CARBOXYLASE               (319)    1041   245    1.6e-63
16   Q9K842        ACETYL-COA CARBOXYLASE               (325)     915   216    5.3e-55
13   ACCA_SYNP7    ACETYL-COA CARBOXY                   (327)     878   208    1.7e-52
 8   Q05914        HYPOTHETICAL PROTEIN                 (320)     852   202    9.3e-51
10   P71532        MAGNETIC PARTICLE SURFACE            (317)     840   200      6e-50
17   BAB57862      ACETYL-COA CARBOXYLASE CA            (314)     837   199    9.4e-50
35   CAC99650      ACCA PROTEIN.                        (318)     829   197    3.3e-49
36   ACCA_BACSU    ACETYL-COA CARBOXY                   (325)     827   197    4.6e-49
11   AAK89767      AGR_L_2394P.                         (317)     826   196    5.2e-49
 9   Q9A448        ACETYL-COA CARBOXYLASE               (320)     819   195    1.6e-48
21   ACCA_PORPU    ACETYL-COA CARBOXY                   (324)     788   188      2e-46
12   Q98FX7        ACETYL-COA CARBOXYLASE               (316)     774   185    1.7e-45
18   Q9RV16        ACETYL-COA CARBOXYLASE               (316)     746   179    1.3e-43
23   Q9PI62        ACETYL-COA CARBOXYLASE               (312)     742   178    2.4e-43
29   ACCA_ANTSP    ACETYL-COA CARBOXY                   (322)     731   175    1.4e-42
28   Q9Z8C9        ACCOA CARBOXYLASE/TRANSF             (324)     726   174      3e-42
19   Q9LLQ9        CARBOXYL TRANSFERASE                 (690)     729   175    3.3e-42
27   Q9PKC9        ACETYL-COA CARBOXYLASE               (324)     723   173    4.8e-42
26   ACCA_HELPJ    ACETYL-COA CARBOXY                   (312)     721   173    6.3e-42
20   Q9LD43        CARBOXYLTRANSFERASE                  (769)     715   172    3.2e-41
14   Q41008        CHLOROPLAST INNER MEMBRANE           (875)     710   171    7.6e-41
37   Q97DB2        ACETYL-COA CARBOXYLASE               (274)     688   166    9.6e-40
22   Q99YE1        PUTATIVE ACETYL-COA CARBOX           (256)     657   159    1.1e-37
25   Q9FBB7        ACETYL-COA CARBOXYLASE               (255)     642   155    1.2e-36
24   Q9CHF1        ACETYL-COA CARBOXYLASE CAR           (260)     578   141    2.4e-32
38   Q9LCG6        CARBOXYLTRANSFERASE                  (257)     280    74    3.1e-12
39   Q9GQQ8        PUTATIVE ACETYL-COA CARBOX          (1338)     257    69    3.9e-10
42   ACCD_MYCTU    PUTATIVE ACETYL-CO                   (495)     244    66    1.4e-09
41   Q9L0A1        PUTATIVE ACETYL COA CARBOX           (458)     225    62    2.5e-08
43   O28066        METHYLMALONYL-COA DECARBOX           (516)     168    49    0.00019
52   AAK48272      PROPIONYL-COA CARBOXYLASE            (522)     158    47    0.00091
51   Q9A3J0        CARBAMOYL-PHOSPHATE SYNTH...        (1078)     157    47     0.0018
44   BAB65588         523AA LONG HYPOTHETICAL M         (523)     150    45     0.0032
53   Q9I3U4        PROBABLE PYRUVATE CARBOXYL          (1095)     151    46     0.0047
49   PCCB_PIG      PROPIONYL-COA CARBOXYLASE            (539)     146    44      0.006
48   PCCB_HUMAN    PROPIONYL-COA CARBOXYLASE            (539)     145    44      0.007
54   AAK57534      PUTATIVE DECARBOXYLASE PG            (521)     143    44     0.0093
55   Q9ZGC9           DECARBOXYLASE HOMOLOG.            (524)     142    43      0.011
50   O05766        PUTATIVE PROPIONYL-COA CAR           (210)     136    42      0.014
45   AAL03498      PROPIONYL-COA CARBOXYLASE            (514)     138    42       0.02
47   PCCB_SACER    PROPIONYL-COA CARBOXYLASE            (546)     137    42      0.025
```

Notes This is an edited list, with some entries omitted. The numbers in the left-hand column are added for cross-referencing to other figures. Those numbered 52–55 are not present in Figure 11.12. Some entries in Figure 11.12 are not found in this search.

Figure 11.13 Results of a database search using FASTA (protein search)

FASTA and BLAST, although they work in different ways, have one thing in common: they are essentially shortcuts to enable database searches to be carried out rapidly without demanding too much computer power. The price to be paid is the possibility of missing something significant. A possibly more sensitive method is provided by the Smith–Waterman algorithm, which is used by a program known as MPsrch.

The comparison between the methods highlights an important message. Many matches feature in all searches, but not in the same order of score. On the other hand, some matches were found by one method and not by another. With a less well-conserved protein, these differences would be greater. Furthermore, these searches were carried out using the default values for parameters like gap penalties; carrying out the same search with different values of these parameters might produce further differences in the list of matches. The take-home message is that the list of matches resulting from any program is not a definitive list of all matching sequences, nor the last word in how well those sequences match.

As we have mentioned above, only a small minority of the proteins in the databases have been sequenced as such. Most of them have been deduced from the corresponding cDNA and genomic DNA sequences, with varying degrees of confidence. These predictions are not necessarily either accurate or complete – for example a product may be translated from a different reading frame, or transcription (from a genomic sequence) may occur in the opposite direction. Conversely, and even more likely, if your own DNA sequence that you wish to compare is incomplete, you will not be able to accurately predict intron/exon boundaries (if applicable) or the location and direction of the ORF. However, it is possible to instruct the computer to compare the predicted products of all potential reading frames of your DNA, even in both directions, against the protein sequences deposited in the database – or even all six reading frames of your query sequence against all six reading frames of the DNA sequences in the databanks.

GenBank also has sections that are based on sequence variation, rather than the canonical sequence for each given species. One of these, the Taxonomy Database, lists sequences by species, organized according to taxonomic boundaries. Another section, the SNP database, is dedicated to cataloguing single nucleotide polymorphisms between individuals of one species. More specifically, the OMIM database (Online Mendelian Inheritance in Man) catalogues information, both genetic and phenotypical, about known genetic diseases.

11.3.3 Sequence alignments: CLUSTAL

As indicated in the above discussion, search methods such as FASTA and BLAST do not necessarily display the optimal alignment between your query sequence and the target. Furthermore, they only show pairwise alignments, whereas you are likely to want to see how your sequence lines up with a collection of other proteins. (Similar arguments apply to nucleic acid comparisons, but we will just look at protein alignments.) In order to produce optimized multiple alignments, the most commonly used program is CLUSTAL. (Many suites of programs incorporate a version of CLUSTAL for sequence alignments, although not necessarily identifying it as such.)

CLUSTAL starts by comparing all the sequences submitted, to produce a matrix of pairwise alignment scores. This can be displayed as a dendrogram, a 'family tree' of sequences. This information is then used to organize the alignment process, following the branching order of the dendrogram, until a multiple alignment of all the proteins is produced. The comments above about gap penalties and amino acid substitution matrices also apply to CLUSTAL alignments. In addition to the multiple alignment, the program will also generate a consensus sequence. An example of the output from CLUSTAL, using a selection of the protein matches from Figure 11.12, is shown in Figure 11.14. It can be seen that a substantial number of amino acids (indicated by an asterisk) are conserved in all the sequences shown, whether from bacterial, plant or mammalian sources.

The alignment produced should not be regarded as absolute – indeed a visual inspection may show places where the alignment could obviously be improved, for example by introducing an extra gap into one of the sequences. Remember that there is no such thing as a perfect alignment, and the alignment produced does not necessarily have any absolute meaning; this is mainly a tool to display features of the sequence.

Alignments of sequences can be used, cautiously, as aids towards the establishment or confirmation of evolutionary relationships. Some of the conserved amino acids that are identified by the alignment may have real significance, such as indicating a substrate-binding motif for example, and it is then likely that the aligned amino acids do actually represent an evolutionary relationship. However, in many cases, to be able to draw such a conclusion, you would need to look at the position of this amino acid in the three-dimensional structure of the proteins you are comparing.

```
ACCA_ECOLI     ----------------------------SLNFLDFEQPIAELEAKIDSLTAVSRQDEKLD   32
Q9KPW8         ---------------------------MSLNFLDFEKPIVELETKIQALRDVSRHSTSAS   33
ACCA_HAEIN     ---------------------------MNQEYLDFELPIAELEAKIEALRAASDDK----   29
BAB57862       -------------------------------MLDFEKPLFEIRNKIESLKESQDKN---D   26
Q9LLQ9         PWPANPGPNVKGGVLSHLSLFKPLKGEPKPVTLDFEKPLVDLQKKIIDVQKMANET---G   115
Q41008         PWSSNPDPNMKGGRLRHLSTFQPLKQPPKPVILEFEKPLINMEKKINDFRKVAEKT---G   117
PCCB_HUMAN     ---------------------MAAALRVAAVGARLSVLASGLRAAVRSLCSQATSVN-ER   38
PCCB_PIG       ---------------------MAAAVRVTAARARLRVVVRSLHAGVRSLCTQPVSVN-ER   38
                                                 :       :.  :  .

ACCA_ECOLI     INIDEEVHRLREKSVELTRKIFAD-LGAWQIAQLARHPQRPYTLDYVRLAFDEFDELAGD   91
Q9KPW8         VDLDKELEQLEKKSLELKKKIFSD-LGAWQVAQLARHPQRPYTLDYLKHIFTEFDELAGD   92
ACCA_HAEIN     VDLTDEIKRLQKKSNELTKKTFAN-LDAWQVSRMARHPNRPYTLDYIEHIFTEFEELAGD   88
BAB57862       VDLQEEIDMLEASLERETKKIYTN-LKPWDRVQIARLQERPTTLDYIPYIFDSFMELHGD   85
Q9LLQ9         LDFSDQILSLENKYQQALKDLYTH-LTPIQRVNIARHPNRPTFLDHVFNITEKFVELHGD   174
Q41008         VDLSDQILALEAKYQKALVELYTN-LTPIQRVTVARHPNRPTFLDHMYNMTEKFVELHGD   176
PCCB_HUMAN     IENKRRTALLGGGQRRIDAQHKRGKLTARERISLLLDPGSFVESDMFVEHRCADFGMAAD   98
PCCB_PIG       IENKRQAALLGGGQRRIDSQHKRGKLTARERISLLLDPGSFIESDMFVEHRCADFGMAAD   98
               ::   .   *     .   .     * . :   :          * .         : .*

ACCA_ECOLI     RAYADDKAIVGGIARLDGRPVMIIGHQKGRETKEKIRRNFGMPAPEGYRKALRLMQMAER   151
Q9KPW8         RAYADDKAIVGGIARLEGRSVMVIGHQKGRETREKVKRNFGMPKPEGYRKALRLMEMAER   152
ACCA_HAEIN     RAFADDKAIVGGLARLDGRPVMVIGHQKGRSVKEKVQRNFGMPAPEGYRKALRLMEMAER   148
BAB57862       RNFRDDPAMIGGIGFLNGRAVTVIGQQRGKDTKDNIYRNFGMAHPEGYRKALRLMKQAEK   145
Q9LLQ9         RAGYDDPAIVTGLGTIDGRSYMFIGHQKGRNTKENIQRNFGMPTPHGYRKALRLMEYADH   234
Q41008         REGYDDPAIAAGLGSIDGKTYMFIGHQKGRDTKENIKRNFAMPTPHGYRKALRLMEYADH   236
PCCB_HUMAN     KNKFPGDSVVTGRGRINGRLVYVFS ------ QDFTVFGGSLSGAHAQKICKIMDQAIT   150
PCCB_PIG       KNKFPGDSVVTGRGRINGRLVYVFS ------ QDFTVFGGSLSGAHAQKICKIMDQAMT   150
               :    .  :: * . ::*    .:.        :..    .       :*  ::*. *

ACCA_ECOLI     FKMPIITFIDTPGAYPGVGAEER-GQSEAIARNLREMSRLGVPVVCTVIGEGGSGGALAI   210
Q9KPW8         FNMPIITFIDTAGAYPGVGAEER-GQSEAIAKNLKVMSGLKVPVICNVVGEGGSGGALAI   211
ACCA_HAEIN     FKLPIITFIDTPGAYPGIGAEER-GQAEAIARNLREMAQLTVPVICTVIGEGGSGGALAI   207
BAB57862       FNRPIFTFIDTKGAYPGKAAEER-GQSESIATNLIEMASLKVPVIAIVIGEGGSGGALGI   204
Q9LLQ9         HGFPIVTFIDTPGAYADLKSEEL-GQGEAIAHNLRSMFGLKVPVISIVIGEGGSGGALAI   293
Q41008         HGFPIVTFIDTPGAFADLKSEQL-GQGEAIAHNLRSMFALKVPVISIVIGEGGSGGALAI   295
PCCB_HUMAN     VGAPVIGLNDSGGARIQEGVESLAGYADIFLRNVTASG--VIPQISLIMGPCAGGAVYSP   208
PCCB_PIG       VGAPVIGLNDSGGARIQEGVESLAGYADIFLRNVSASG--VIPQISLIMGPCAGGAVYSP   208
                  *:. : *: **      *.  * .: :  *:      : * :. ::*  ..*... .
```

Key

ACCA_ECOLI	Escherichia coli
Q9KPW8	Vibrio cholerae
ACCA_HAEIN	Haemophilus influenzae
BAB57862	Staphylococcus aureus
Q9LLQ9	Glycine max (Soybean)
Q41008	Pisum sativum (Garden pea)
PCCB_HUMAN	Homo sapiens (Human)
PCCB_PIG	Sus scrofa (Pig)

Note that the alignments have been truncated.
In the line under the alignments,

* indicates identical or conserved residues in all sequences in the alignment
: indicates conserved substitutions
. indicates semi-conserved substitutions.

Figure 11.14 CLUSTAL multiple sequence alignment: this shows a multiple alignment of protein sequences selected from Figure 11.12

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12

Analysis of Genetic Variation

12.1 Nature of Genetic Variation

The basic structure and organization of genes, in eukaryotes and prokaryotes, was considered in Chapter 2. It is a central dogma of molecular biology that the inherited characteristics of an organism are a reflection of the structure and organization of its genes. However, it is important to realize that this includes an extremely complex set of interactions between different genes, and their products, as well as environmental factors. The gene(s) directly responsible for the observed characteristic may be absolutely identical but the effects may be different because of variation in other genes that affect their expression, or because of alteration in other cellular components that affect the activity of the proteins encoded by those genes. Environmental influences will therefore have a major role in determining the observed characteristics of the organism. Excessive reductionism – ascribing every change to one single gene – is an ever-present pitfall in the analysis of variation, as many traits are much more complex than that.

We can use the study of genetic variation to examine differences between members of the same species, ranging from the study of bacterial characteristics (such as antibiotic resistance) to investigation of human genetic diseases, or to differentiate between individuals (for example in forensic analysis). Alternatively, we can compare the genetic composition of members of different species – even over wide taxonomic ranges – which can throw invaluable light on the processes of evolution as well as helping to define the taxonomic relationship between species. Many of the concepts involved are similar, but of course the differences are (usually) much greater when we are comparing widely different species.

12.1.1 Single nucleotide polymorphisms

The simplest form of genetic variation consists of a change in the sequence of bases at a single point. Such differences are called *single nucleotide polymorphisms* (SNPs). It is worth reflecting briefly on the consequences of such a simple change. The first possibility is that the polymorphism is not even within a gene; indeed in eukaryotes that is the most common occurrence (this is both because most eukaryotic DNA is not a part of any gene, and because a non-coding sequence is where a polymorphism is least likely to have a negative effect). Second, if the change is within a gene, the chances are (again in eukaryotes) that it is in an intron and (unless it affects a splice site) does not affect the resulting gene products. Even if it is within an exon, there may be no change at all in the sequence of the protein encoded by that gene. A single base change may alter for example a leucine codon such as UUA into CUA, which also codes for leucine (Figure 12.1). This is referred to as a *synonymous* substitution. This would be expected to have little or no effect on the organism (and hence is also referred to as a silent mutation) – although the change in codon usage could have some effect on the translation of the mRNA. Other changes may also be silent, although not synonymous. A change from UUA to GUA would replace the leucine with valine at that position in the protein, which may have little consequence for protein structure and function (depending on how critical that specific position is), since leucine and valine are similar amino acids. Other changes, such as UUA to UCA (serine), are more likely to have an effect, although such a change (and even more radical changes) can be tolerated at some positions. These non-synonymous (or missense) substitutions therefore may or may not give rise to changes in the characteristics of the cell.

Most commonly these changes will either have no significant effect or will simply impair or destroy the function of the product of the gene. Very rarely, they will lead to a protein that can function in a slightly altered way – for example, an enzyme that may be able to use a different substrate. An

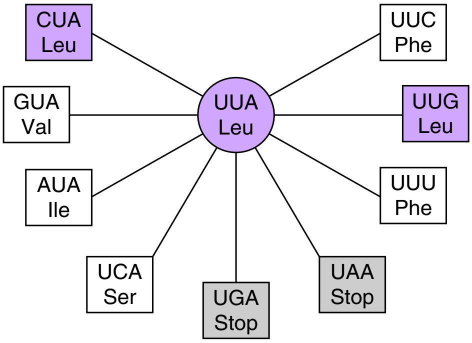

Figure 12.1 Codons arising by single-base substitutions from UUA

accumulation of such mutations may eventually give rise to an enzyme which carries out quite a different reaction. This is of course the basis of the gradual process of evolution. It is also important to keep in mind the differences that *diploidy* makes in species such as our own. Clearly, having two copies of each gene allows more leeway for evolutionary drift in one allele, as long as the other one functions normally. This is why carriers of a recessive disease gene generally are not affected by it. When analysing genetic variation in diploid organisms, it is obviously paramount to keep in mind that the organism has two copies of each gene (with exceptions such as the X chromosome genes in mammals), and that the individual can be either homozygous or heterozygous for any given genetic variation. There are potential pitfalls here; for example, you may screen an individual for a particular SNP, find only the wild-type allele and conclude that it is a homozygote. However, if that individual had a large deletion in one allele deleting a large part of or the whole gene, then you would, with many methods, only see one allele, and might erroneously conclude that the individual is homozygous.

However, it is much easier to obtain a mutation that results in the loss of function than one which changes the characteristics of the product, so although we can study the products of changes that have accumulated over a long time scale, we rarely see these changes in action. The main exception to this is where there is a strong selective pressure in favour of such a change – the best example being some types of antibiotic resistance in bacteria. For example, rifampicin resistance can be caused by specific mutations in the gene (*rpoB*) for one of the subunits of RNA polymerase. Although this is an essential enzyme, and therefore there are constraints on the mutations that can be tolerated, the use of rifampicin as an antibiotic provides a selective pressure under which only those rare mutants with an appropriate *rpoB* mutation can survive.

A further possibility is exemplified by the change from UUA to UGA, in Figure 12.1. This is a *stop codon* (in the standard genetic code), i.e. it causes termination of protein synthesis (because of the absence of a tRNA that could recognize it). Such a change will therefore cause a truncated protein, which will usually have lost activity.

If instead of changing a single base we envisage a situation where a base is removed, then we have a *frameshift mutation*. Once the ribosomes pass that point in the mRNA, they are reading the message in the wrong frame – and the translation product will be totally different from that point onwards. Usually they will encounter a stop codon quite quickly, as the unused reading frames are generally well supplied with stop codons.

A point mutation may also occur within an exon but outside the coding region in the 5′ or 3′ untranslated regions (UTR). One might assume that such mutations would not cause any discernable differences in the phenotype, but they may have an effect, because the UTRs have a function, too, although a less easily predicted one than the coding region. They serve as a type of ‘bar code' specifying parameters such as sorting and stability of the RNA, which is accomplished by the action of RNA-binding proteins. An alteration to the mRNA in this region may therefore affect its recognition by such a protein, which may have a significant effect on the translatability of the mRNA.

12.1.2 Larger-scale variations

In addition to these *point mutations*, we now recognize that there is a wide range of larger-scale variations in the structure of the genome. These include *deletions*, *duplications*, *insertions*, *transpositions*, *inversions*, and other rearrangements. Of particular importance in the context of this chapter is the activity of mobile genetic elements, including insertion sequences and transposons. Genome sequencing projects have disclosed large numbers of such elements in most species, where they play an important role in the generation of variation by inactivation of the genes into which they are inserted; they also provide a convenient tool for the differentiation of distinct strains and species. Extrachromosomal agents (plasmids and bacteriophages in prokaryotes, retroviruses in eukaryotes) are also a significant component of variation. In eukaryotic cells, we also have to recognize the contribution of the prokaryote-derived mitochondria and chloroplasts to the overall genetic make-up of the cell.

12.1.3 Conserved and variable domains

A typical protein can be considered to be composed of a number of regions with different functions. In many cases, these different regions fold into semi-autonomous structures known as *domains*. Some of these regions are concerned directly with binding to the substrate(s); since the structure of these regions is essential for the function of the enzyme, it is not surprising that they show relatively little variation from one species to another. They are referred to as *conserved* regions. In Chapter 11, we saw how you can compare protein sequences to identify conserved domains (or smaller regions known as *motifs*). On the other hand, some regions, especially those which do not play a vital role in the folding of the protein, are much more variable. These often include the terminal regions, as well as regions connecting separate domains, and other sequences that form loops protruding from the main body of the folded protein. The latter are especially important in some viruses (notably HIV) where they are both *hypervariable* and *immunodominant*, i.e. they vary very extensively and rapidly, and constitute the principal antigen 'seen' by the body's immune system. This makes it extremely difficult to produce effective vaccines.

In some genes, in eukaryotes, there is a clear correlation between the exons in the gene and the domains in the protein – for example in immunoglobulins, each exon in the gene corresponds to a separate functional domain in the protein. It is likely that the exons evolved, originally, coding for separate small polypeptides, and subsequently became combined into a single gene (separated by introns) coding for a larger protein with identifiable domains corresponding to the originally independent polypeptides. This is supported by the observation that some proteins, known as *mosaics*, are composed of several domains that are related to regions of different proteins. For example, tissue plasminogen activator (TPA) contains four domains, two of which are related to a region of plasminogen, and the others to domains in epidermal growth factor and fibronectin respectively. The occurrence of different proteins (or genes) formed by various combinations of domains (or exons) is referred to as *domain shuffling* (or *exon shuffling)*.

Although bacterial genes do not usually have introns, a similar phenomenon can occur and give rise to a high degree of variability. In this case, the mosaic protein in one strain contains a domain that is not related to the sequence found in other strains of the same species, but is closely related to the sequence found in a different organism (see Figure 12.2). The inference is that these organisms have swapped information, so that a part of the gene is replaced with information from a different source. This contributes a further dimension to our overall understanding of the dynamic nature of the genome (sometimes referred to as *genome plasticity*) – rather than the genome being an essentially

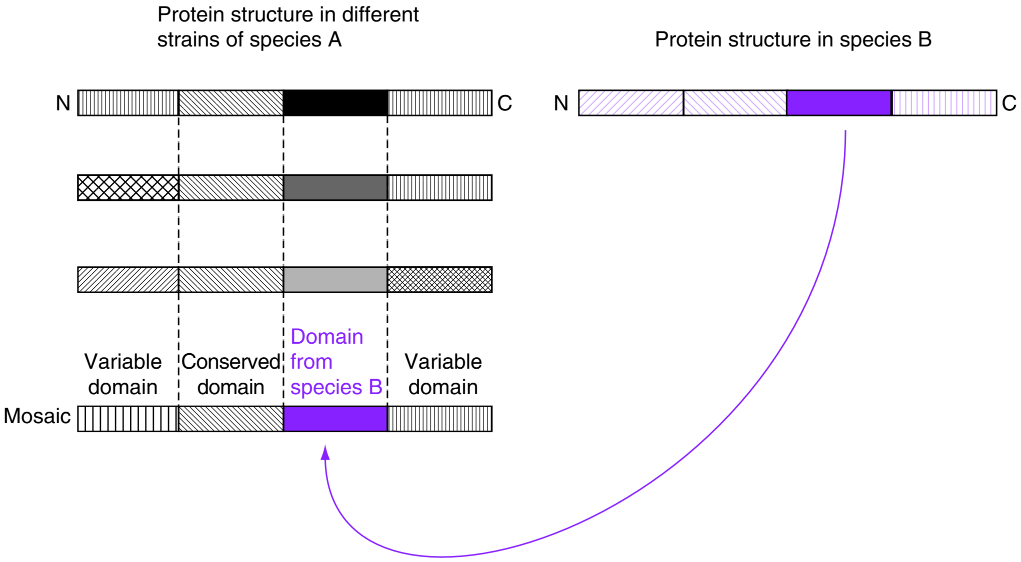

Figure 12.2 Conserved domains and domain shuffling

static structure evolving slowly through minor changes to its sequence, it is subject to occasional quite major changes and rearrangements.

12.2 Methods for Studying Variation

Ultimately, comparison of complete genome sequences is the only way of detecting all the possible types of variation that may occur. This may seem to be an extravagant and unrealistic suggestion, but it is a measure of the speed with which genome sequence technology is advancing that complete sequences are already available for more than one strain of several bacterial species (to say nothing of two human genome sequences), and the global comparison of the sequence between strains, species, and genera is playing a major role in our understanding of the evolution of the genome as a whole. So we are not far from a position when a serious analysis of the differences between, for example, two closely related bacterial strains would rely on a comparison of the complete genome sequences – although we should remember that the genome sequence by itself does not directly explain which of the differences is responsible for the observed variation in the characteristics of the organism. Indeed, if you had enough spare money, there are no technical obstacles to comparing your own complete genome sequence with that of your best friend. Again, you would no doubt find many interesting differences but none that would be likely to explain why you like different kinds of music.

However, we are not quite at the point yet where genome sequencing would be an effective *routine* method of identifying variations between two organisms. We need quicker methods which are targeted at certain types of changes.

12.2.1 *Genomic Southern blot analysis – restriction fragment length polymorphisms (RFLPs)*

One of the mainstays of the analysis of genetic variation is the Southern blot, which enables us to detect which band on an agarose gel is able to hybridize to a specific probe. The technique was described in Chapter 8 as a way of verifying the nature of the insert in a recombinant clone. In that case, there were only a few bands on the gel. If we digest a total DNA preparation from an organism, we will usually get so many fragments that they will appear as a smear on the gel rather than as discrete bands. (We will consider later on, under *Pulsed-field gel electrophoresis*, what happens if you use an enzyme that cuts so rarely that you get only a few very large fragments.) In this situation, Southern blots not only enable us to identify specific gene fragments, but the method also enables us to compare the DNA samples according to the pattern of hybridizing bands.

If you use a probe that hybridizes to a DNA sequence that is present as a single copy in the genome, then you will detect a single band on the Southern blot. (This assumes that the enzyme used to digest the genomic DNA does not cut within the region that the probe hybridizes to; if it does, you will get more bands.) The size of that band (or bands) will be determined by the position of the restriction sites flanking the detected sequence. If the structure and location of that gene is the same in each organism tested, then the band will be in the same position. On the other hand, if there is variation in the distance separating the two restriction sites, there will be a difference in the size of the fragment, and you will see a difference in the position of the detected band. This is referred to as a *restriction fragment length polymorphism* or *RFLP*.

There are a variety of changes that can give rise to such a polymorphism (see Figure 12.3). The most obvious one – a point mutation causing loss (or gain) of a specific restriction site – will usually happen at a very low frequency. Insertions, for example of mobile genetic elements such as insertion sequences, will make the fragment larger (or paradoxically smaller, if the inserted element contains a cleavage site for the enzyme used), but this is also likely to be infrequent unless the flanking sequences contain a preferred target site for

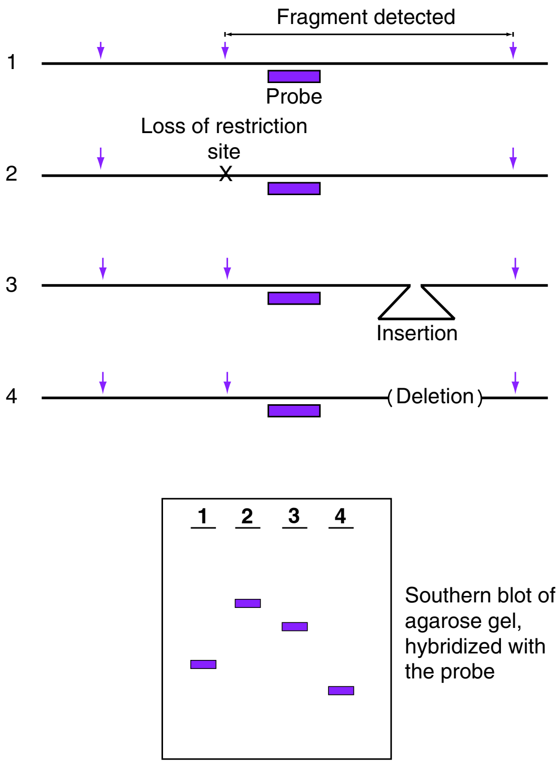

Figure 12.3 Restriction fragment length polymorphism

such an element. Deletions will also alter the restriction fragment size, and since the deletion may remove the restriction site as well, the fragment detected can be either larger or smaller than the original. In some regions of the genome, deletions may be relatively common, usually arising from recombination between two homologous, or partly homologous, sequences.

The most useful polymorphisms arise through the duplication or transposition of repetitive sequences. Duplication is often associated with the occurrence of tandem repeated sequences, i.e. a short sequence of bases occurs twice (or more) in succession. When this region is replicated, mistakes can occur: the replication machinery may slip back from the second copy to the first, giving rise to an extra copy of the repeat; or it may (less commonly) slip in the other direction, causing a reduction in the number of copies. The repeat sequence can be quite short – for example just a pair of bases, or even a run of the same base – in which case the change in the length of the restriction fragment will be small. PCR-based techniques are then more useful in detecting these changes (see the section below on *VNTR and microsatellites*).

If we use a probe that hybridizes to a mobile DNA element such as a transposon or insertion sequence, then when a copy of that element moves to a different site on the chromosome we will pick up a new band on the Southern blot (Figure 12.4). This is a widely used technique for typing bacterial strains for epidemiological purposes. It should be noted that in this case we are mainly looking at changes arising from the location of the sequence detected by the probe, rather than alterations in a specific region of the genome, although the latter can contribute to the overall polymorphism. We will return to the use of RFLP typing in Chapter 16.

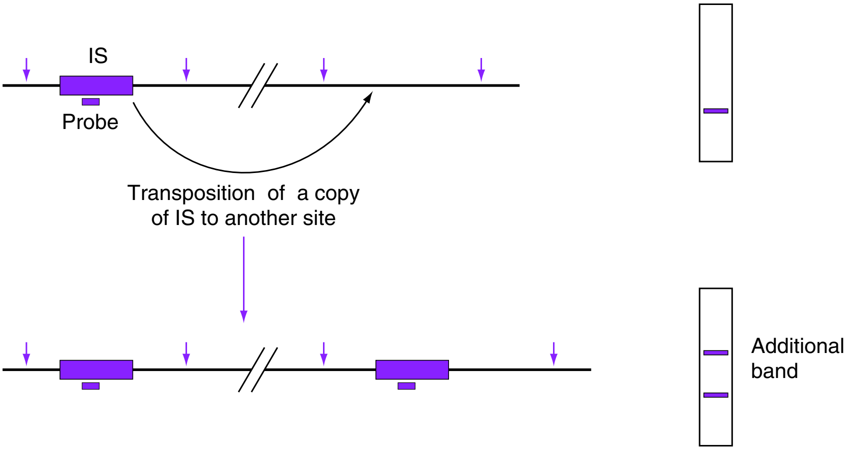

Figure 12.4 Restriction fragment length polymorphism arising from transposition of an insertion sequence

From the above discussion it will be evident that using Southern blots to detect RFLPs will mainly identify larger-scale changes in the structure of the genome, or a specific part of it, and are much less likely to identify minor sequence changes in a specific gene (unless that change destroys or creates a restriction site). However, Southern blots, or other hybridization-based techniques, can be used to detect specific sequence changes. If you use a small oligonucleotide probe, and high stringency conditions, a single base change in the target sequence can cause a detectable change in the ability of the probe to hybridize to the target DNA. This has applications as diverse as the diagnosis of human genetic disease and the detection of bacterial mutations causing antibiotic resistance. Generally, however, the probe would be used in combination with PCR (see below) rather than being applied directly to genomic DNA.

12.2.2 PCR-based methods

One of the practical limitations of the conventional RFLP method described above is that it requires a relatively large amount of DNA for the signal to be detectable. Exactly how much we need depends on the size of the genome. For a large genome such as ours, you would need 6 μg or so to have enough of each individual gene to bind enough probe to visualize it. This is acceptable for many research purposes where it is not difficult to obtain sufficient DNA from tissues from an animal, or from cells grown in culture. However, for many practical applications, including forensic examination and many types of clinical diagnosis, this approach is not possible. We have to use PCR to amplify the tiny amount of DNA that is available, and we then need a different approach to identify variations.

VNTR and microsatellites

One approach exploits the tandem repeats referred to previously; but instead of using a probe and a genomic Southern blot, we can amplify the region containing the tandem repeat, using primers that hybridize either side of the tandem repeats. If we run the product on an agarose or (preferably) acrylamide gel, we will see that the size of the product varies in a stepwise fashion according to the number of copies of the repeated sequence (see Figure 12.5). This gives the technique its name, VNTR standing for Variable Number Tandem Repeats. Each strain can then be given a number corresponding to the number of that tandem repeat that is present. By identifying several loci that contain VNTRs, and determining the number of copies at each position, we arrive at

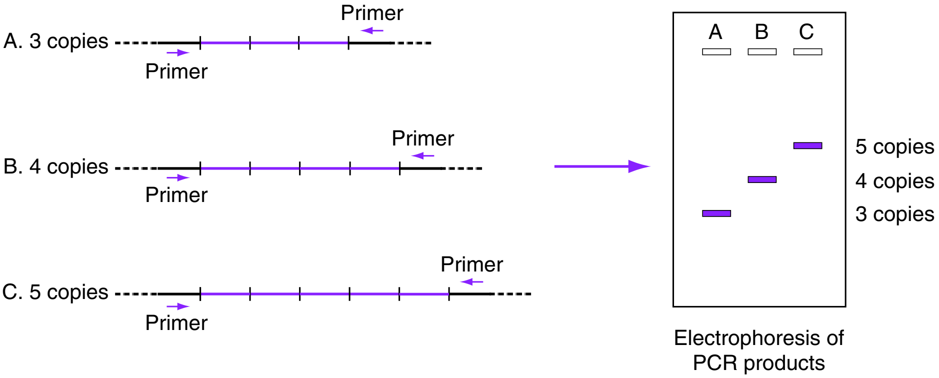

Figure 12.5 Variable number tandem repeats (VNTR)

a composite designation such as 3433243. This is now increasingly used in genotyping as an alternative to RFLPs.

A very similar concept that has proved extremely useful in genotyping involves the use of shorter tandem repeats known as *microsatellites*, or *short tandem repeats* (STRs). These are curious short stretches of sequence of 200 bases or less. They look almost as if the genome got bored with randomly distributed bases and instead decided to go for a straight stretch of one or two (less commonly three or four) nucleotide bases repeated 100 times or so (such as TTTTTTTTT. . . . or GTGTGTGTGTGTG. . .). Nobody knows what these repeats do, but it cannot be particularly important, because they are extremely variable in length (no doubt because of 'slipping' during replication) as well as randomly distributed throughout the genome, and it is this that makes them so useful. By performing PCR with a battery of primers flanking microsatellite markers, a genetic fingerprint can be obtained that makes it possible to draw conclusions about an individual's identity and genetic relationship with others with great confidence.

Sequence polymorphisms

For typing or fingerprinting, methods based on RFLPs or VNTR and microsatellites are often satisfactory. However, for other purposes, their inability to specifically detect minor changes at sequence level is a serious limitation. In addition, much of the time we are looking at the structure of DNA sequences that do not carry any coding information, or changes that are neutral in their affect on the phenotype. Indeed, the neutrality of the changes is, unsurprisingly, connected with the frequency of occurrence which makes those methods useful for typing. However, if we want to look at the structure of genes that code for important functions – such as testing for the presence of genetic diseases – we will often need other methods to test for the presence of those changes.

There are other reasons, too, for wanting to detect these *sequence polymorphisms*, related to the use of these techniques for studying evolutionary relationships between organisms. For molecular typing, where we are considering whether two samples match or not, we do not need to know the rate at which the fingerprint changes (the 'molecular clock') – the rate is likely to be so low that an individual's 'fingerprint' remains constant. We do need to know how likely it is that a match might occur by chance, but that estimate can be obtained empirically by examining large numbers of fingerprints. However, for evolutionary analysis, and the study of the phylogenetic relationships between organisms (e.g. how far in the past did they diverge from a common ancestor?), we do require an estimate of the clock speed – or at the very least confidence that there is a constant molecular clock, which may well not be true for some data such as that based on RFLP patterns for insertion sequences. We can illustrate this by reference to Figure 12.6. Here we can see that A and B are more similar to one another than either is to C, and a computer program will generate a dendrogram to display that relationship visually. However, unless we know that the rate of variation is constant, we should not jump to the conclusion that C diverged from a common ancestor of A and B, and that the difference between A and B happened more recently. If for some reason variation has occurred more rapidly in C (and its ancestors) than in A and B, then C might have diverged more recently, but has undergone more rapid variation since.

We are on somewhat safer ground with sequence polymorphisms, where we can get some estimate of the current rate of variation in each organism, and the assumption of a constant rate is rather more meaningful (although it should be treated with caution as we know that mutation rates do vary between organisms). Note that for evolutionary analysis we would normally focus on synonymous substitutions (to avoid complications arising from advantageous or

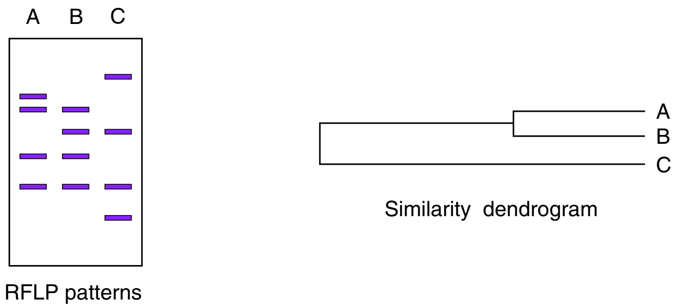

Figure 12.6 Analysis of similarity of RFLP patterns

disadvantageous mutations) whereas for other purposes such as study or diagnosis of human genetic disease we would by definition be looking for mutations that affected the function of that gene. We should stress that the construction of a true phylogenetic tree for a number of strains or species is much more complicated than the simple example shown; there will be a very large number of possible trees, and more complex forms of analysis are needed to be able to produce a tree that can be claimed to represent the true evolutionary relationship between the strains or species.

Direct sequencing

The principle here is to amplify the gene, or a suitable fragment of it, and to sequence the PCR product. For studying a human genetic disease, you would then compare this sequence in individuals with or without the condition. However, things are not necessarily that simple. For example, you may find a change at one position in all the patients compared to all the controls. That does not necessarily mean that you have identified the cause of the disease. Assuming this is an inherited condition, that change may be genetically linked to another elsewhere on the same gene or on a neighbouring gene. On the other hand, you may find that not all the patients have the same mutation. What presents as a single condition may in fact be due to any one of several mutations. For example, the human eye disease retinitis pigmentosa can be caused by no less than 90 different mutations in the rhodopsin gene; but it could also be caused by any one of hundreds of other mutations in dozens of other genes.

Similar considerations would apply to studying for example the genetic basis of rifampicin resistance in bacteria. It is known that this is commonly caused by mutation in the *rpoB* gene (coding for a subunit of RNA polymerase). Sequence analysis of the PCR-amplified *rpoB* gene from different strains identifies a number of variations, some of which are only present in rifampicin-resistant strains. If this remains true after sequencing a larger number of strains, you can become increasingly confident that these mutations include those responsible for rifampicin resistance. In this case you have the advantage of being able to test the hypothesis experimentally, by deliberately introducing each of those mutations (by allelic replacement, see Chapter 14) into a rifampicin sensitive strain and seeing if it does become resistant. There is still the limitation that you are likely to find some strains that are resistant to rifampicin but do not have any of these mutations; this indicates that there is another mechanism that you have not identified.

Another possibility that you will have to keep in mind in a diploid organism is that the mutation may affect the binding site for one of the PCR primers. In that case, in a heterozygote, the mutant allele will fail to amplify. On the other hand, the wildtype allele will amplify perfectly, and give you the incorrect impression that you are dealing with a homozygous wildtype. In these cases, RFLP analysis remains a valuable tool to ensure that there are no major deletions or other rearrangements in your gene of interest.

Allele-specific oligonucleotide probes

If you are confident that you have identified all the possible mutations, then using sequencing to identify them is rather unnecessary. In this case it is possible to make a pair of short oligonucleotides, one of which will hybridize (under stringent conditions) only to the mutated form, and the other only to the corresponding wild type. The pair of probes can then be used to test the PCR amplified product for the presence of that mutation. This is normally done using yet another variation of filter hybridization – *dot* or *slot blot*, where you pipette all your PCR amplicons from the individuals you are testing in a row on one membrane for each probe that you are using. You can either do this by hand, each pipetted drop forming a dot, or you can use a dot or slot blot manifold, through which your sample will be transferred onto the membrane by vacuum suction in a geometrically defined pattern. Since a defined amount is applied to the membrane, it is possible to obtain a quantitative result, using densitometry to assess the strength of the signal obtained after hybridization with the probe. If the number of known mutations is too large to make this approach feasible, you can produce an *array* (see later in this chapter), with each oligonucleotide immobilized on a membrane or a glass slide, and hybridize it to your PCR-amplified sample. In this case, the PCR product would be labelled, rather than the oligonucleotides.

Quicker and more sensitive results can be obtained by combining this concept with that of real-time PCR, in which specific fluorescent probes are used to monitor the progress of the PCR reaction as it happens. If these probes are designed to detect a specific mutation, you have a sensitive and rapid test for that specific lesion. Real-time PCR is described more fully in Chapter 13.

SSCP

The mobility of a DNA (or RNA) molecule in an electrophoresis gel is determined not solely by its length but also by its conformation, or shape. Most electrophoresis of DNA fragments is concerned with linear double-stranded DNA (such as restriction fragments or PCR products), in which case the conformation is relatively uniform, and the assumption that DNA length is the determining factor is sufficiently valid. However, single-stranded nucleic acids are a different matter. The bases are hydrophobic, but are removed from the aqueous environment by folding the nucleic acid into a complex series of stem-loop structures and even more complex forms (see Chapter 2). The way in which it folds will be influenced by its sequence; even a single base change can have a significant effect on the overall conformation – and hence on its mobility in gel electrophoresis. This is why sequencing gels (Chapter 10) are run under denaturing conditions, as in this case separation strictly according to molecular weight is required. Conversely, if we run this single-stranded fragment on a non-denaturing gel (i.e. we keep the temperature low enough to *prevent* it from unfolding), we can detect even single base substitutions in the DNA by a change in the position of the resulting band. This technique is known as *single-strand conformational polymorphism*, or *SSCP*.

SSCP can be especially useful for detecting the presence of sequence polymorphisms in specific regions of the genome, although the increase in the power of automated sequencers has reduced the necessity for using SSCP – it is often easier now to sequence the region directly (and this is what you would have to do anyway to confirm the findings from the SSCP analysis). However, the throughput of SSCP is higher.

12.2.3 Genome-wide comparisons

All the above methods are based on the examination of variation in limited regions of the genome. For example, even with a method such as RFLP fingerprinting of bacteria using an insertion sequence, where the insertion sites giving rise to the polymorphism may be more or less randomly distributed over the whole chromosome, the actual regions involved in the comparison of two strains represent less than 1 per cent of the whole chromosome. Short of complete sequencing of all the genomes to be compared (see the discussion earlier in this chapter), how can we look at the global structure of the genome?

Pulsed-field gel electrophoresis

In principle, you might expect that since a restriction endonuclease will cut genomic DNA at defined positions, you would get a characteristic pattern of restriction fragments from a given strain. Any deletions, insertions or transpositions (and some base substitutions) would give rise to a change in that pattern – and in principle you would be correct. However, if we consider an enzyme that requires a six-base recognition sequence (a ‘six-base cutter’) then, assuming an equal proportion and random distribution of all four bases, we would expect this enzyme to cut on average once every 4096 (4^6) bases. If you are working with a bacterial genome of, say, 4 million (4×10^6) bases, you would expect about 1000 fragments. Agarose gel electrophoresis does not have enough resolving power to separate out such a large number of fragments (although it is possible, albeit extremely difficult, to obtain characteristic patterns for typing using only a part of the range of sizes). For organisms with larger genomes, the problem is of course correspondingly greater.

There are enzymes available that will cut DNA less frequently because they have a longer recognition sequence and/or a recognition site that occurs less frequently than expected on a random distribution. Such an enzyme will produce fewer fragments, but the problem now is that the fragments are too big to be separated by conventional gel electrophoresis. These large fragments *will* actually move through the gel, but in a size-independent manner. This is most easily visualized as considering the DNA fragments as very long and very thin structures; once they eventually get lined up end-on, parallel to the direction of the electric field, they can then slide through the pores in the gel. As long as the field direction is constant, they will keep moving. The limiting factor is the time taken to orientate the molecules.

The technique of *pulsed-field gel electrophoresis* (PFGE) exploits this. The simplest version to describe is one where the polarity of the electric field is briefly reversed periodically throughout the run (Figure 12.7). Each time the field is reversed, the molecule has to be re-orientated in order to move through the pores in the opposite direction, and the longer the molecule is, the slower this process will be. Consequently, the net migration of the fragments through the gel will be size-dependent just as a gel with smaller fragments would have been. (In reality, this approach leads to unacceptable distortions of the lanes,

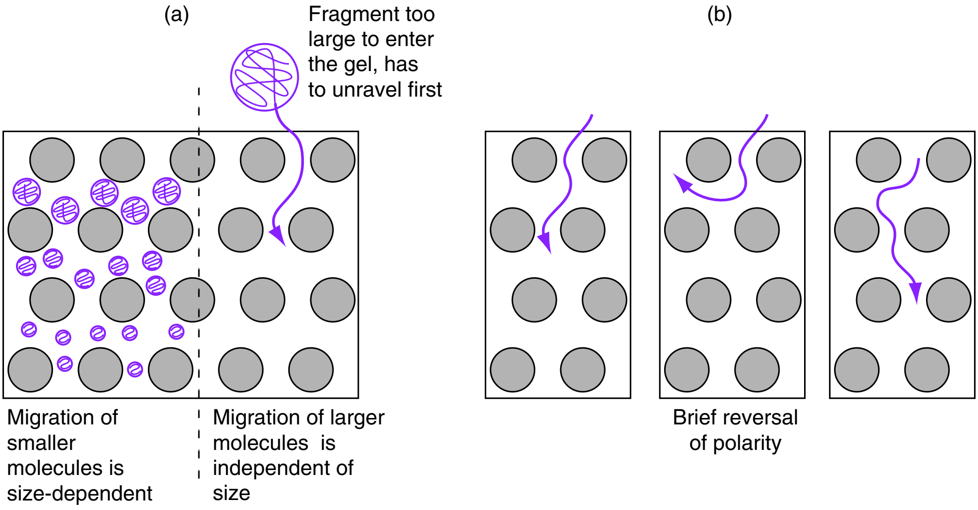

Figure 12.7 Pulsed-field gel electrophoresis: (a) conventional electrophoresis; (b) PFGE

and the apparatus actually used for PFGE employs more complex systems for changing field direction in order to achieve straight and coherent lane patterns.)

Successful application of PFGE requires the isolation of genomic DNA intact or at least as extremely large fragments. This is virtually impossible by conventional means, as the DNA tends to fragment when the cells are lysed. Instead, intact cells are embedded in agarose, and both lysis and restriction digestion are carried out in a plug of agarose excised from the initial block. The agarose plug is then inserted into the gel and the DNA fragments migrate from the plug into the gel proper during electrophoresis.

PFGE has been employed for bacterial typing, but the technical demands of the process make it less popular than the other methods described. It should be noted, however, that it does provide a method (although not the only one) for the preparative separation of chromosomes from eukaryotic cells.

Microarrays

A more recent, and much more widely useful, approach to the analysis of whole genomes is to employ *microarrays*. This involves the production of a series of PCR products (or synthetic oligonucleotides) which correspond to all the predicted genes from a specific organism. Or, for analysis of small genomes, you could use sequences spaced at regular intervals over the whole genome. To provide reasonable coverage of the whole genome you would need thousands of such fragments for a bacterial genome, or millions for an organism with a larger genome. A robot is used to produce tiny spots of each fragment in a precisely defined pattern on a treated glass slide. This is the microarray. Once the fragments are made and the machine is set up, it can produce many identical copies of the same microarray.

For genomic analysis, you would then produce labelled genomic DNA from the organism to be tested, and allow it to hybridize to the microarray. All the sequences in the microarray that are represented in the target organism will result in a positive signal; deletions will show up as the absence of a signal (Figure 12.8). This is therefore a very effective way of detecting insertions and deletions (*indels*) in different strains or individuals, and identifying the genes, or chromosomal regions, that are affected by each deletion.

An alternative to this global approach is to use a microarray of oligonucleotide sequences representing all the known mutations or polymorphisms in a gene, allowing a quick screen in cases where there are many potential differences such as the range of rhodopsin mutations as described above.

Microarrays are also used extensively for genome-wide analysis of gene expression, including the effect of different growth environments, or to trace

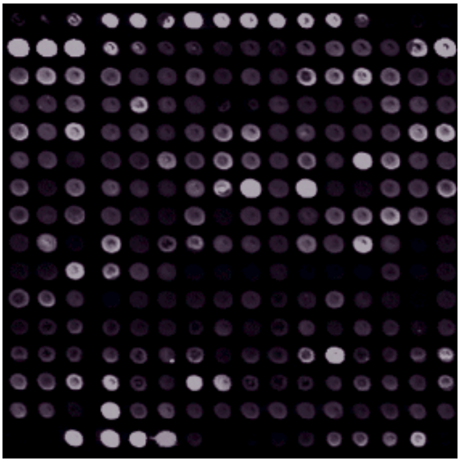

Figure 12.8 Use of a microarray: the illustration shows a representation of a microarray, hybridized with a fluorescent-labelled probe; in practice, two probes with different labels would normally be used and the results displayed in red, green or yellow, depending on which probe hybridized to the greater extent

the expression of different genes during different stages in development of the cell. These applications are considered further in Chapter 13, along with a more detailed account of microarray production and use.

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13

Analysis of Gene Expression

Gene expression can be divided into two main phases: *transcription* (copying DNA into RNA), and *translation* (production of a protein or polypeptide to the specifications of an mRNA template). For the purpose of this chapter, we will assume that the polypeptide chain is the end product of gene expression: this ignores subsequent post-translational modification, including the folding of the polypeptide into the correct conformation for biological activity, as well as other modifications such as the specific addition of carbohydrate groups. Assessment of this part of the process requires specific assays for functional activity, and the factors involved are considered further in Chapter 15.

The first methods that were developed allowed investigators to study the expression of a specific RNA or a specific protein. Gradually, methods became available that also made it possible to compare increasingly complex mixtures of gene products. Recent advances in technologies have increased these possibilities even further, prompting scientists to create new terms for these types of analysis, although conceptually they are not new. The complete collection of mRNA transcripts in a cell, tissue, or organism at a particular time is referred to as the *transcriptome* (by analogy with the *genome*, which is the complete set of genetic information). Thus, the comparative study of mRNA transcripts would be called *transcriptomics*. The complete set of translated proteins is called the *proteome* and the global study of translated proteins is called *proteomics*. Both for the study of mRNA and protein, we will begin by discussing methods for the study of the products of single genes, and then extend these to the study of complex samples.

13.1 Analysing Transcription

The first group of techniques for examining gene expression consists of various ways of assessing the amount of a specific transcript in a specific sample at a specific time. Other methods, described later in the chapter, are concerned with studying the transcriptional activity associated with the gene in question. Two points concerning the measurements of mRNA levels should be noted. First, the amount of a specific mRNA in a cell at a point in time is influenced not only by the level of transcriptional activity but also by the stability of that message. A gene that is transcribed at a low level but gives rise to a stable product may result in a higher amount of mRNA than a more active gene with an unstable message. Second, the amount of mRNA present does not necessarily correlate with the amount of protein made. This is most obvious with bacterial cells, where a single polycistronic message may be translated into many different polypeptides; although these are all translated from the same mRNA template, the levels of the different proteins made can be widely different. Translation efficiencies can vary considerably.

13.1.1 Northern blots

The oldest methods for detecting specific mRNA rely on hybridization to specific, labelled, probes. A common manifestation of this is known as a *Northern* blot. A Northern blot involves electrophoretic separation of a purified extract of RNA from the cell, followed by immobilization onto a membrane and hybridization with a specific probe, as shown in Figure 13.1. This enables us to detect the presence of a specific mRNA, assuming that the probe we use is sufficiently specific, and to estimate its size. By careful calibration, for example by comparison of the strength of the hybridization signal with that of other genes that are expressed at known levels, we can get an idea of the *relative* level of expression of the gene in question (or more accurately, the relative amount of mRNA present). In Figure 13.1, we can see that sample B gave a

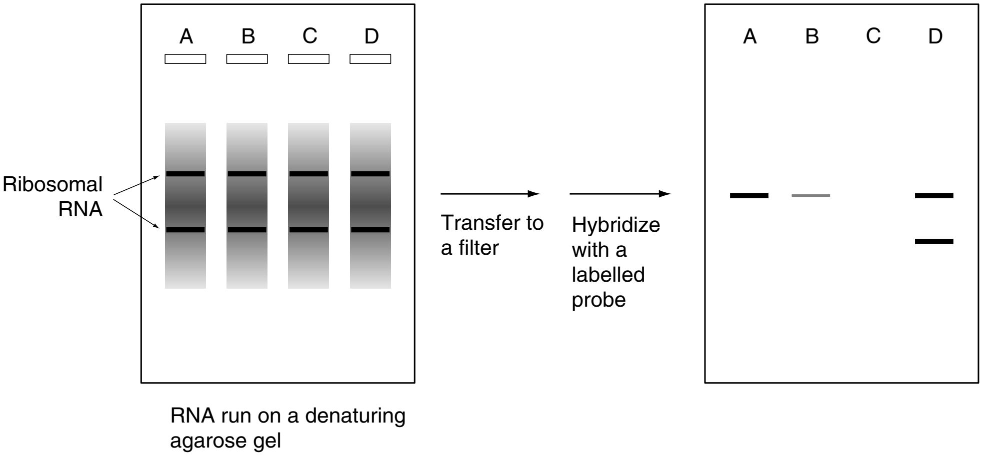


Figure 13.1 Northern blotting

weaker signal than sample A, and therefore contained much less of the specific mRNA, while in sample C expression of the gene was not detected. (However, we have to bear in mind that RNA is readily degraded, especially by ribonuclease contamination, so a weak or absent signal may be due to loss of the mRNA).

However, there are some limitations to this technique. First, although we can get a *relative* quantitative estimate of the strengths of the signals, it is generally not possible to obtain an *absolute* measurement of the amount of specific mRNA.

Second, the technique is not very sensitive, and requires fairly large amounts of RNA (normally at least a microgram of poly-A-enriched mRNA or ten micrograms of total RNA). It may not be possible to obtain sufficient quantities of starting material. For example, if we want to look at the expression of specific genes in a pathogenic bacterium when it is growing within an infected host (rather than the artificial situation of laboratory culture), or levels of mRNA in material obtained by biopsy, it is likely to be extremely difficult to get large enough quantities of mRNA to be able to do a Northern blot.

On the other hand, Northern blotting has some lasting advantages. The risk of artefacts is much smaller than with the PCR-based methods described below and, above all, it remains the only method for determining the size of a specific transcript and for detecting the presence of different transcripts for a specific gene (e.g. in Figure 13.1, we can see the presence of a second, smaller, transcript in sample D). This is becoming more important as the human genome project teaches us that the number of genes is considerably smaller than the number of gene products. An important part of the explanation for this is that many genes are differentially spliced, and Northern blotting is a very important tool for detecting this.

13.1.2 RNase protection assay

This method is based on *solution hybridization* rather than filter hybridization. The target is a specific RNA species in a complex RNA mixture purified from a biological sample. The probe is a radiolabelled antisense RNA. Such RNA probes can be produced quite easily from plasmid cloning vectors, such as the pGEM series (Chapter 6) that are equipped with binding sites for RNA polymerases (T3, T7, and/or Sp6). In the example shown (Figure 13.2), the gene (or part of it) has been inserted into the vector in the correct orientation for transcription from the T7 promoter. Addition of T7 RNA polymerase, and the NTP substrates, will therefore produce the normal (sense strand) RNA. However, the gene is in the opposite orientation with respect to the SP6 promoter. So, if you use SP6 RNA polymerase, *in vitro*, it will transcribe the gene in the wrong direction – or in other words it will copy the opposite strand.

The resulting RNA (the antisense RNA) will be complementary to the true (sense strand) RNA, and will therefore hybridize to it.

When you add the (labelled) antisense RNA to your mRNA preparation, it will anneal to the specific RNA you want to measure, forming double-stranded RNA, while non-specific mRNA molecules remain single-stranded. When ribonuclease (RNase) is added, it will degrade all the single-stranded RNA, while the double-stranded hybrids formed by annealing the antisense RNA probe to the specific mRNA are protected (Figure 13.3). After RNase digestion, the hybridization mixture is separated in a polyacrylamide gel. The gel is then dried and left to expose an X-ray film.

This assay has some distinct advantages over Northern blotting. It is more sensitive, it is more quantitative, and it is more tolerant of partial degradation of the RNA samples. The reason for the latter is that only part of the RNA molecule needs to be intact to take part in the hybridization. This is simultaneously one disadvantage compared with Northern blotting. It does not allow

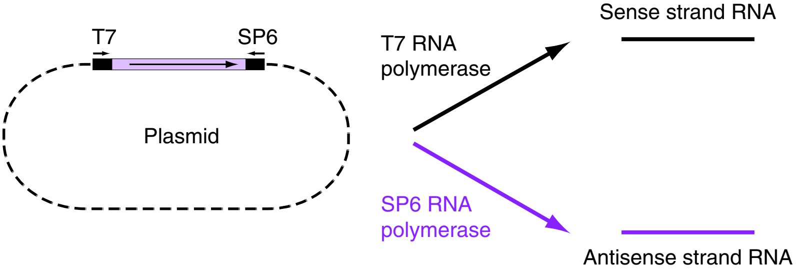


Figure 13.2 Production of antisense RNA

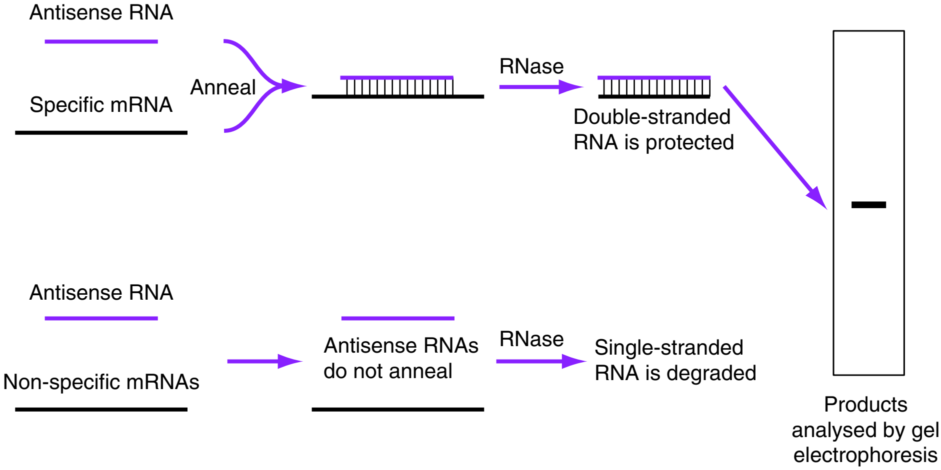


Figure 13.3 RNase protection assay

you to determine the size of the transcript(s), because the band you see in the gel is only defined by the length of the probe. The other disadvantage is that it demands more work than Northern blotting in the production of the probe. However, it certainly is an attractive method if you study one particular gene extensively.

13.1.3 Reverse transcription PCR

A much more sensitive way of detecting specific mRNA is to adapt the polymerase chain reaction (PCR, see Chapter 9) so as to be able to amplify the specific message. Since this requires copying the mRNA into cDNA using the enzyme reverse transcriptase (see Chapter 7), it is described as reverse transcription PCR (RT-PCR). The principle of the method is illustrated in Figure 13.4.

The sensitivity of this procedure is such that it is possible to detect a specific mRNA in a single cell. This has obvious advantages, in being able to detect low abundance mRNA (i.e. mRNA that is present at extremely low levels), or to

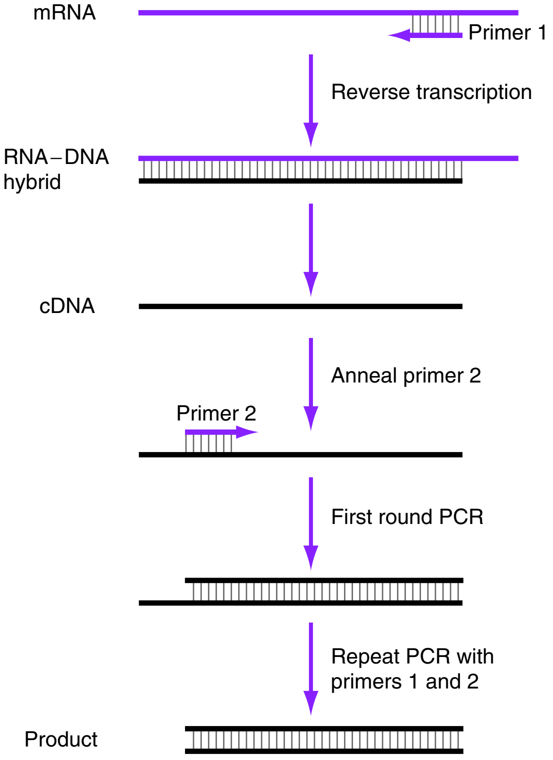


Figure 13.4 Reverse transcription PCR

analyse gene expression in cells that are difficult to obtain in large numbers. An example of the latter is the analysis of gene expression in bacteria from lesions in an infected animal (or human) to find out which genes are expressed under those circumstances. Culturing the bacteria is no use, as the gene expression would change. RT-PCR enables us to analyse gene expression in the limited number of bacteria recoverable from the lesion.

The sensitivity of RT-PCR confers a further, rather less obvious, advantage. If we want to look at tissue-specific expression, or at expression in cells under different physiological conditions (whether bacterial or eukaryotic), we have to try to ensure that all the cells from which we extract the mRNA are indeed expressing the same repertoire of genes. The relatively large amounts of mRNA required for a Northern blot mean that we have to use mRNA extracted from a number of cells, and this population may actually be heterogeneous. RT-PCR requires much less mRNA, and hence far fewer cells. It is therefore much more likely that all the cells are in the same physiological state, and we will therefore get a much more accurate picture of the true profile of gene transcription in those cells. Reporter genes (see below) provide an alternative way of addressing this question.

Conventional RT-PCR still suffers from the other limitation, namely quantification. It is difficult to be sure that you are getting reliable results by simply amplifying the template and measuring the amount of product. There is no simple and reliable relationship between the amount of template you start with and the amount of product formed, unless the PCR reaction is truly proceeding exponentially. It will cease to do so when the availability of reagents becomes limiting. This can be overcome, to a point, by adding a known amount of a second template that is almost identical (and amplifiable with the same primers) but still distinguishable on an electrophoresis gel. Normally, this is produced by cloning the PCR product into a plasmid vector and deleting a small part of it. Adding a known quantity of this modified DNA to the PCR reaction will allow you to achieve much better quantitative accuracy by comparing the relative amounts of the two resulting PCR products. In practice, this has now been superseded by the more accurate and simpler method of real-time PCR, as described in the next section.

Real time RT-PCR

As mentioned above, there is no reliable relationship between the amount of template added and the ultimate amount of product detected after a fixed number of cycles. However, there is a more reliable relationship between the amount of template and the number of cycles of amplification needed to produce a detectable product. So you could in theory set up a number of amplification reactions, and stop them after different numbers of cycles and see which reactions give detectable product. This would be tedious and expensive. However, if you have a way of detecting the product without interrupting the series of amplifications, then you have an invaluable way of quantifying the procedure. This is the basis of real-time PCR.

For real-time PCR, you therefore need a way of detecting the product as it is formed, without having to stop the reaction and run the products out on a gel. The simplest method to understand is to add, to the PCR mixture, a dye that fluoresces when it binds to double-stranded DNA. However, this can lead to artefacts, as you will get a signal produced even if the wrong product is made. Greater specificity can be achieved by using probes that will only bind to the specific product. Using a combination of dyes to label these probes, it is possible to ensure that fluorescence will only be observed when the probe is annealed to the target, and not when the probe is free in solution. There are several quite different ways of doing this.

If you carry out the PCR reaction in a machine that not only performs the temperature cycling needed for a PCR reaction but will also detect the fluorescence of your samples, then you can monitor the progress of your PCR in real time. Initially, the template is single-stranded, and so there is no signal. As amplification proceeds, double-stranded product is formed, and eventually enough of this product is made to allow the resulting fluorescence to be detected by the machine (see Figure 13.5). The level of the fluorescence will increase over a number of cycles, and by extrapolation of the resulting curve back to zero you can determine the number of cycles needed for formation of a detectable amount of product. This value, known as the C_T value, is related to the initial amount of template: higher amounts of initial template initially will

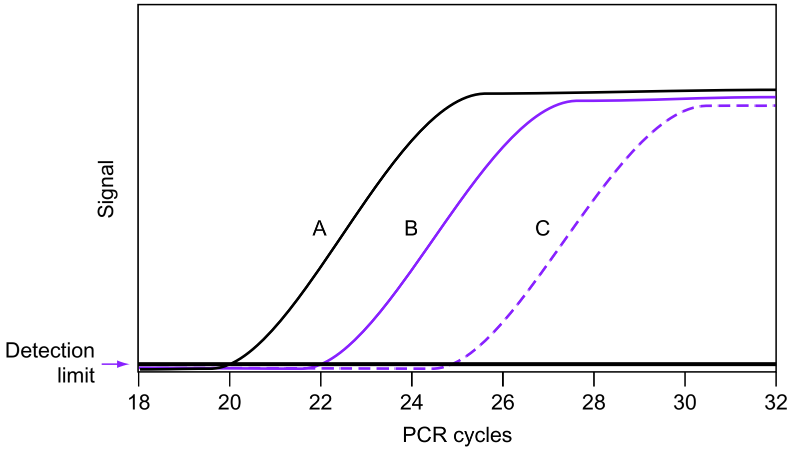


Figure 13.5 Real-time PCR

result in lower C_T values. In the figure, sample A produces a detectable signal after 20 cycles, so we say it has a C_T value of 20. This represents more template than in samples B ($C_T = 22$) or C ($C_T = 25$).

If you are comparing the levels of mRNA in different samples, you have to make sure that you start with the same number of cells, or more specifically with the same overall amount of mRNA. Standardizing the amount of *total RNA* (rather than specifically mRNA) is not completely reliable, as the ribosomal content of the cells can vary considerably. The best way of standardizing the template is to carry out a parallel RT-PCR using another gene which is known to be constitutively expressed (or at least expressed at the same level under the different conditions you are testing).

This enables testing for differences in the specific expression of an individual gene under different conditions. Can you use the same method to compare the expression of different genes? Subject to certain safeguards, yes, you can. The problem to be dealt with is that PCR is not equally efficient with all genes. Even if the amounts of template are the same, a more efficient PCR will result in earlier detection (a lower C_T value) than for another gene with a less efficient PCR. Some preparatory work is therefore necessary to ensure that the genes are amplified equally efficiently, possibly including using different primers to change the efficiency so that they match. (It should be noted that this consideration also applies to the comparison of your gene with the standard template as referred to above.)

Real-time PCR is also an important technique in the quantitative detection of specific DNA sequences, and in the identification of specific alterations in DNA sequence, including medical diagnostic applications (see Chapters 12 and 16).

13.1.4 In situ *hybridization*

This is the third type of hybridization that we cover in this book (the other two being solution and filter hybridization). In this method, the target is found immobilized within a cell on a microscopic slide. The target could be either DNA or RNA. The former is often used for gross chromosomal mapping of genes. A labelled probe is hybridized to cells with metaphase chromosomes. By using a differentiating counterstain, the investigator can identify the localization of the gene to a specific region of a specific chromosome (see Chapter 16 for a more detailed account). What we are concerned with here is RNA *in situ* hybridization which, by contrast, can be used to identify which cells, for example in a tissue section, produce mRNA for a specific gene. *In situ* hybridization is also used for the detection of viruses. In this case, the target is either DNA or RNA depending on the pathogen.

Although it is possible to obtain some quantitative information by counting the number of grains (when using radioactive labels), quantification is not the strength of this method. Rather, its strength is to tell us which particular cell types express a specific transcript. By using a panel of RNA samples from different tissues, other methods such as Northern blot hybridization, RNAse protection assay, and RT-PCR can tell us in which tissue(s) expression takes place. *In situ* hybridization allows us to refine this analysis even further. In addition, the size of the sample required is, of course, minimal.

13.1.5 Primer extension assay

As part of the study of gene expression, we are likely to want to know, not only how much of a specific mRNA is made, but where does it start and stop. Studying the 3′ end of a eukaryotic mRNA species is rarely a problem. Most cDNA libraries are constructed by oligo-dT priming of poly-A-enriched mRNA, so by definition this is the part of the mRNA molecule that is always employed for cDNA synthesis (although, occasionally, the primer ends up binding to an adenosine-rich stretch within the mRNA molecule instead). However, as mentioned earlier (Chapter 7), the 5′ end is not always incorporated into the cDNA. The reverse transcription of the full mRNA may not occur because of degradation of the template, or because of secondary structures or the presence of RNA-binding proteins that stop the reverse transcriptase. Moreover, studying the sequence of a cDNA will not necessarily tell us if its 5′ end goes all the way to the transcription start site or not. We can establish whether the whole open reading frame for the protein product is present, but that is pretty much it. Comparing the length of your cDNA sequence with the size of the full transcript as seen on a Northern blot will give you an idea of whether you are dealing with a gene with multiple transcripts (which may vary in which exons are included or in the length of their untranslated regions), and the approximate size of the transcript(s).

The primer extension assay is used for the exact mapping of the 5′ terminus of an mRNA. Poly-A RNA is purified from a tissue or cell type known to be expressing the gene in question at as high a level as possible. This complex mixture of all the mRNA species present is then mixed with a radiolabelled oligonucleotide primer complementary to a region close to the 5′ end of the sequence (as you know it), which will also need to be as specific as possible for the transcript you are studying. Reverse transcriptase is then added and used to extend the primer to the very 5′ end of the mRNA, at which point the polymerization automatically terminates. You will then need to run out the new radioactive cDNA strand in a sequencing gel for optimum resolution, and by running alongside a sequencing reaction of the same gene you are studying, starting with the same primer, you will be able to identify the transcription start site down to the very first base (see Figure 13.6). Fluorescent labels can now be used to perform the same procedure in an automated sequencer.

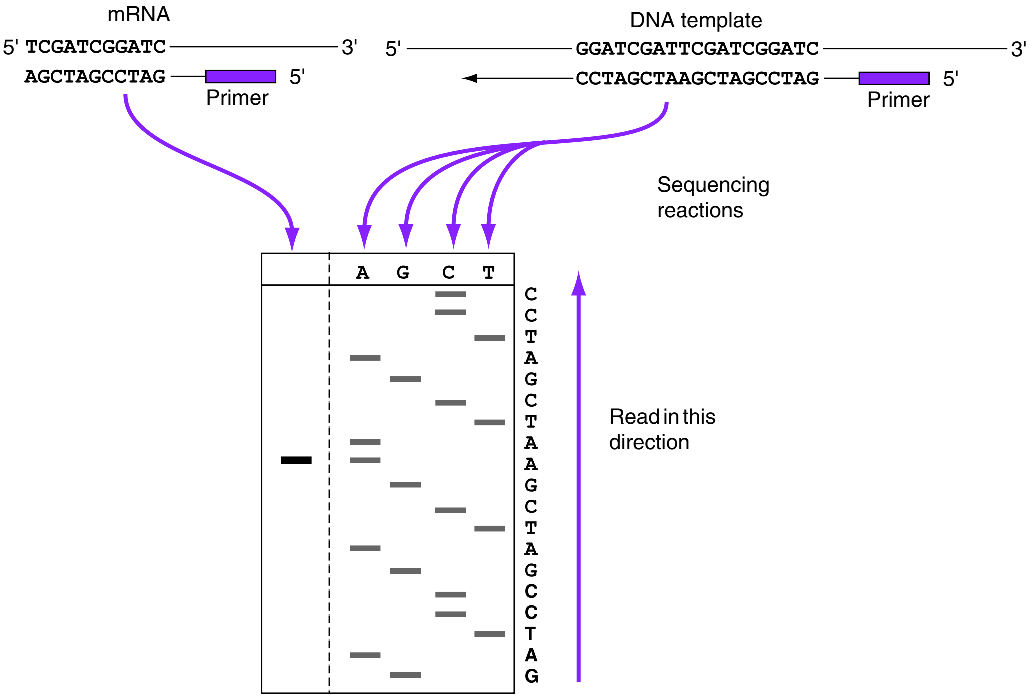


Figure 13.6 Determination of the 5′ end of mRNA: primer extension

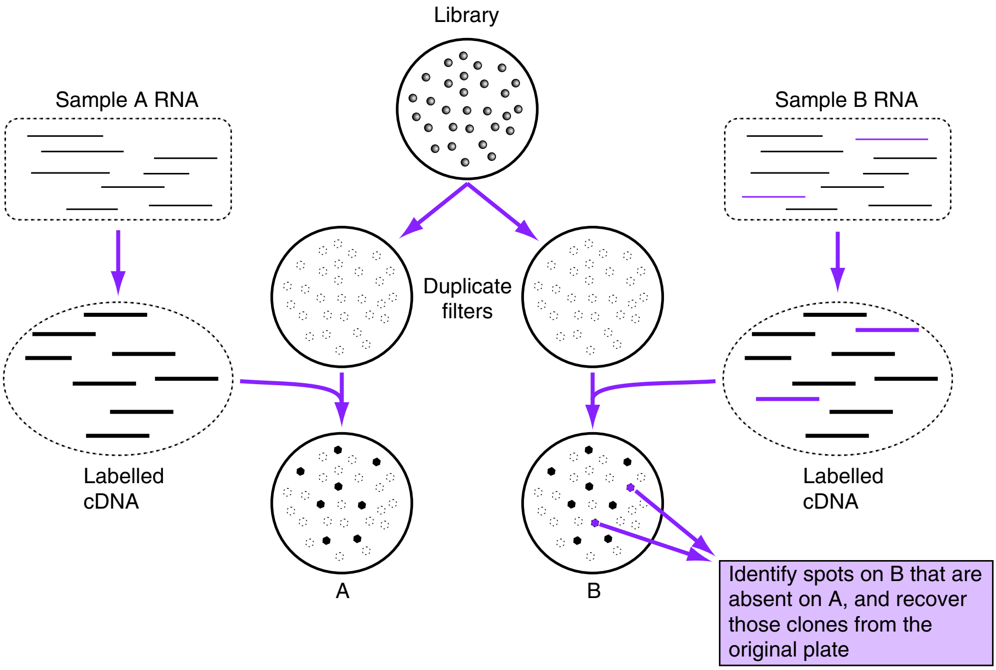
13.2 Comparing Transcriptomes

Methods such as Northern blotting are really only suitable if we want to compare the expression of a small number of genes, which would usually be genes that we were already specifically interested in for some reason. Northern blotting can be – and has been – used for the identification of disease genes when a limited number of strong candidate genes were known. We commonly want to identify genes that are expressed in one organism but not in another, or are tissue-specific, or are differentially expressed under varying environmental conditions. Over the years a number of techniques have been developed for comparing which mRNA species are expressed in different samples. Such methods are able to answer some very important questions. They can tell us which particular gene is causing a degenerative disease by comparing diseased and healthy tissue. They can tell us which genes of a pathogenic bacterium are expressed during infection of a suitable host, as opposed to those that are expressed in laboratory culture. In some cases, these methods have been superseded by the advent of array-based hybridization techniques (see below); in other cases, they are still in use.

13.2.1 Differential screening

The most primitive method for identifying cloned genes that are differentially expressed is by using *differential screening* of a gene library (see Figure 13.7). This involves producing two identical filters containing DNA imprints of the gene library (either by repeated transfer from the same plate, or by transfer from two identical plates produced by replica plating). Each of the two complex mRNA mixtures that we wish to compare is used to produce a probe by performing a reverse transcription in the presence of radiolabelled nucleotides. One of the filters is hybridized with one of these pools of radioactive cDNA, while the second pool is used to probe the second filter. Clones that show a positive signal with one probe but not the other are candidate clones of differentially expressed genes.

This description assumes that the specific transcript is completely absent from one sample (sample A in Figure 13.7). However, genes are not necessarily turned off that absolutely; there may still be a small amount of the specific mRNA present. This might result in a different intensity of some spots with the two probes, and we could use that for our differential screening. However, the situation is rather better than that. If there is only a very small amount of a



Identify spots on B that are absent on A, and recover those clones from the original plate

Figure 13.7 Differential screening of a gene library specific mRNA species (a very low abundance), then the amount of the corresponding cDNA in the probe will be too low to produce a visible signal. Furthermore, the synthesis of cDNA is likely to be more efficient for the high-abundance mRNA, leading to even further depression of the signal from the low-abundance species. So any low-abundance mRNA in sample A will give, not just a weak signal, but no signal at all. This actually causes a problem in some cases, in that mRNA species that are less abundant, but are still differentially expressed, may not be detectable in either sample. RT-PCR can be used to generate more representative cDNA probes, but the more representative the probes become, the more difficult it is to pick up differential expression where the ratio between the two sources is not high.

13.2.2 Subtractive hybridization

An alternative procedure is to remove the common sequences from the probe, leaving only those which are present in the test material; this is known as *subtractive hybridization*, and is illustrated in Figure 13.8. We are looking for transcripts that are present in one sample (which is called the *tester*) but are absent from the other (the *driver*). For example, the driver may be derived from diseased cells so we would like to identify the gene that is unable to be expressed

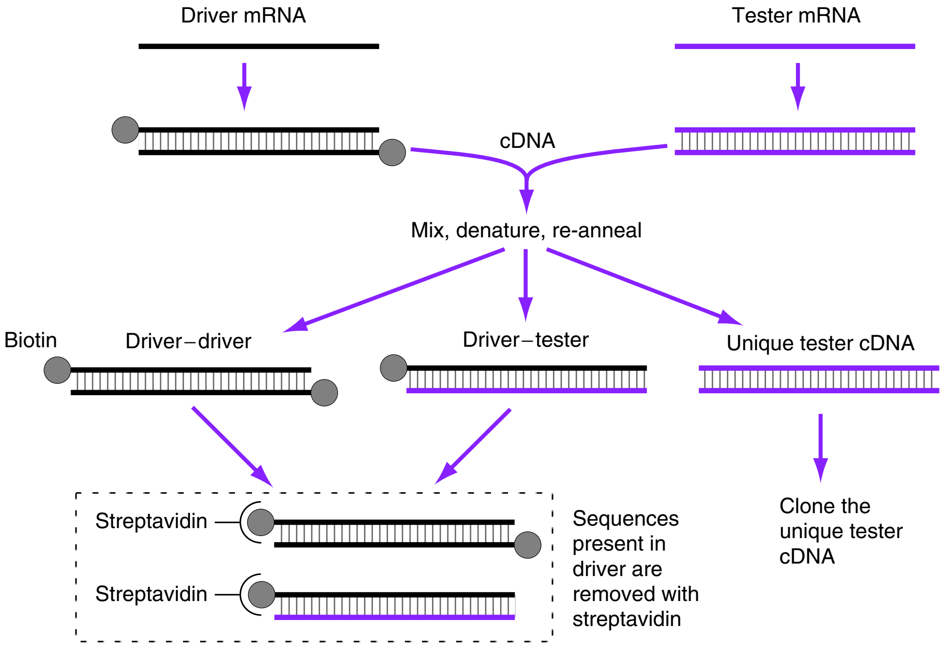


Figure 13.8 Subtractive hybridization

in those cells; the tester would then come from normal cells. Alternatively, we could be interested in genes that are only expressed under certain conditions, for example bacterial genes that are switched on during anaerobic growth, in which case the driver is the ‘normal’ sample. Whichever way round we look at it, the procedure is designed to find mRNA that is present in the tester and not in the driver.

The details of the procedure vary, but in the example shown the first step is to produce cDNA copies of the mRNA from both samples. The cDNA from the driver is labelled with biotin (in the figure it is shown as end-labelled for clarity, whereas in practice the biotin will be incorporated throughout the molecule). Biotin has strong affinity for streptavidin, so all the molecules containing biotin can be easily removed. The next step is to mix a large excess of the biotin-labelled driver cDNA with the tester cDNA, and to denature and reanneal the mixture. Any tester cDNA that is complementary to driver cDNA will form hybrid molecules, with one tester strand and one driver strand. Since the driver strand carries biotin, it can be removed by streptavidin (together with any excess driver–driver double-stranded DNA). You will then be left with a mixture that is enriched for those tester cDNA molecules that are unique, i.e. they are not present in the driver population.

The subtracted tester transcripts can be used as a probe for screening a gene library. However, that way you only have one shot at enjoying the fruits of your labour. Moreover, rare transcripts are unlikely to yield any detectable signals even though they may be the ones you are looking for. A better method is to produce a subtractive cDNA library by adding adaptors, and ligating the tester cDNA into a vector. This library is then available for repeated testing, either by systematic analysis of all clones, or by comparative screening of duplicate lifts with labelled tester and driver cDNA. This method has been used successfully on a number of occasions for the identification of disease genes, by using a wild-type source as the tester and cells from the disease phenotype (lacking a specific gene product) to generate the driver.

One disadvantage of this procedure is that it requires relatively large amounts of mRNA. The use of PCR, combined with the principles of the above procedure, provides a range of more powerful methods. One such procedure, known as representational difference analysis (RDA) is illustrated in Figure 13.9. In this example, linkers are ligated to the ends of the cDNA to provide priming sites for PCR analysis. In the first part of the procedure, the tester and driver samples are treated in the same way. Then after removing the initial linkers from the PCR products, a different pair of linkers is added to the tester PCR product, but not to the driver product. When the two products are mixed and allowed to anneal (again using a large excess of driver), any DNA strands that are present in both samples will anneal to form a hybrid molecule, while the unique tester strands, which will not be able to anneal to the excess driver DNA, will form specific tester–tester molecules. These can be

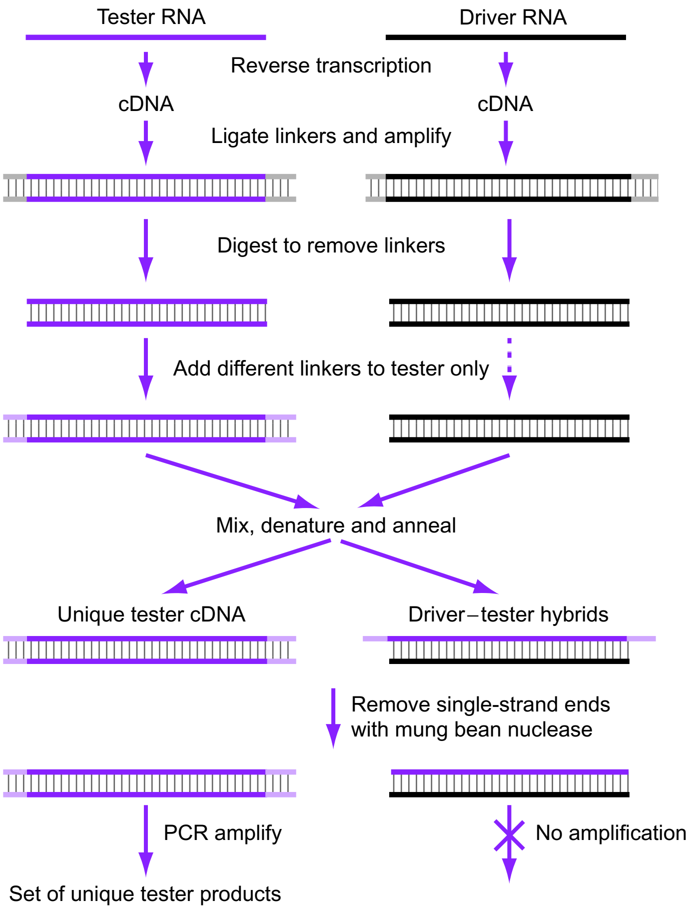


Figure 13.9 Representational difference analysis (RDA)

amplified by subsequent PCR using primers that are specific for the linkers that were added only to the tester DNA, which will thus undergo exponential amplification. Any driver–driver molecules (not shown) will fail to amplify, because they lack this primer-binding site. The tester–driver hybrids carry unpaired ends (from the linkers that were added to the tester molecules), and these can be removed by digestion with a nuclease that is specific for single-stranded DNA (such as mung bean nuclease).

13.2.3 Differential display

Differential display is a PCR-based method of comparing two cDNA populations. One primer is designed to bind to the poly-A tail, while the other is a random sequence. By running PCR at a very low annealing temperature, a subpopulation of the cDNA molecules in the sample is amplified. This subpopulation will be small enough to be resolved in a polyacrylamide gel. If you perform identical PCR reactions with two cDNA populations, you will be able to identify cDNA species that are expressed at different levels in the two samples, or even completely absent in one of them. These can then be excised from the gel, reamplified, and sequenced. If novel, their full sequence can be obtained by library screening or by RACE (see Chapter 9).

The presence of these cDNA species amongst the amplified mixture is a random event governed by the degree of sequence similarity with the primers; the method itself does not favour the amplification of differentially expressed messages. Thus, a considerable number of primer combinations will have to be tried, and even so, the analysis will be far from exhaustive. Nonetheless, although nicknamed ‘differential dismay’ by some frustrated practitioners, the PCR-based nature of this method carries some specific advantages, including the use of small amounts of starting material, and the ability to identify differential expression even of very rare transcripts. However, this procedure has been largely supplanted by more sophisticated techniques such as representational difference analysis (RDA) as described above, or by array technology (see below).

13.2.4 Array-based methods

The development of methods such as subtractive hybridization allowed scientists to pan for differentially expressed gene products, rather than merely examining the expression of a specific gene. Often, the objective has been to search for a specific disease gene, or one or a few genes whose products fulfil a particular role. Methods such as Northern blotting or RT-PCR can be used to investigate if one or a few candidate genes fulfil our criteria, but they are not suitable for analysing a large number of genes. Even more importantly, they can only be used for genes that we already know about. With the rapid development of genome sequencing, it becomes possible to ask not only such specific questions, but also global ones: can we identify the complete spectrum of genes that are expressed under certain conditions and not otherwise?

This can be done using *arrays*, as described previously (Chapter 12) . In this case, however, the spots of DNA are allowed to hybridize with labelled cDNA. Those DNA spots corresponding to an expressed gene will yield a positive signal. Using machines to produce many identical copies of the array, you can test for the presence of a wide range of specific cDNA molecules derived from cells from different environments.

Conceptually, this is not really different from differential screening. Subtractive cDNA libraries in plasmid vectors have often been painstakingly picked clone by clone and inoculated in a grid formation on an agar plate, and duplicate colony lifts used for their analysis. The advent of robotic picking and high-density spotting technology, and of electronic image analysis, not only made it technically simpler to produce such arrays, but also largely eliminated the desirability to limit the array to a subpopulation of particularly suspect transcripts. Furthermore, knowledge of the complete genome sequence of an organism makes it possible to produce arrays of small DNA fragments (either synthetic or generated by a series of PCR reactions) that will identify every predicted ORF. Such whole genome arrays are now available for a number of bacterial species (see below).

Expressed sequence tag (EST) arrays

The simplest way to produce a cDNA array is to plate an unamplified cDNA library, say from the pancreas, and pick the clones one by one. This has its distinctive disadvantages, however. First, the use of your array will be restricted to samples from one tissue. Your array will have limited use with samples from brain tissue, for example, because the brain and the pancreas will have their specific repertoires of genes which are expressed in either tissue but not in both.

Second, when you made your library, you were wise to base it on poly-A-enriched mRNA (if you were working with a eukaryote), thus avoiding a library where 95 per cent of the material consists of endlessly repeated rRNA and tRNA sequences. However, even so you will have quite a lot of repetition. This is because the frequency of any given gene in a cDNA library (unlike a genomic library) is biased according to the levels of expression of that particular gene. Thus, you will find major components such as structural genes (e.g. tubulin), housekeeping genes (e.g. metabolic enzymes), and major tissue-specific products (e.g. insulin) greatly over-represented in your library.

A project that has simultaneously helped us towards independence of tissue-specific libraries and overcoming the problem of over-representation is the development of *expressed sequence tags* (ESTs). ESTs are cDNA clones where a laboratory equipped with a robot has done just what we outlined above, picked every clone from a library. This has been done with libraries from many different tissues, creating a sort of transcriptome fingerprint for each one of them. They have all been subjected to one sequencing run, yielding a few hundred (unconfirmed) bases from one end, numbered, and archived jointly in large public facilities. The sequence tag database is available through GENBANK, so that you can search it for similarity to a gene you are interested in, and obtain the clones for a nominal charge.

Systematic computer comparison has made it possible to identify which cDNA clones are unique. It is thus possible to produce cDNA arrays with non-redundant clones. By spotting these onto a membrane (macroarray) or a glass slide (microarray), resources have been created that enable simultaneous study of the majority of the genes in the human, mouse, and rat genomes, independently of the sequencing of these genomes. The label of choice of macroarrays is ^{33}P (providing better resolution than ^{32}P), and the labelling is then captured by a phosphoimager. For microarrays, fluorescent labels are used, and the data are collected by specialized microarray readers. A virtually unlimited number of copies of each array can be produced at a negligible marginal cost. Thus, by obtaining a pair of identical arrays, and screening them with labelled tester and driver cDNA populations, you can in one experiment collect more data than many months of differential display analysis would have given you.

PCR product arrays

One objective of the EST programme was to try to obtain at least one clone representing each gene. Although, in the case of the human genome, this objective was not quite achieved, ESTs have been immensely helpful in the task of identifying those genes that were represented, and their open reading frames. Some genes, however, lack EST correlates. In addition, in the cases of those that do, the method of spotting bacterial clones carrying plasmids is far from ideal. Inevitably, some eukaryotic probes will have high enough sequence similarity to these to bind non-specifically to the array.

In bacteria, by contrast, ESTs are not available. However, we now know the entire genome sequence for a number of bacteria (see Chapter 10), making possible another even more efficient approach for array production. Computer methods are used first of all to identify all the open reading frames above a certain size; these are the predicted genes. For each predicted gene, we use the computer to design a pair of primers that will amplify a suitably sized region of that gene. Automated DNA synthesizers are used to make each of these primers, and robotic methods are used to carry out the thousands of PCR reactions needed to make the DNA fragments, which are then robotically spotted onto macro- or microarrays.

Mammalian genomes are much larger, of course, but the same method will make it possible to produce a complete array of PCR-amplified products covering all the genes in the entire human genome now that it has been sequenced. A virtually complete array for the human genome may be available by the time this book is published.

Important factors in array hybridization

Note that array hybridization is in effect the reverse of conventional hybridization. The conventional techniques involve attaching the target DNA or RNA to a filter (e.g. by Southern or Northern blots, or by using dot blots), and then hybridizing that filter with a specific labelled probe. With macro- or microarrays, the specific fragments are attached, unlabelled, to the solid phase, and they are hybridized with the labelled target molecule.

The production of arrays requires a high initial investment. Not only does it require expensive equipment, but also the synthesis of the large number of PCR primers, and the carrying out of large numbers of PCR reactions, is costly. However, once you (or someone else) has got to that stage, large numbers of arrays can be made; the subsequent hybridization and reading of the results is much less expensive, in particular for macroarrays which can even, to a degree, be interpreted with the naked eye without access to special imaging equipment. The actual production of arrays therefore tends to be limited to a few public and commercial centres, while a larger number of laboratories are capable of using the microarrays for their own projects.

All the variants of differential and subtractive hybridization and screening are subject to unreliability and artefacts. They are unlikely to ever completely replace conventional hybridization methods, which still need to be employed for unambiguous confirmation of array data, and for providing information about the corresponding transcripts.

13.3 Methods for Studying the Promoter

13.3.1 Reporter genes

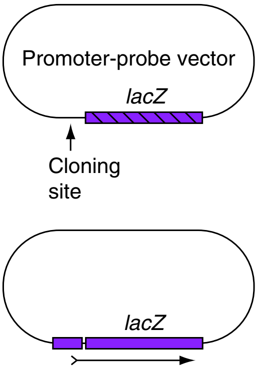
There are a variety of situations where even the most sophisticated of the above approaches is inadequate or inconvenient for following the nature of gene expression. For example, if we are investigating cellular differentiation in a multi-cellular organism, we might want to ask in which cells, or in which tissues, certain genes start to be expressed, and at what stage of development. An alternative approach involves the use of *reporter genes*. Reporter gene studies are also very important in the analysis of a promoter and its transcription factors (see below), and are a crucial preamble to the construction of a transgenic organism (Chapter 17).

Reporter gene analysis involves the use of gene cloning technology to make a construct in which a DNA sequence upstream from a specific gene, including the promoter and some potential regulatory signals, is attached to another gene that codes for a readily detectable product (the reporter). Examples of commonly used reporters include beta-galactosidase (detected using a chromogenic or fluorogenic substrate), green fluorescent protein (a naturally fluorescent protein, originally from a jellyfish), or firefly luciferase (where enzymatic activity is detected by the emission of visible light). The simplest procedure is then to introduce this construct, carrying a reporter gene driven by a promoter, into the organism in question. As the cells start to differentiate, those cells in which the relevant gene is switched on will also switch on the transcriptional signals attached to the reporter gene, leading to detectable expression of the reporter.

Reporter gene technology therefore provides a powerful way of assessing the transcriptional state of selected genes in cells or tissues under different conditions or at different stages of growth. Even if expression occurs only transiently, so that detection of the mRNA may be difficult, the greater stability of the reporter protein may allow the identification of the time at which expression is activated. (It follows from this, however, that it is less easy to determine when expression is switched *off*.)

13.3.2 Locating the promoter

Reporter genes also provide a useful way of locating the sequences that are necessary for promoter activity, and for assessing the regulatory functions associated with upstream sequences (see later). In order to locate the promoter of a specific gene you can start by cloning fragments of DNA around and upstream from the transcription start site, using a vector that carries a promoterless reporter gene (this is known as a *promoter-probe vector*; see Figure 13.10). Alternatively, you can do genome-wide screening for promoters with specific function, for example by making a library of random DNA fragments in a promoter-probe vector, and selecting those clones which exhibit expression of the reporter under the chosen conditions.



Promoterless *lacZ* gene
No expression of beta-galactosidase
White colonies on X-gal

Promoter fragment insert next to promoterless *lacZ* gene
Expression of beta-galactosidase
Blue colonies on X-gal

Figure 13.10 Use of a promoter-probe vector

Promoter-probe vectors are more commonly used in bacterial systems; other approaches that are more suitable for locating regulatory elements in eukaryotes are considered subsequently. In Chapter 14, we describe the related technique of *enhancer trapping*, which involves the use of a transposable element carrying a reporter gene with a weak promoter. Other approaches described in this chapter include the yeast one-hybrid system, which is analogous to the promoter-probe approach in enabling the identification of DNA sequences that interact with known transcription factors. It is also used to identify DNA-binding proteins that interact with a defined DNA sequence, and it is in that context that we consider it in this chapter.

The question addressed by promoter-probe analysis is similar to that in microarray studies of gene expression, except that the latter looks at mRNA levels while the promoter-probe approach tests for promoter activity. The two approaches are to some extent complementary; microarrays are a more direct measure of what actually happens, and directly identify the genes, while promoter-probes may be more successful in detecting transient and low level activity. The promoter-probe approach has the further advantage of not requiring knowledge of the genome sequence, since you can use random DNA fragments – but it does not identify the genes concerned. Once you have detected positive clones, you have more work to do in identifying the DNA fragment in those clones.

One serious disadvantage of using reporter genes on a plasmid is that the system is prone to artefacts. Fragments of DNA, taken out of context, may show quite different effects in their ability to promote transcription. Some promoters do not work properly (or their regulation is quite different) when located on a plasmid; conversely, some DNA fragments that produce positive signals on a promote-probe vector are subsequently found not to be ‘real’ promoters. Reporter genes, used in either of the above ways, often give more reliable results when inserted into a specific chromosomal position rather than in the artificial environment of a plasmid.

One important example of the use of the promoter-probe approach is the IVET (*in vivo* expression technology) method for identifying potential virulence genes in pathogenic bacteria (see Figure 13.11). In the original version of this procedure, used for identifying *in vivo* expressed genes in *Salmonella typhimurium*, the vector contained two promoterless genes (*purA* and *lacZ*) and the host strain was a *purA* mutant of *S. typhimurium* (i.e. it had a defect in purine biosynthesis, which makes it unable to grow in experimental animals). Random fragments of *S. typhimurium* DNA were inserted in the vector to produce a gene library, and the constructs were integrated into the chromosome by homologous recombination (see Chapter 14). When animals were challenged with the mixture of clones, only those clones with a promoter that was active in this situation were able to express the *purA* gene – thus providing direct selection for promoters that were active during infection. The promoterless *lacZ* reporter

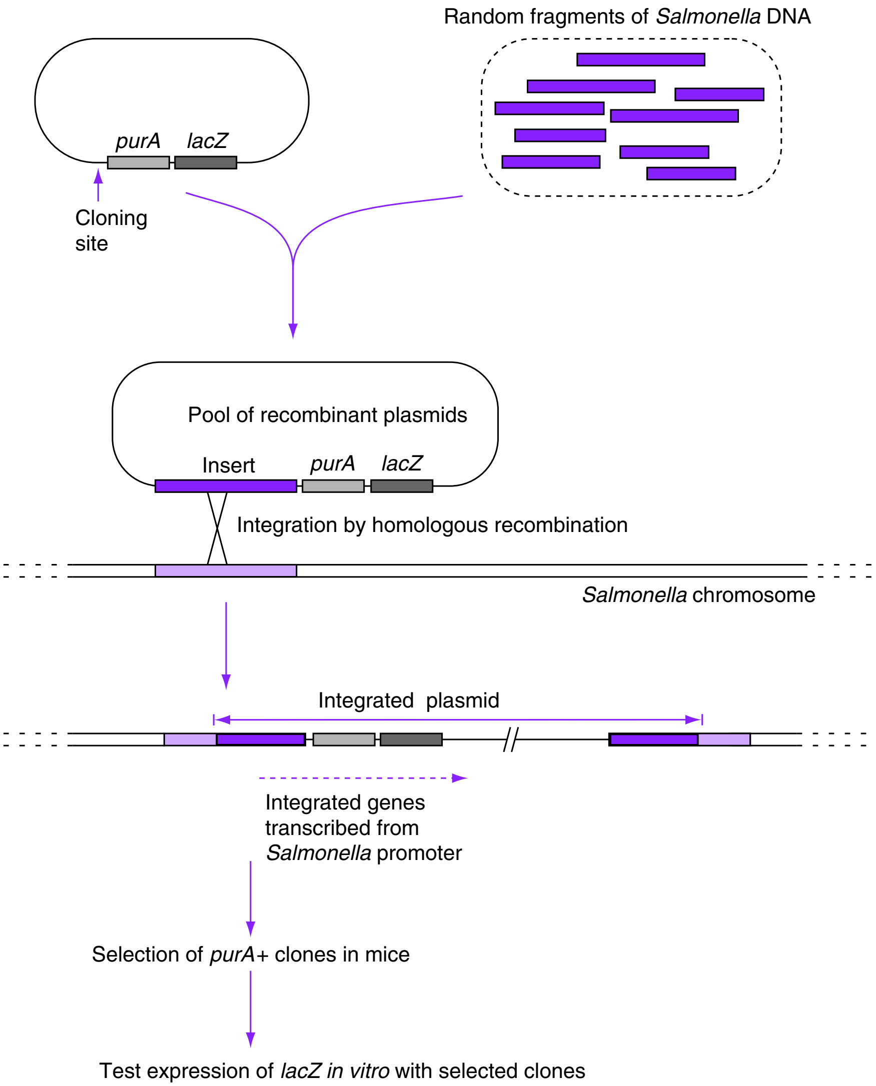


Figure 13.11 *In vivo* expression technology (IVET)

gene allowed confirmation of the results; most of the recovered bacteria contained promoters that were also active in the laboratory, but about 5 per cent were specifically activated during infection.

Another elegant method for identifying virulence genes, known as *signature-tagged mutagenesis*, is described in Chapter 14.

The techniques above will identify a DNA fragment that has promoter activity, but will not tell you which part of that fragment is responsible. You can extend the approach to provide a more precise identification of the sequences that are necessary for promoter activity. At the simplest level, this could involve making a series of deletions from one or both ends of the fragment, and seeing how much you can remove without affecting promoter activity. Other techniques that we will describe later (Chapter 15) can be used to alter specific bases within the promoter in order to assess the sequence requirements for initiation of transcription. This approach can also be used to identify regulatory sequences that affect the activity of the promoter, usually through binding of regulatory proteins. Subsequent sections in this chapter examine other methods for investigating the DNA binding of RNA polymerase, transcription factors, and regulatory proteins.

13.3.3 Using reporter genes to study regulatory RNA elements

Reporter gene assays based on joining a promoter to a reporter gene have been very useful both for dissecting the components of the promoter and for quantifying its activation in different situations; but of course there are limitations. If you couple your promoter to a gene that encodes a completely different protein, your readout will be a function of the intrinsic properties of that mRNA and of that protein. These include the stability of the mRNA and the protein, as well as the efficiency of translation. However, as long as you compare like with like – the activation of the promoter, *as measured by its effect on reporter gene levels*, under different circumstances – you can make a convincing case that your observations are comparable as a measure of promoter activation because you are keeping all other parameters constant.

You may, however, especially in eukaryotic systems, wish to study other parameters that affect the levels of the transcript encoded by your gene, such as the 5′ untranslated region (UTR) and its effect on translation efficiency, or the 3′ UTR and its effect on mRNA stability. In these cases, you will wish to keep all other factors constant, including the promoter. Thus, you choose a vector that contains your reporter gene under the control of a constitutively active promoter without cellular or organismal specificity, such as that of the large T antigen of the SV40 virus. You can then slot the whole or part of your 5′/3′ UTR on either side of your reporter gene. By comparing the amount of reporter gene product in different constructs, and in the native vector, you are able to gauge the positive or negative impact of different elements on mRNA *translatability*.

13.3.4 Regulatory elements and DNA-binding proteins

The most direct, precise and reliable way of locating a promoter, or any other DNA sequence that influences transcription through the binding of specific proteins (which includes many but not all regulatory elements) is to detect the binding of those proteins to specific DNA fragments. Three methods are worth describing briefly: yeast one-hybrid assays, DNase I footprinting and gel retardation assays.

Yeast one-hybrid assays

The purpose of the yeast one-hybrid system is to identify proteins that interact with a defined DNA sequence, or conversely to locate DNA regions that will bind to known transcription factors. In the example illustrated in Figure 13.12, tandem copies of the DNA sequence under investigation (known as the *bait*) are inserted upstream from a reporter gene. This construct is then integrated into the yeast genome. In the absence of transcription factors that bind to the bait, the reporter gene will not be expressed (Figure 13.12(a)). However, if you transform the recombinant yeast strain with a cDNA expression library, any clones that express a transcription factor that can interact with the bait will activate transcription of the reporter gene (Figure 13.12(b)).

The versatility of the system can be extended by exploiting the bipartite nature of eukaryotic transcription factors. This means that in general they consist of two domains: one part of the protein (the DNA-binding domain) makes specific contacts with certain DNA sequences, while the other part (the activation domain) is responsible for activating transcription. These domains can be separated, and function independently, so the activation domain (AD)

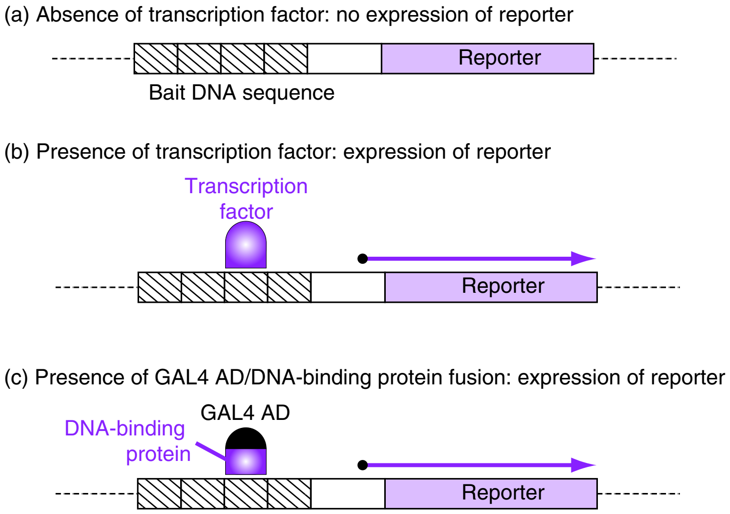


Figure 13.12 Yeast one-hybrid system

can be joined to other DNA-binding proteins, when it will activate transcription from other sites according to the specificity of the new DNA-binding domain. The best example is the yeast activator GAL4, which normally controls the transcription of genes involved in galactose metabolism. If the DNA-binding domain of GAL4 is removed and replaced with another DNA-binding domain, the hybrid protein will activate transcription of a different set of genes.

To take advantage of this, the cDNA library is made using a special vector containing the GAL4 AD so that the products will be expressed as fusion proteins with the GAL4 AD. If the product binds to the bait sequence, the GAL4 AD will activate transcription of the reporter (Figure 13.12(c)). Since the activation is provided by the GAL4 AD, this system can detect a wider range of DNA-binding proteins, and is not limited to those that are themselves transcription factors.

An extension of this concept, the yeast two-hybrid system, is used to study protein–protein interactions, and is considered in Chapter 14.

DNase I footprinting

In *DNase I footprinting* (or just *footprinting*), you identify which part of the suspected promoter sequence binds to transcription factor proteins. Nuclei are isolated from whole cells (whether a cultured cell line or a whole tissue), and proteins isolated from the nuclei. These proteins, or a specific protein purified from the mixture, are then mixed with the DNA fragment you wish to analyse. A parallel control reaction contains DNA without protein. In both reactions, the DNA is radiolabelled at the 3′ end. You then add the nuclease DNase I to both reactions. DNase I will cleave all the phosphodiester bonds in the DNA chain that it can reach. The reaction is not allowed to go to completion; thus, as a result, the DNA in your control reaction will be fragmented randomly into a continuous ladder of DNA fragments of all possible sizes between full size and one base. However, in the other reaction, DNase I will be unable to access its substrate wherever a *cis*-acting DNA element has been protected by a bound *trans*-acting protein. Thus, if you run these two samples out on a sequencing gel side by side, you will find that the continuous ladder as seen in the control is interrupted in these protected regions (Figure 13.13). Since DNase digestion is random, there will of course be a lot of other products as well as those shown – but as you are using autoradiography to detect the label attached to the DNA, you will only detect those fragments that contain the end of the original DNA molecule. By running out a sequencing reaction as well, you will be able to tell exactly which regions of DNA were protected. Note, however, that the extent of the DNA that is protected by a specific protein is usually considerably larger than the region that is necessary to make specific contacts with the protein.

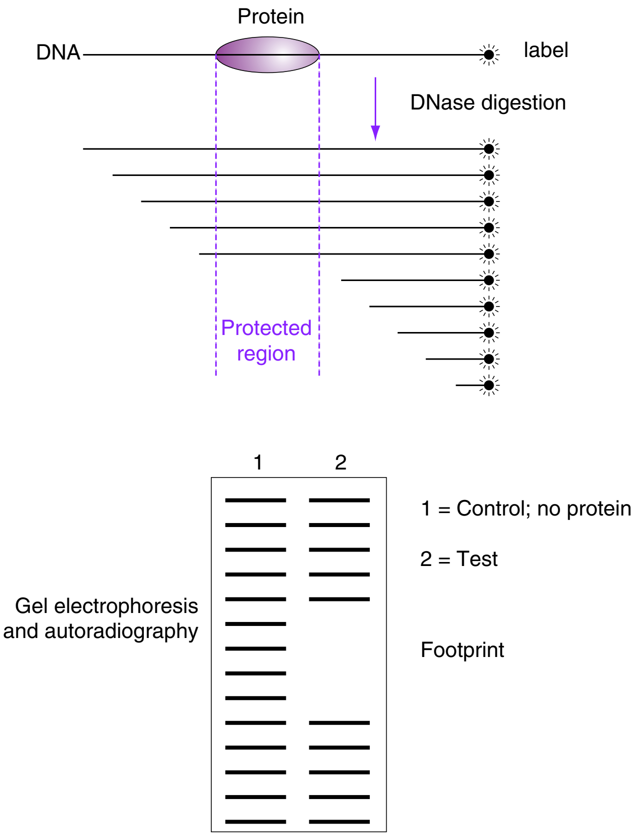


Figure 13.13 DNase I footprinting

Gel retardation assays

Another way of determining if a particular sequence of DNA is involved in transcription factor binding is *gel retardation* or *gel shift* assays. Here, the DNA fragment is mixed with nuclear proteins. If one or more of these bind to the DNA fragment, its mobility in a polyacrylamide or agarose gel will be decreased. This can be easily detected by comparing it with a control sample without added protein (Figure 13.14). To investigate the specificity of the reaction, we can add different amounts of protein to ensure that there is a linear dose relationship.

Both footprinting and gel retardation assays are applicable to any protein that binds to specific DNA sequences, and are not limited to those proteins that regulate transcription of the DNA.

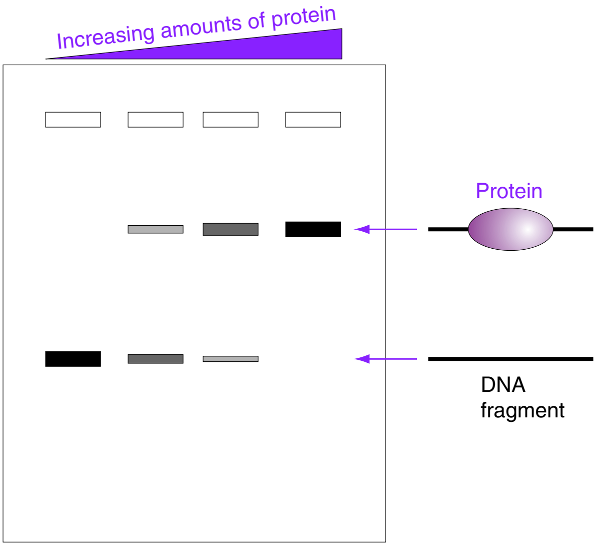


Figure 13.14 Gel retardation assay

13.3.5 Run-on assays

Above, we have discussed several methods for determining the amount of an mRNA species in a sample 'frozen' in time (what is called, not always accurately, 'steady-state levels'). These, as we have seen, have the disadvantage of only showing us the net effect of promoter activation and mRNA degradation at one given moment. Using reporter gene assays, we are able to start disentangling the actual effects of the promoter and of the other regulatory elements, but they have the disadvantage of being 'contaminated' by the fact that the read-out is a different protein. All of these methods are very useful within these limitations, but sometimes you want to obtain a more accurate read-out. This is found in the *run-on assay*, also known as *nuclear* or *transcriptional* run-on assay (also, confusingly, called run-off assay!). This assay quantifies the actual transcription of the specific gene in a sample. Nuclei are harvested either from cultured cells or whole tissue and supplied with radiolabelled UTP. They are then left to proceed with transcription, for a short period of time, of all active genes according to their degree of activation in the situation when the nuclei were harvested. The radiolabelled mRNA created by transcription of your gene of interest can then be specifically quantified by immobilizing it through hybridization to 'cold' complementary DNA.

13.4 Translational Analysis

The methods for studying protein expression were developed earlier than DNA cloning methods. For example, it was possible to sequence proteins (if they could be purified in large enough amounts) before gene sequencing was developed. When gene cloning techniques became available, the characterization of DNA and mRNA became much easier than sequencing their protein products. However, many protein biochemists are gleefully witnessing the renaissance of their craft, now renamed proteomics. Not least the realization that the human genome contains many fewer genes than there are proteins has reminded molecular biologists that gene products must be studied on both levels (to say nothing of the actual effect on the cell or organism as a whole) in order to understand how a gene works.

13.4.1 Western blots

The conventional way of analysing the proteins produced by a cell involves electrophoresis through a polyacrylamide gel in the presence of sodium dodecyl sulphate (SDS polyacrylamide gel electrophoresis, or SDS-PAGE). In general, there are too many proteins present to give a clear picture of the complete protein profile if you simply stain the gel with a general protein stain, although much higher resolution can be obtained by two-dimensional gel electrophoresis (see later in this chapter). In a one-dimensional SDS-PAGE, you will usually only be able to see the major bands, and even then it is not always easy to identify any specific protein.

The generally applicable technique for the detection of a specific protein, and analysis of its expression, relies on the use of specific antibodies, in combination with SDS-PAGE. Following separation of the cell extract by SDS-PAGE, the proteins are transferred to a membrane by running a perpendicular current through the gel into the membrane, and a specific protein or proteins detected using *antibodies* (see Chapter 8). These antibodies can be either labelled themselves or, more commonly, detected by a second labelled antibody. For example if your primary antibody is a mouse antibody that recognizes the required protein, the binding of that antibody can be detected by a labelled rabbit anti-mouse antibody. The analogy with a Southern blot led to this technique being termed *Western blotting*. The technique will identify not just the presence or absence of a protein that reacts with the antigen, but also its size and an estimate of relative levels of expression. In Figure 13.15, we see that expression was not detected in sample 2, and only weakly in sample 1, while the

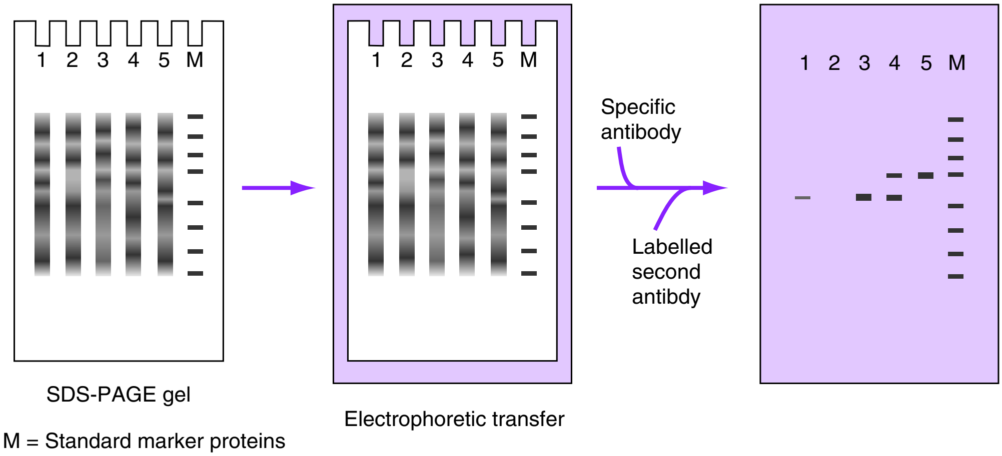


Figure 13.15 Western blotting

protein detected in track 5 was a different size, perhaps due to post-translational modification; both bands were present in track 4.

The major considerations in the use of antibodies for molecular biology were covered in Chapter 8 (see Box 8.2). The main point to remember is that proteins in an SDS-PAGE gel are denatured, so the antibody must be capable of recognizing a linear epitope. For recombinant proteins, you also require an antibody that binds to a non-glycosylated epitope. The complex mixture of antibodies in a conventional antiserum is virtually guaranteed to contain some suitable antibodies, but an individual monoclonal antibody may fail one or both criteria. On the other hand, monoclonal antibodies have the advantage of reproducibility, whereas the antisera from different animals may differ in sensitivity and specificity.

13.4.2 Immunocytochemistry and immunohistochemistry

This method (differently described depending on whether we are working with cells or tissues) relates to Western blotting the way *in situ* hybridization relates to Northern blotting. The blotting method allows us to determine whether the probe (whether antibody or nucleic acid probe) is specific and, if it is, how many polypeptides or transcripts it recognizes, and what size they are. The cytological/histological method, by contrast, allows us to determine which cell type the gene product is localized to. Apart from being much easier to perform and less prone to artefacts than *in situ* hybridization, the study of the translated protein product has an added bonus. The *intracellular* localization of an mRNA, as determined by *in situ* hybridization, is rarely particularly revealing –

a transcript lingers in the vicinity of the nucleus whilst being translated, and that is that. However, the destination of the translated protein shows much greater variation. Some are cytoplasmic, some are membrane-bound; some gather at one pole of the cell (which, in a nerve cell, could be many centimetres away from the nucleus), and others re-enter the nucleus. The localization of a protein can give us important clues to its function, and indeed the incorrect transport of a protein can give us important information about a disease phenotype.

13.4.3 Two-dimensional electrophoresis

Western blotting is suitable for analysing the production of a single protein (if you have a suitable antibody to it), but if you want to examine the overall protein composition you need a different technique. This can be done by *two-dimensional gel electrophoresis*, which involves isoelectric focussing (IEF) in one direction and SDS-PAGE in the other (Figure 13.16). Isoelectric focussing consists of applying an electric field across a stable pH gradient. All proteins have a characteristic *isoelectric point* (or pI), that is a pH value at which they have no net negative or positive charge. At pH values above the pI, the protein will be negatively charged; below the pI the net charge will be positive. Negatively charged proteins will therefore move through the pH gradient towards the positively charged electrode (anode) until they reach a pH equal to their pI,

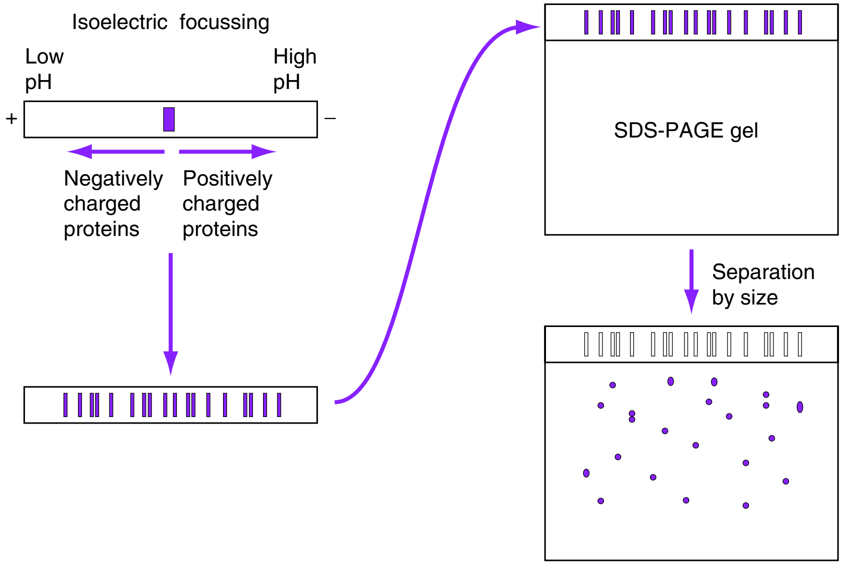


Figure 13.16 2D protein gel electrophoresis

at which point they are no longer charged ; similarly the positively charged proteins will move towards the cathode until they reach their pI. The end result is that each protein will focus at a characteristic point on the pH gradient. The second dimension separates the proteins according to their size.

After 2D gel electrophoresis, you will get a pattern of spots that can be compared with other patterns to enable the identification of proteins that are present in one strain but not another, or proteins that are differentially expressed according to changes in environmental conditions or phase of growth. It is necessary to exercise careful control of the conditions used to enable a reliable comparison to be made. It should also be noted that a single protein can occur at different positions in a 2D gel, for example as a consequence of differences in post-translational modification.

13.4.4 Proteomics

Rather than focussing on a limited number of specific spots on a 2D gel, you may want to determine the complete profile of proteins that are made by the organism in question under defined conditions. The distinction is analogous to the difference between studying individual genes and sequencing a whole genome. The profile of expressed proteins is referred to as the *proteome* (by analogy with the *genome*), and hence the study of the spectrum of proteins produced is known as *proteomics*. It should be emphasized that while the genome of an organism is a fixed entity (give or take a bit of variation), the profile of expressed proteins will be different according to factors such as growth conditions and cellular differentiation.

The most commonly used way of attempting to define the proteome is to try to identify all the spots on a 2D protein gel. The best way of identifying and characterizing a protein, whether it is purified by conventional means or it is a spot on a 2D gel, is to determine its sequence. The technology for protein sequencing has advanced very markedly, so that it is now possible using microsequencing techniques to obtain protein sequences from individual spots. However, this would be a rather cumbersome way of identifying all the spots on a 2D protein gel. Fortunately, if the genome sequence is available, we can use quicker methods.

For example, the protein can be eluted from the gel and digested with a proteolytic enzyme that will cut the protein only at specific positions determined by the amino acid sequence. This will produce a set of peptides. The precise molecular mass of each peptide in that mixture can readily be determined by mass spectrometry. From the genome sequence, we can predict the sequence of all potential proteins, and from that information we can predict the sizes of all the peptides that would be generated from each protein (since we know the specificity of cleavage of the protease). The computer can then compare the sizes of the peptides we have found with the peptides that would be generated from each predicted protein – and hence identify the protein.

Even this procedure is time-consuming, if you have to rely on recovering each spot from the gel by hand. For large-scale proteomics, it is possible to use automated equipment that will recognize spots on the gel, excise each one individually, and carry out the mass spectrometry of the peptides. This gives high-throughput identification of proteins and is capable of defining extensive proteomes virtually completely.

If the genome sequence is not available, alternative forms of mass spectrometry are capable of identifying at least part of the amino acid sequence of the protein. This information can then be used to predict the likely sequence of part of the DNA of the corresponding gene, which enables you to design a nucleic acid probe to recover that gene from a gene library (see Chapter 8).

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14 Analysis of Gene Function

14.1 Relating Genes and Functions

We have already, in Chapter 11, discussed how we can use the primary DNA sequence of a gene to make predictions about the structure and function of its protein products. However, this approach has limitations. First, a substantial portion of the genes identified in any genome sequence are not related to any known gene. Second, in many cases where there is similarity to another gene, that gene itself is of unknown function. Also, there is always the cautionary note that bioinformatics can make predictions about function, but does not provide definitive answers. This chapter deals with some of the methods that can be used to identify or confirm the function of specific genes, which includes an integration of molecular (*in vitro*) and classical (*in vivo*) genetic techniques. The subsequent chapters also contain material relevant to this question. In Chapter 15 we will look at the use of systems to express specifically modified (or wholly synthetic) genes, while Chapter 16 includes the use of molecular techniques to identify the gene changes responsible for human genetic diseases. Finally, in Chapter 17 we will discuss how genes can be removed, inserted, or modified in living multicellular organisms, including the use of these procedures in the analysis of gene function.

14.2 Genetic Maps

14.2.1 *Linked and unlinked genes*

The classical approach to genetics starts with the identification of variants which have a specific *phenotype*, i.e. they are altered in some way that can be seen (or detected in other ways) and defined. For Mendel, this was the appearance of his peas (e.g. green versus yellow, or round versus wrinkled). One of the postulates he arrived at was that these characteristics assorted independently of one another. For example, if you cross a strain that produces yellow round peas with another strain that produces green wrinkled peas, the first generation

(F1) are all round and yellow (because round is dominant over wrinkled, and yellow is dominant over green). Let us assume that the original parents are *homozygous*, i.e. they carry identical versions of both alleles; these are described as GGWW and ggww respectively (where G = yellow, g = green; W = round, w = wrinkled). The F1 generation, although they all appear round and yellow, are actually *heterozygous* (GgWw). If you then cross the F1 peas with themselves, the next generation (F2) will contain yellow and green peas in a 3:1 ratio. The ratio of round to wrinkled peas will also be 3:1, irrespective of whether the peas are yellow or green. This is explained in more detail in Figure 14.1. In

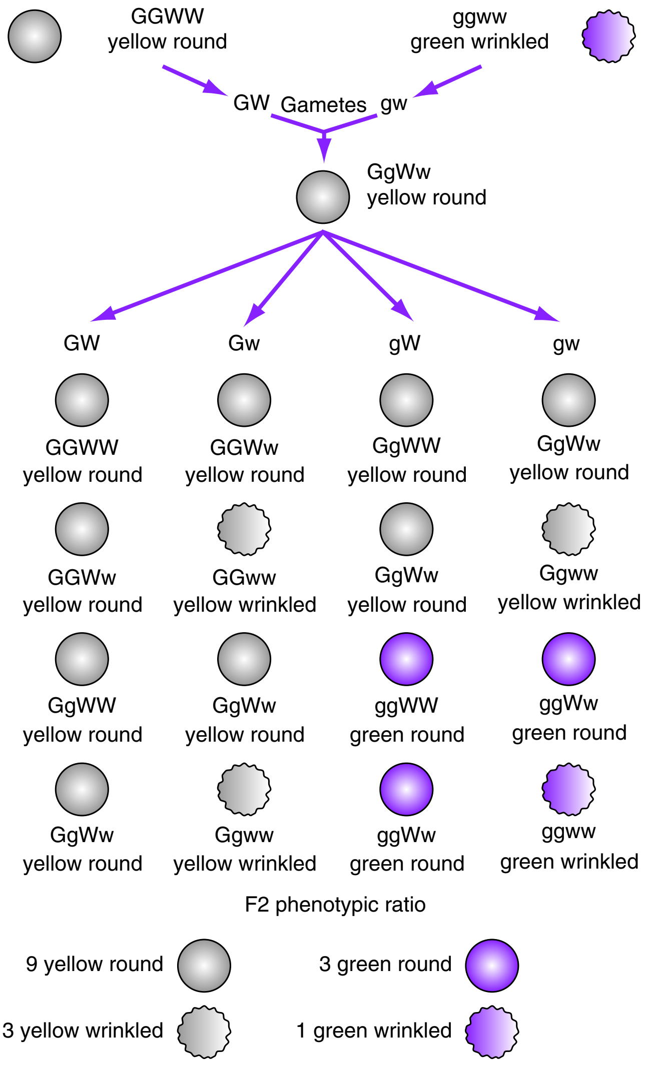

Figure 14.1 Independent assortment of unlinked genes

other words, the green/yellow character is assorted independently of the round/wrinkled property. We would described these characters (and the genes responsible) as *unlinked*.

Of course Mendel did not know why this happened. We now know that if genes are located on different chromosomes, they will segregate independently during meiosis, and will thus be distributed independently amongst the progeny. The same can happen if the two genes are on the same chromosome, but are so far apart that the recombination between the homologous chromosomes will be sufficient to reassort them independently. On the other hand, if they are quite close together, they will tend to remain associated during meiosis, and will therefore be inherited together. We refer to genes that do *not* segregate independently as *linked*; the closer they are, the greater the degree of linkage, i.e. the more likely they are to stay together during meiosis. Measuring the degree of linkage (*linkage analysis*) is a central tool in classical genetics, in that it provides a way of mapping genes, i.e. determining their relative position on the chromosome. Furthermore, it provides us with an important method for correlating genetic and physical maps, as described below.

Bacteria and yeasts provide much more convenient systems for genetic analysis, because they grow quickly, as unicellular organisms, and on defined media. You can therefore use chemical or physical mutagens (such as UV irradiation) to produce a wide range of mutations, and can select specific mutations from very large pools of organisms – remembering that an overnight culture of *E. coli* will contain some 10^9 bacteria per ml. These mutations may simply affect the ability to produce a specific amino acid, manifested as a requirement for that amino acid to be added to the growth medium, or to use a particular carbon source such as lactose. Alternatively, it may be a more complex phenotype, such as loss of motility, or inability to divide into two cells leading to production of filaments. So we can use genetic techniques to investigate detailed aspects of the physiology of such cells, including identifying the relevant genes by mapping the position of the mutations. Although the techniques in bacteria differ from those in higher organisms, forms of linkage analysis still play a major role.

For multicellular organisms, the range of phenotypes is even greater, as we now have questions concerning the development of different parts of the organism. However, animals have much longer generation times than bacteria and, depending on the species, using millions of animals to identify the mutations you are interested in is either cumbersome, impossible, or indefensible. Human genetics is even more difficult as you cannot use selected breeding to map genes; you have to rely on the analysis of real families. Nevertheless, classical genetics has contributed extensively to the study of developmental processes, notably in the fruit fly *Drosophila melanogaster*, where it is possible to study quite large numbers (although nothing like the numbers that can be used in bacterial genetics), and to use mutagenic agents to enhance the rate of variation.

14.3 Relating Genetic and Physical Maps

In any of these systems, the question addressed by classical genetics is the same: can we locate the gene that is associated with a specific phenotype? This produces a *genetic map*, in which the position of these genes on the chromosome is determined, relative to other genes. However, the genetic map does not directly tell us what these genes actually do.

On the other hand, as we have seen in previous chapters, molecular techniques are, in the first place, concerned with the structure of genes and their sequence. This could start with cloning and sequencing a fragment of DNA; you could then use hybridization techniques to find the position of that sequence on the chromosome, producing a *physical map* of the chromosome. Physical maps can take other forms as well, including maps of restriction sites. Ultimately, you can determine the complete sequence of the genome, which is the ultimate physical map. It tells you exactly the DNA sequence at any position on the genome but, taken in isolation, tells you nothing about the nature or function of the genes or their products. In order to advance our understanding further, we have to be able to relate the genetic and physical maps.

To some extent we can tackle the problem from either end. For example, we could start with the classical approach, i.e. isolating specific variants and mapping the genes concerned. We would then follow that with the techniques described in the previous chapter to isolate and clone the DNA region that is different in the mutant and wild-type organisms – in this way linking the genetic map to the structure of a specific gene. Later in this chapter we describe techniques that are specifically designed for this purpose.

Alternatively, we can start from the other end. If we know the sequence of a piece of DNA, or the entire genome, we can (as described in Chapter 11) infer the likely nature of the enzyme or other product coded for by each gene, by comparison with the sequence of known genes from other organisms. So we can work backwards from the physical map towards the genetic one. However, this approach has limitations. First, a substantial proportion of the predicted genes identified in any genome sequence are either not related to any known gene, or are related to another gene of unknown function. Second, even where there is a good degree of similarity with another gene that is labelled as coding for an identified enzyme, that identification is only as good as the identification of the gene we are comparing it with. As discussed in Chapter 11, there is a risk of setting up a chain of increasingly unreliable similarities. Furthermore, we cannot be sure in all cases that enzymes with a similar structure actually carry out the same biochemical reaction. The enzyme beta-lactamase, responsible for ampicillin resistance (as used in many cloning vectors) is similar in some respects to a serine protease, but it is not a proteolytic enzyme.

There is a final limitation to this approach that is more fundamental in nature. We may have correctly identified the biochemical reaction carried out by the enzyme for which our gene is responsible. However, this does not necessarily tell us what role that gene plays in the characteristics of the cell. For bacteria, and other unicellular organisms, it may be relatively straightforward to understand the role of enzymes that are components of a simple metabolic pathway, such as synthesis of an amino acid – but, even at this simplest of levels, such understanding is not always completely straightforward. The organism may have more than one gene coding for enzymes that carry out the same reaction, so we would have to ask under what conditions each of those genes is used. With more subtle processes, it may be very difficult to ascertain the role of specific proteins – and if we consider complex processes such as the regulation of cell division it is likely to be impossible to determine the role of individual proteins just by examining their structure. If we then move on to consider a multicellular organism such as an animal, there is an even bigger jump from knowing the biochemical function of the enzyme to understanding its role in the whole animal.

We can now look at some of the techniques that are available for constructing more direct links between genetic and physical maps, i.e. for establishing (or confirming) more directly the actual function in the cell of specific genes.

14.4 Linkage Analysis

For the first of these techniques, we can return to the topic of linkage analysis. As described earlier in this chapter, this is a classic technique for establishing how close two genes are on the chromosome. So if our genetic mapping data tell us that the gene we are interested in is closely linked to another marker that has been characterized, we can narrow down the search for the gene of interest to a much smaller region of the chromosome. However, this would require the mapping of a very large number of genes if we are to be sure that there will be a mapped gene very close to our unknown gene. The distances separating known linked genetic markers in mammals commonly run to thousands of kilobases (1 per cent recombination corresponds to about 1000 kb, or 1 Mb, of DNA). However, the second marker does not have to be a functional gene; it can be a polymorphic marker such as the microsatellites described in Chapter 12. If the unknown gene is often co-inherited with such a polymorphism, linkage analysis can identify its position to a comparatively short region, which can then be cloned and characterized to identify the nature of the mutation that is responsible for the observed variation. This technique, known as *positional cloning*, has been used for the identification of important human genes such as *BRCA1* the presence of which predisposes to breast cancer. The identification of genes associated with human diseases is covered further in Chapter 16.

14.4.1 Ordered libraries and chromosome walking

In Chapter 7, we described the construction of a special type of gene library known as an *ordered library*, which consists of a set of overlapping clones so that the position of each clone is known with respect to the clones on either side on the genome. This provides, in essence, a form of physical map of the genome (and ordered libraries have played a significant role in some of the genome sequencing projects). Any gene that has been cloned can easily be located to one of these clones by hybridization. (Of course if the genome sequence is known this is not necessary.) We can then use that as a starting point for locating other genes that are known to be linked to the first marker.

Construction of ordered libraries, especially of large genomes, is a laborious undertaking. A more generally applicable version of the technique is that known as *chromosome walking* (see Figure 14.2). Again, this requires as a starting point a marker that is known to be linked to the gene in question. This marker is used to identify a clone from a gene library, by hybridization. That clone is then used to screen the gene library in order to identify overlapping clones; one (or more) of these clones is then in turn used as a probe to identify other clones that overlap with it. These steps are repeated until the required sequence is reached.

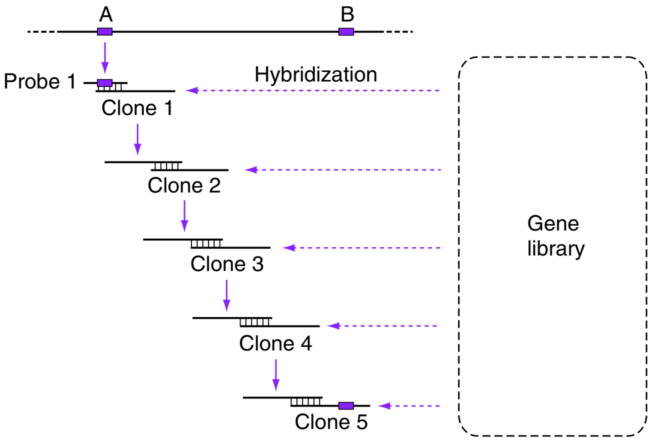

Figure 14.2 Chromosome walking

14.5 Transposon Mutagenesis

Although these methods can be applied to bacteria, the smaller genome and the availability of additional tools makes it unnecessary.

Once again, the starting point is a mutant with an interesting phenotype, and we want to identify the gene that has been altered. If the change is a major one, such as deletion, this is straightforward; we can use array technology (Chapter 12) to identify the region of the chromosome that has been deleted, by the absence of hybridization to a specific DNA fragment on the array. If the mutation results in the failure of expression of the gene concerned, we can use differential or subtractive hybridization, as described in Chapter 13. However, many of our mutants are likely to be *point mutations*, i.e. alterations of a single nucleotide, which result in production of an inactive protein. Such mutations would not be detected by those techniques. An alternative is to carry out the mutation with a procedure that would at the same time label the affected portion of the DNA sequence. This can be done using *transposon mutagenesis*.

Transposons are DNA sequences that have the ability to move from one DNA site to another. Part of the DNA of a transposon codes for an enzyme (transposase) that is capable of carrying out a special form of recombination, involving inverted repeat sequences at each end of the transposon, that results in insertion of the transposon at a new position, either on the same DNA molecule or on a different one. Transposons can thus move from one site to another on the chromosome, or they can move from a plasmid to the chromosome, or from one plasmid to another. The details of the process can vary quite considerably from one transposon to another, but that does not need to concern us. We should, however, note two features: some transposons, but not all of them, replicate in the process (i.e. one copy stays at the original site and a new copy is inserted elsewhere), and some transposons can insert more or less at random while others have varying degrees of specificity. One of the most commonly used transposons is Tn5 (or derivatives thereof), which is not very specific in its insertion site requirements, and hence can insert at a large number of positions.

A further feature of transposons that is relevant here is that they generally carry antibiotic resistance genes. Indeed they play, together with plasmids, a major role in the spread of antibiotic resistance genes amongst pathogenic bacteria. However, transposition not only moves genes between different sites; insertion of a transposon within a coding sequence will usually inactivate that gene, thus producing a mutation. The site of that mutation is now marked by the presence of the resistance gene, which makes it relatively easy to clone, and thus to identify, the affected portion of the DNA.

The procedure in practice (illustrated in Figure 14.3) is to use a plasmid, carrying the transposon, which is unable to replicate in the host species being

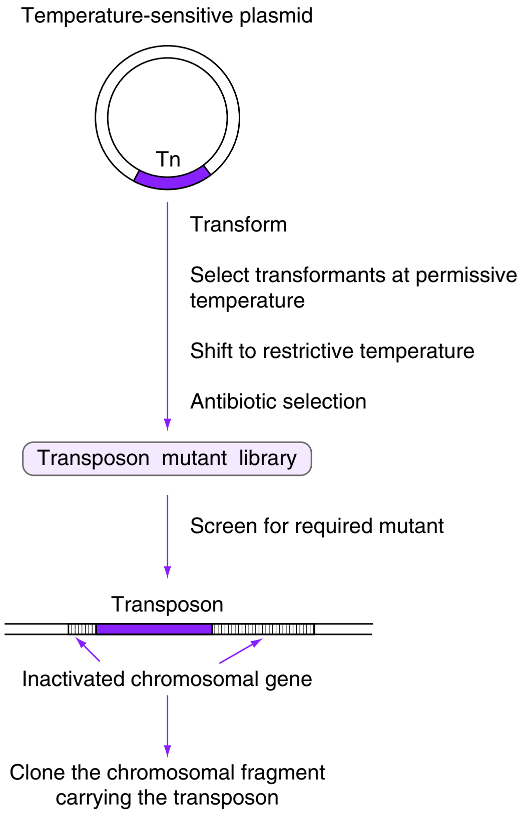

Figure 14.3 Transposon mutagenesis

investigated; this is known as a *suicide* plasmid. Even better is to use a plasmid that is temperature-sensitive for replication, so you can establish the plasmid at a low temperature (e.g. 30°C, the *permissive* temperature) and subsequently prevent its replication by shifting the incubation temperature to say 42°C (the *restrictive* temperature). Inside the bacterial cells, the plasmid is unable to replicate at the restrictive temperature. Thus, if we plate the transformed bacteria on a medium containing the relevant antibiotic, only those cells in which the transposon has hopped onto the chromosome will be able to survive and grow to form colonies. If this happens, the transposon will be replicated as part of the bacterial chromosome.

Of course we do not know where the transposon will have jumped to, but there are a large number of possibilities. We can store this collection of cells as a *transposon mutagenesis library*. Within this library we hope there will be some bacteria in which the transposon has ended up within the gene(s) we are interested in.

The procedure now requires a method of testing for the mutation that we are interested in. In some cases this is relatively easy. We can identify *auxotrophs* (mutants that require a specific supplement, such as an amino acid, in the growth medium) for example, by replica plating. With other types of mutants, we may have to employ more ingenuity in screening our library to identify the mutants that we need.

Assuming we can identify a number of mutants that may be of interest, the next step is straightforward. We can extract genomic DNA from those cells, digest it with a restriction enzyme, and ligate these fragments with a suitable vector. In effect, we create a genomic library, but we do not need the complete library; we are only interested in those fragments that carry the transposon. We can identify these quite easily because they will contain the antibiotic resistance gene that is part of the transposon. So we just need to plate the library onto agar containing the relevant antibiotic, and only those clones that carry the transposon will be able to grow.

These clones will contain not only the transposon but also a portion of the DNA either side of the insertion side. Determining the sequence of this flanking DNA will therefore enable us to identify the gene into which the transposon has inserted, and we thus have a direct link between the sequence and the phenotype, i.e. we know (subject to certain limitations that are discussed below) that inactivation of that gene gives rise to that phenotype, and hence we can infer the function of that gene in the normal life of the cell.

As is so often the case, PCR provides us with an alternative to cloning for this purpose. We cannot do a straightforward PCR, because that would require knowledge of the flanking sequence, for designing the primers, and that is exactly what we do not know. One strategy that we can adopt in such circumstance is known as *inverse PCR* (see Figure 14.4). If we cut the DNA with a restriction enzyme (using one that does not cut the transposon itself), then, instead of ligating these fragments with a vector, we can carry out a ligation in the absence of a vector and under conditions that promote self-ligation (intramolecular rather than intermolecular ligation). Reference to Chapter 5 will show this requires ligation at *low* DNA concentrations, whereas usually we do ligation at high concentrations of DNA to promote ligation of DNA fragments with the vector DNA. The consequence of self-ligation is that, amongst a lot of other fragments, we have circular molecules containing the transposon and the flanking sequences. Although this will be only one amongst thousands of products, the flanking sequences can be amplified by PCR, using primers derived from the known sequence of the transposon and directed outwards from the transposon.

In transgenic multicellular organisms (Chapter 17), similar situations are sometimes encountered when an introduced transgene happens to lodge itself inside another gene. Because only a small fraction of eukaryotic genomes

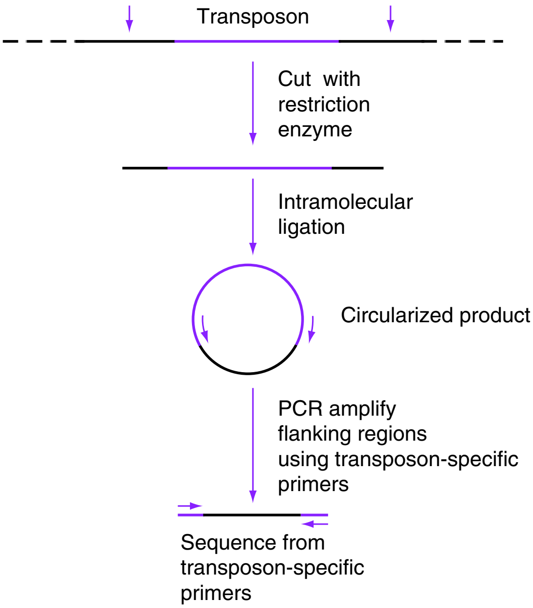

Figure 14.4 Locating an integrated transposon by inverse PCR

actually code for gene products, this is a much less likely occurrence than in bacteria. However, although useless from the point of view of the intended experiment, these strains are sometimes useful for gene mapping and/or as disease models.

In addition to using transposons for mutagenesis of host genes, we can also use transposon mutagenesis to obtain a library of mutations in a cloned gene, since transposition can occur between plasmids, as well as into the chromosome. In Chapter 15 we discuss ways in which specific alterations can be made in a cloned gene. However, if we want to study the functions of different parts of the gene, it can be useful to introduce random mutations within the gene. Transposon mutagenesis enables us to do this quickly and easily.

14.5.1 *Transposition in* Drosophila

The discussion of transposons and transposition has so far focussed on bacteria. However, transposable elements of one sort or another are common in all types of organisms. The family of transposable elements known as P elements, which occur in the fruit fly *Drosophila melanogaster*, are especially important – both in providing vectors for the integration of foreign genes into the *Drosophila* genome and in providing a system for transposon mutagenesis of *Drosophila*.

Transposition of P elements, as with bacterial transposons, requires the action of a transposase, acting on inverted repeat sequences at the ends of the element. In a P strain, which carries multiple copies of the P element dispersed throughout the genome, the transposase is repressed and so no further transposition occurs. However, if sperm from a P strain fertilizes an egg from a strain that does not contain a P element, the temporary absence of the repressor causes extensive transposition, resulting in a high rate of mutation.

The P element is also able to transpose into the genome from an injected piece of DNA. Therefore, if we insert a piece of foreign DNA into a P element contained on a plasmid vector, and then inject that construct into a fruit fly embryo, the P element will transpose into the genome, carrying our inserted DNA fragment with it. However, it is not easy to insert DNA into a P element without disrupting the transposase gene. Figure 14.5 shows how we can get round this problem. The transposase can act *in trans*, i.e. it can be expressed from a different piece of DNA. In the example shown, the foreign DNA fragment has replaced most of the P element genes, leaving the inverted repeat ends intact. The transposase is expressed from a second copy of the P element, and it will recognize the inverted repeats flanking the foreign DNA, resulting in transposition of the insert into the chromosome (with the IR ends). At the same time, we do not want the element with the intact transposase to be inserted as well, as it would cause additional mutations. So we remove the inverted repeat ends from the P element that has the transposase, rendering it non-mobile; this is referred to as a 'wings-clipped' element.

The main applications of this approach lie not in the expression of genes from other organisms in *Drosophila*, but in identifying or confirming the relationship between specific genes and identified phenotypes. As a simple

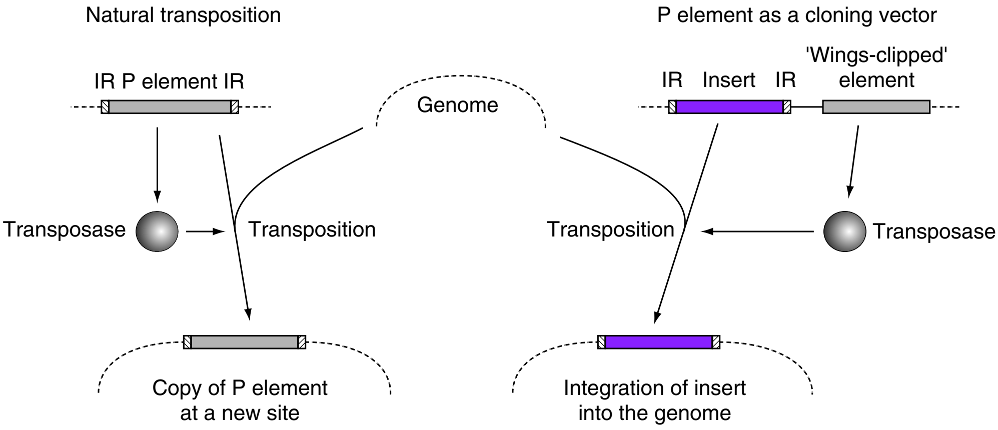

Figure 14.5 Transposition of P elements in *Drosophila*

example, Rosy$^-$ flies have brown eyes rather than red ones. Insertion of a DNA fragment coding for the enzyme xanthine dehydrogenase will restore the wild-type eye colour, thus confirming the function of the *rosy* gene; this is an example of *complementation* (see later in this chapter). Fruit flies have been used extensively as a model system, especially for research into differentiation and development of multicellular organisms, and the ability to link phenotypes with specific DNA sequences in this way has been an important component of these advances.

The applications of P elements do not end there. Insertion of a P element into the chromosome can cause a mutation, and since the affected gene is tagged with the transposon, it is readily identified, as in bacterial transposon mutagenesis described above. Another application involves a P element containing a reporter gene (such as the beta-galactosidase gene, *lacZ*) with a weak promoter. Random insertion of this element into the genome will occasionally result in integration adjacent to an enhancer element, resulting in activation of expression of the reporter gene. This technique, known as *enhancer trapping*, enables the identification of enhancers and their specific activity in certain cell types; related ways of using reporters to identify regulatory sequences, such as bacterial promoter-probe vectors, were described in Chapter 13.

These applications to fruit flies represent an example of *transgenics*, in that they include the manipulation not just of individual cells but of the whole organism. Further examples of transgenics, as applied to higher animals and plants, are discussed in Chapter 17.

14.5.2 Other applications of transposons

The description above of the use of P elements in *Drosophila* exemplifies how we can use the ability of transposons to insert more or less randomly within the chromosome in a variety of ways. In a bacterial system, as in *Drosophila*, we can use a transposon incorporating a reporter gene to identify genes that are expressed under a specific set of conditions. We can expose the transposon library to the environmental conditions in question, such as anaerobic growth, and identify those clones that show expression of the reporter gene. Alternatively, if we include an alkaline phosphatase gene as a reporter (Tn*pho* transposon) we can identify secreted proteins. This depends on the fact that alkaline phosphatase activity is only exhibited if the enzyme is secreted, and not if it remains in the cytoplasm. Insertion of Tn*pho* within a gene coding for a secreted protein can give rise to a fusion protein carrying the secretion signals from the native protein joined to the alkaline phosphatase. If this product is secreted, then phosphatase activity will be detected.

A form of transposon mutagenesis can also be used to identify genes that are necessary for the virulence of pathogenic bacteria. Insertion of the transposon into such a gene will attenuate the organism, i.e. it will destroy (or reduce) its virulence. This will be manifested by a reduced ability to grow or survive following administration to an experimental animal, or in some cases by a reduction in its ability to survive attack by macrophages in culture. It is not possible to select directly such a mutation, and testing the thousands of mutants in a transposon library is impractical.

However, we can modify the transposon by incorporating a highly variable sequence tag so that each copy of the transposon is uniquely identifiable (Figure 14.6). We then produce a transposon mutant library, with these tagged transposons, and infect mice with a pool of transposon mutants. Those clones in which the transposon has inserted into a gene that is essential for virulence will be unable to replicate in the mice and will therefore be absent when we recover the bacteria from the infected mice. We then use PCR to amplify all the tags that are present in the recovered bacteria, and label the collection of PCR products for use as a probe. Identification of the tags that are absent in this mixture is carried out by probing a membrane that contains a gridded array of each of the clones from the original transposon mutant library. Absence of

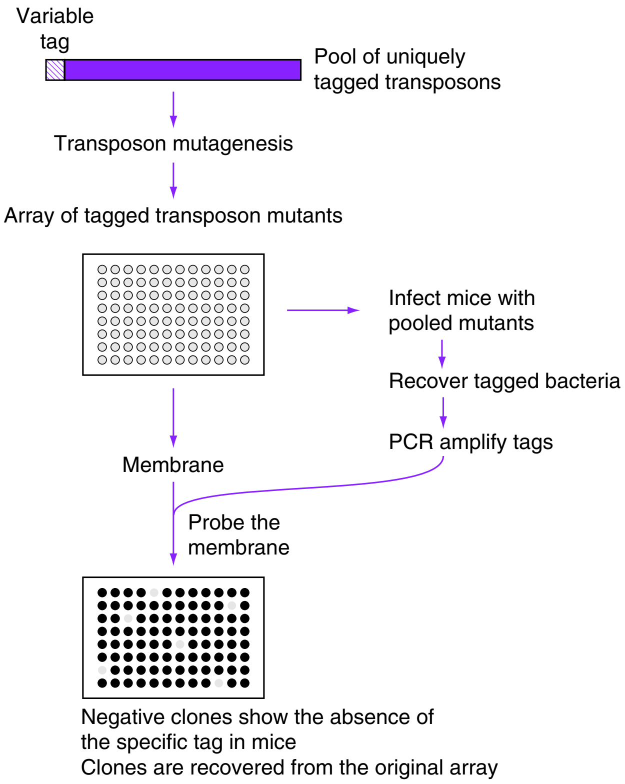

Figure 14.6 Signature tagged mutagenesis

hybridization means that the clone concerned was not present in the material recovered from the mice – and hence identifies this as a mutant in which an essential virulence gene has been inactivated by the transposon. The gene can then be recovered and identified as described above. This technique, known as signature tagged mutagenesis, has proved to be an extremely powerful tool for the identification of virulence genes – or, in principle, for the identification of any gene that is essential for the growth of the bacteria under defined conditions.

Transposons can also be used to facilitate the sequencing of a large insert. You can normally only obtain half a kb or so of high-quality sequence in each run. As discussed in Chapter 10, there are different ways around this problem. The most straightforward one is to make new, customized primers for walking down the gene. A recently developed method uses a donor vector that leaves a universal primer sequence embedded in a transposon, which will allow you to create easily a library of clones that can be sequenced for the rapid assembly of the full sequence of the insert.

14.6 Allelic Replacement and Gene Knock-outs

Transposon mutagenesis provides a useful method for identifying unknown genes that are connected with a selectable (or at least readily identifiable) phenotype. What of the reverse situation? That is, when we have a gene, and we know its sequence, but we do not know what its function is. One of the most powerful strategies in such a situation is known variously as *allelic replacement* or *gene replacement* or *gene knock-out*. This relies on the natural process of *homologous recombination*, which means that when there are two identical pieces of DNA in the cell, enzymes within the cell may break the two DNA chains, cross them over and rejoin them. (This is a highly simplistic version of a more complex process, but it will do for our purposes.) We can exploit this process to replace a specific gene (or part of a gene) in the chromosome with a version that we have inactivated *in vitro*, thus destroying the function of that gene. This allows us to test the consequences of inactivating an individual gene, and thus make deductions about its function.

A typical procedure for allelic replacement in a bacterial host would be to manipulate the cloned gene so as to replace the central part of the gene with an antibiotic resistance gene (see Figure 14.7). We would do this using a suicide plasmid (as described above, for transposon mutagenesis) so that when we transform the bacteria with the construct, only those cells in which the resistance gene has become incorporated into the chromosome will become antibiotic resistant. We can select these on agar containing the antibiotic. If things go well, incorporation into the chromosome will have occurred by homologous recombination at the required position, thus inactivating the gene concerned.

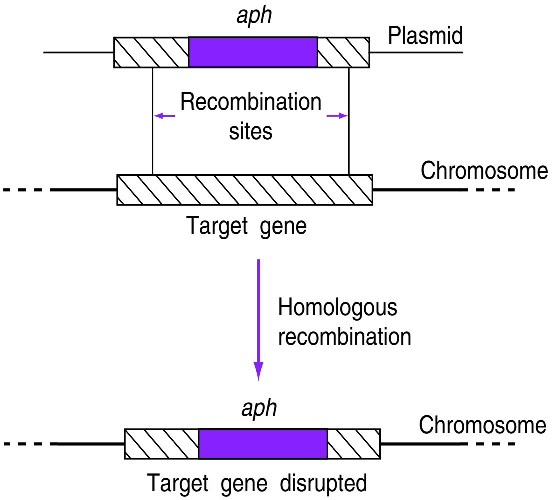

Figure 14.7 Gene disruption by allelic replacement: *aph* = aminoglycoside phosphotransferase, causing kanamycin resistance

It should be noted that replacement of the gene actually requires recombination at two positions, one either side of the gene. This is referred to as a double cross-over event. A single cross-over, in the homologous region at one side or the other of the construct, will produce resistant bacteria by incorporation of the entire plasmid into the chromosome rather than replacing the gene. Single cross-overs may result in gene inactivation, depending on the details of the construct, but will usually be unstable, as further recombination may eliminate the plasmid again, restoring the original intact gene. We can select against single cross-overs by incorporating a counter-selectable marker into the plasmid. In other words, we put a gene on the plasmid that will, if it is still present, confer a disadvantage on the cell. A gene known as *sacB* is commonly used for this purpose, as the presence of *sacB* renders the cells sensitive to sucrose in the medium. Plating the cells on a sucrose containing medium will result in any cells containing *sacB* (which includes the single cross-overs but not the doubles) being unable to grow and form colonies.

An obvious limitation of gene replacement technology is that the inactivation of the gene concerned may be a lethal event. Nevertheless, it is a valuable approach for identifying the function of specific genes.

Although we have described gene replacement in terms of identifying gene function, its applications extend far beyond that. It can be used to inactivate genes that are necessary for the virulence of a pathogenic bacterium, thus producing attenuated strains that may be useful vaccine candidates. In addition, essentially identical procedures can be used to knock out genes in other organisms, including experimental animals (especially mice). Many strains of mice, lacking individual genes, have been produced in this way, and are invaluable for research purposes. This is considered further in Chapter 17.

It should be noted also that the technique is not confined to the inactivation of the genes concerned. The technique can be readily adapted to the replacement of a gene by an altered version of the same gene, thus conferring novel properties on the organism.

14.7 Complementation

In the discussion of both transposon mutagenesis and allelic replacement, it has been implicitly assumed that the phenotypic consequences of the mutation are due solely to the effect of the loss of that gene. This is not always true. In particular, some mutations (especially those caused by insertion of a transposon) may show an effect known as *polarity*. This means that the mutation affects not only the altered gene, but also those adjacent to it. In bacteria, this can arise from the arrangement of genes into operons which are transcribed into a single mRNA. Mutation of one gene may interfere with transcription of the operon, and thus affect the expression of the genes downstream from it. Furthermore, genes and their products interact in many complex ways within the cell, so that disruption of one gene may have unexpected effects on the activity of other genes and their products.

It is therefore necessary to interpret carefully the results arising from these experiments. The standard way of checking that the altered phenotype is a direct consequence of the inactivation of a specific gene is by *complementation*. This involves introducing into the mutant cell a fully active version of the affected gene (usually a cloned version on a plasmid). If the alteration in the phenotype is indeed due solely to the loss of the affected gene and its product, then the mutation will be complemented by the plasmid, i.e. the wild-type phenotype will be restored. This is not entirely foolproof. For example, if the original mutation disrupts the regulation of other genes, complementation may be successful in restoring the wild-type phenotype, even though the gene product is not directly responsible for the observed characteristics. Nevertheless, complementation does provide an element of confirmation of the consequences of the original mutation.

14.8 Studying Gene Function through Protein Interactions

Genes do not, of course, ‘function’ at the DNA level. Some genes function solely by producing specific RNA molecules (such as rRNA, tRNA). Most, however, exert their function by the production of protein. Thus, to a large extent, studying gene function means studying protein function. Most of the methods for this fall outside the scope of this book. However, we will cover two methods which are frequently used both to identify genes and to characterize their function.

14.8.1 Two-hybrid screening

One important clue as to the function of a protein is its ability to interact with other proteins. Two-hybrid screening is a commonly used method to identify such interactions; since this method is normally used with yeast cells as a host organism, it is therefore often called *yeast two-hybrid screening* (although versions are now available for bacterial and mammalian cells as well).

The basis of this procedure is similar to yeast one-hybrid screening, as described in Chapter 13, in that it depends on the modular nature of transcriptional activators such as GAL4; the activator domain (AD) does not have to be covalently attached to the DNA-binding domain (BD). Provided the activator domain can interact with a protein that is bound to DNA adjacent to a reporter gene, transcriptional activation will occur.

The basis of the technique is illustrated in Figure 14.8. As implied by the name, two recombinant plasmids have to be constructed. The first is made so that one of the proteins that we wish to study (the *bait*) is expressed as a fusion protein with a specific DNA-binding domain, while the second plasmid expresses another protein (the *prey*) as a fusion with an activator domain. The host is an engineered yeast strain that contains, inserted into the genome,

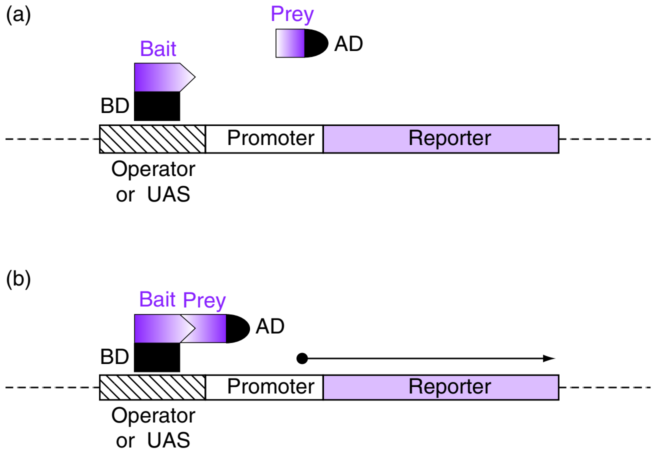

Figure 14.8 Yeast two-hybrid system: (a) non-interacting proteins, no expression of reporter; (b) interacting proteins, expression of reporter

a reporter gene with a specific operator or upstream activator sequence to which the BD domain will bind. Expression of the reporter gene will only occur if the two fusion proteins interact.

The system can be set up to test the interaction between the products of two specific cloned genes. Alternatively, using a library of DNA fragments in the prey vector, it is possible to identify those proteins that are able to interact with a specific bait.

Although yeast two-hybrid assays originally employed GAL4 AD and BD domains, it is apparent from the description that, since there is no direct physical interaction between them, either or both can be replaced by domains from other proteins. Indeed there are advantages in using heterologous proteins, that is proteins that are not derived from yeast. For example, in some systems the BD domain is derived from the bacterial LexA protein, with the LexA operator attached to the reporter gene.

14.8.2 Phage display libraries

In this method, DNA fragments are inserted into a cloning site within the gene coding for one of the proteins that make up the surface of a filamentous phage such as M13 (see Chapter 6). The fused gene gives rise to a hybrid protein which (we hope) will be incorporated into the phage particle in such a way as to *display* the foreign protein or peptide on the surface of the bacteriophage particles that result from infection of host bacteria. (Figure 14.9). A phage display library can be created either by cloning DNA fragments that code for actual proteins or parts thereof, or by inserting synthetic oligonucleotides

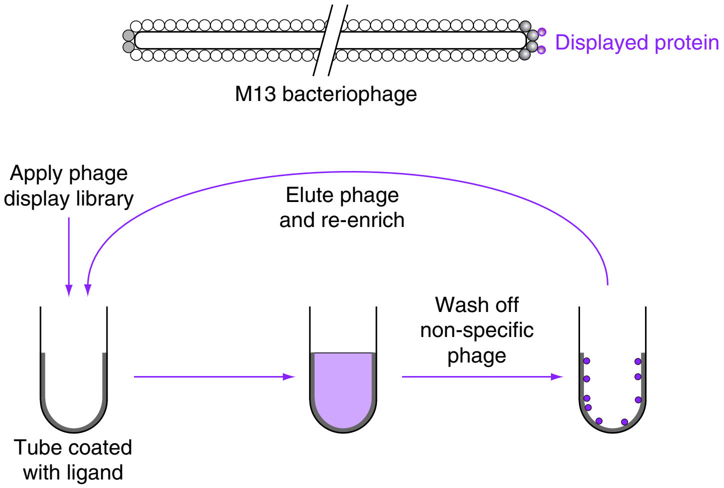

Figure 14.9 Phage display library

designed to give rise to a representative collection of random peptides. The phage particles displaying proteins or peptides with the required properties can be recovered by adsorption to a tube or a well in a microtitre tray coated with the appropriate ligand, such as an antibody, a hormone receptor, or another protein. The non-specific phage are then washed off and the retained phage, enriched for the specific recombinant, are then eluted; further rounds of enrichment can be carried out. The power of this technique lies in the ability to test very large numbers of phage particles – M13 preparations can contain 10^{12} phage particles per ml – and to carry out repeated rounds of enrichment for the phage displaying proteins or peptide with the properties that you want. Compare this with screening a gene library by hybridization or by antibody screening.

Phage display libraries therefore can be used to screen very efficiently for specific proteins or peptides, normally smaller molecules with pharmacological, enzymic, or antigenic properties. Identification of a peptide sequence that binds to the ligand can be of direct use either in itself (such as in the search for a therapeutic agent), or can serve to provide clues about the partial sequence of a longer ligand-binding protein.

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15

Manipulating Gene Expression

We use proteins, or smaller polypeptides, in many ways – ranging from enzymes that can be added to washing powders to hormones that are used for treating medical conditions. Some of these proteins can be extracted easily, although at some cost, from starting material that is readily available, e.g. plant material, or microbial cultures. However, the potential source of such proteins is often scarce or difficult to obtain which severely limits the application of this approach – or may even make it impossible. This applies especially to proteins and polypeptides from human sources, which can be invaluable for treating specific diseases. The classic example here is of human growth hormone (somatotropin), which is used for treating a condition known as pituitary dwarfism, where a child's growth is affected by a deficiency in the production of this hormone by the pituitary gland. For a long time, the only source of this hormone was from pituitary glands removed from the bodies of people who had died from a variety of other causes. The limitations on supply can be imagined; the safety implications, in terms of the potential transmission of disease by this route, were only partly appreciated at the time. Other consequences were only realized more recently when it was discovered that spongiform encephalopathies (CJD) could be transmitted by this material.

Many bacteria and other microorganisms (especially yeasts) can be grown easily and cheaply in essentially unlimited quantity. The expression of the gene coding for the required protein in a microbial host can therefore enable us to obtain that product in large amounts – especially as we can manipulate the gene to maximize its expression. Making a therapeutic product in this way also removes the possibility of transmitting infectious agents, so it is much safer than the 'natural' product. It should also be emphasized that (subject to any post-translational modifications which will be discussed later in this chapter) the purified product obtained from recombinant bacteria is exactly the same as the pure product from its original source. The only way in which the product differs is that it is likely to be purer and will be free from infectious agents. (You may have heard of some difficulties with the use of recombinant human insulin, but these are largely a consequence of the greater purity of the product, rather than an inherent problem of the recombinant product.)

The realization that the new genetic technologies would provide a way of making products that had hitherto been expensive or impossible to produce was a major driving force behind the commercial side of the biotechnology revolution. The research implications are even more dramatic. Some proteins, especially those responsible for regulating cellular activities and for communication between cells, are produced in tiny amounts. You may only be able to detect their presence by extremely sensitive assay methods. It is likely to be effectively impossible to extract and purify such a protein in sufficient quantity to be able to characterize its structure or to analyse the way it works. Yet in principle, as long as you can identify and clone the gene responsible, it will be possible to express that gene in a microbial host and obtain substantial quantities of the product.

15.1 Factors Affecting Expression of Cloned Genes

Expressing a foreign gene in a bacterial cell is not entirely straightforward. The expression signals that control transcription and translation can be quite different from one organism to another, especially if you are moving a gene from a eukaryotic source into a bacterial host. Expression can also be affected by the base composition of the gene, and by the codon usage. These factors were covered in Chapter 2, but are summarized here for ease of reference (see Figure 15.1). We are generally assuming to start with that the host organism is a bacterium; the expression of foreign genes in eukaryotic cells is considered later on.

(*i*) *Transcription*. The main factor is the promoter site, i.e. the region where the RNA polymerase binds to initiate RNA synthesis (Figure 15.2). This is the principal step where bacteria control which DNA regions are to be expressed and how strong that expression should be. The structure of the RNA polymerase (and especially of the sigma factors that determine the specificity) and the sequences of the promoter regions have evolved together to produce this carefully regulated expression. It is therefore not surprising that a gene from one species may not be expressed when inserted into a different host.

Transcription signals in eukaryotes are generally substantially different from bacterial promoters, but that is usually irrelevant. For gene expression you will usually require cDNA rather than genomic DNA (to remove introns), so the region upstream of the transcription start point will not be included in the cloned fragment. You have to provide a promoter, using an *expression vector*. This concept was introduced in Chapter 6 and is considered again later in this chapter.

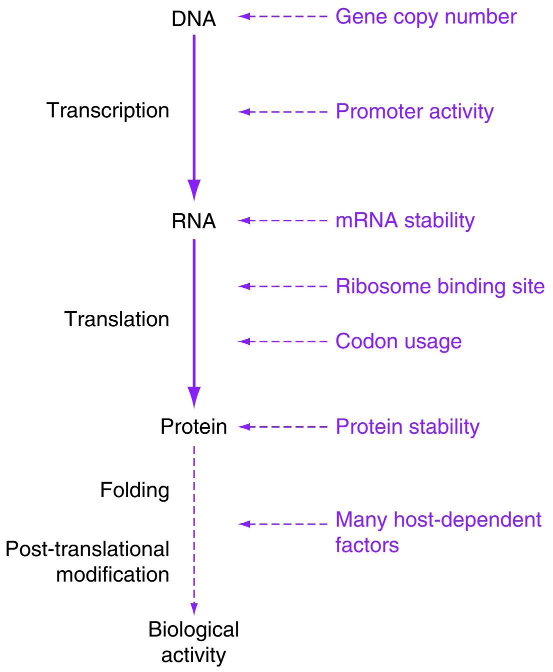

Figure 15.1 Gene expression

Transcription can also be affected by the base composition of the DNA. If the cloned DNA comes from an organism with a higher G+C content than that in the new host, the stronger association of the two strands may hinder transcription, which requires local strand separation and unwinding of the helix.

It should also be borne in mind that in principle it is the *amount* of mRNA available for translation that is important rather than the rate with which it is produced, and that the level of a specific mRNA is determined by its stability as well as by its rate of synthesis. However, in bacteria, the mRNA is typically very short-lived (with a half-life measured in minutes), and translation normally starts as soon as a ribosome-binding site (see below) is available. Differences in stability between mRNAs are therefore less important in bacteria than in eukaryotes. Furthermore, it is easier to manipulate the level of transcription than to alter the stability of a message, so we would generally focus on the former.

(*ii*) *Translation initiation*. In bacteria, ribosomes normally bind to a region of the mRNA adjacent to the start codon, facilitated by a sequence (the Shine–Dalgarno sequence) that is (partially) complementary to the 3′ end of the 16S rRNA (Figure 15.2). This structure is reasonably well conserved in bacteria, so you could expect to get some degree of translation initiation if you move a gene from one bacterium to another (assuming transcription occurs). However, the structure may not be optimal; even within the same organism, genes may not have an optimum ribosome binding site, for example the distance between the

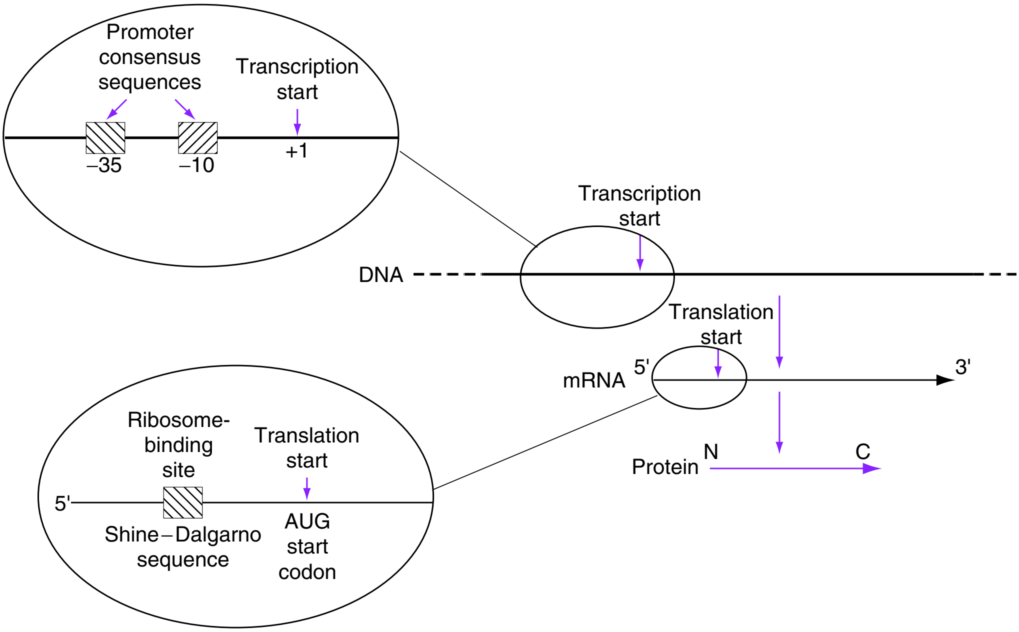

Figure 15.2 Principal factors in bacterial gene expression

Shine–Dalgarno sequence and the start codon can vary, which can affect the efficacy of translation initiation. So you may not get maximum gene expression unless you manipulate this region.

Ribosome binding in eukaryotes works in a different way. Thus, if you choose a bacterial host for expression of eukaryotic genes, you will usually have to provide translational signals as well as a promoter.

The nature of the start codon may also have to be considered. In the standard genetic code, AUG is used to signal the start of translation. However, in some organisms (especially those with a high G+C content), a proportion of genes (sometimes the majority) use alternative start codons such as GUG, UUG or CUG. Trying to express such a gene in an organism with a stricter preference for an AUG start may limit or prevent translation, or give a product with a different N-terminus.

(*iii*) *Codon usage*. In the standard genetic code there are many sets of *synonymous codons*, i.e. several codons that code for the same amino acid. For example, any one of six different codons in the mRNA will result in incorporation of leucine into the polypeptide (see Chapter 8, Box 8.1). These different codons are not completely equivalent. The cell will use some codons more readily than others, depending on the availability and specificity of tRNA molecules that recognize these codons. Any occurrence of the less readily used codons will slow down translation. The sequence of the genes in that organism will therefore have evolved to match the availability of tRNAs, and some synonymous codons will occur more frequently than their synonyms – which we refer to as *codon bias*. In a different organism, the tRNA population is different, and the codon usage will also be different.

In nature, codon bias is much more pronounced for highly expressed genes, such as those coding for enzymes of the central metabolic pathways. There is more evolutionary pressure favouring the optimization of codon usage of such genes than there is for genes coding for proteins that are produced at a lower level. This is reflected in the behaviour of cloned genes. If we take a gene from one organism and try to express it in another, with different codon usage, we will probably get some expression (assuming everything else is working) but the extent of that expression is likely to be limited. Expression may be enhanced by altering the sequence of the gene to improve the codon usage for that host.

In extreme cases, you may actually fail to get expression at all. The host organism may be virtually unable to recognize certain codons in your cloned gene (and these would be absent from natural genes in that host); in effect, these are acting as additional stop codons, and will cause premature termination of protein synthesis. This does not happen in *E. coli*, which is capable of recognizing (to varying extents) all the codons in the standard code. However, some other hosts have a more pronounced bias in their codon usage, with some codons occurring extremely rarely.

The converse can occur too. In some organisms, a codon that is a stop signal in the standard code is actually used by that organism to code for a specific amino acid. For example, in some species UGA codes for tryptophan rather than being a stop codon. If you try to express such a gene in *E. coli*, you are likely to get premature termination at the UGA codon. It is possible to overcome this by using *E. coli* host strains that have been engineered to produce additional types of tRNA.

(*iv*) *G+C content*. Organisms vary very substantially in the base composition of their DNA (ranging, approximately, from 30 per cent G+C to 70 per cent G+C). So the composition of the cloned gene may be substantially different from that of the new host. We have alluded to the possible effect this might have on transcription; it is also likely to be reflected in differences in codon usage. There is a wide range of other effects which could occur, which might affect not only the expression of the gene but also other properties of the recombinant. These include the structure and stability of the mRNA, supercoiling of DNA, and replication and stability of the recombinant vector itself.

(*v*) *Nature of the protein product*. We also have to remember that the nature of the protein product may influence the amount of protein that is recovered. Most obviously (as with mRNA) the stability of the protein can have a major effect: an inherently unstable protein will not be recovered in high yield, even if we have maximized the rate of production. Amongst other factors, the location of the product can have an important influence of the levels obtained. If the protein remains in the cytoplasm, it may become insoluble at high rates of synthesis, leading to the production of *inclusion bodies*, or aggregates of insoluble product. It can be difficult to recover active soluble protein from such inclusion bodies. If the protein becomes inserted into the cytoplasmic membrane, it is likely to have a deleterious effect on the functions of the membrane; expression of such proteins may be lethal, even at quite low levels.

For high levels of protein production, it is often advantageous to express the product in a form that can be secreted into the culture medium, e.g. by attaching *signal sequences*. Since the volume of the supernatant is very much larger than the combined volume of the cytoplasm of all the cells in a culture, even in a very dense culture, problems of product insolubility are very much less likely to arise. Unfortunately, attaching secretion signals may not work. Proteins that are not naturally secreted products may not pass properly through the cytoplasmic membrane, even when directed to do so by a secretion signal.

We also need to bear in mind that our aim is usually the production of a biologically active protein, rather than a simple polypeptide chain. Biological activity often depends on a variety of post-translational effects, ranging from folding of the polypeptide into the correct three-dimensional structure, to post-translational cleavage or modifications such as glycosylation. This can be a difficult barrier to surmount when attempting to express a protein in a foreign host, and is often a major reason for using a non-bacterial host for expression of eukaryotic proteins.

15.2 Expression of Cloned Genes in Bacteria

15.2.1 Transcriptional fusions

When attempting to express a foreign gene in a bacterial host, the first parameter to be considered is the requirement for a fully functional promoter attached to the cloned gene. In principle, this can be addressed by simply adding in a known, characterized promoter in a separate cloning step. A more convenient procedure is to use a ready-prepared vector (usually a plasmid) that already carries a suitable promoter adjacent to the cloning site. This forms a simple type of *expression vector*, as already introduced in Chapter 6 (see Figure 15.3). Insertion of the cloned fragment at the cloning site (in the correct orientation) will then put that gene under the control of the promoter carried by the vector. In other words, transcription initiated at the promoter site will continue through the cloned gene; since this means a fusion of the gene and the promoter into a single transcriptional unit, it is referred to as a *transcriptional fusion* (cf. translational fusions, below). This is very similar to the concept of a reporter gene (Chapter 13), but for a different purpose. A reporter gene is used

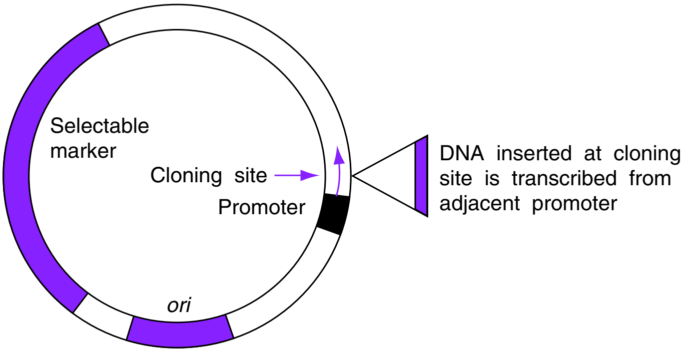

Figure 15.3 Basic features of an expression vector

Box 15.1 Comparison of selected E.coli promoters

Promoter	**–35 region**	**–10 region**
consensus	TTGACA	TATAAT
lac	TT*T*ACA	TATA*T*T
trp	TTGACA	T*TA*A*C*T
P_L	TTGACA	*G*ATA*C*T

to study the activity of the promoter. Here we know what the promoter does, and we are using it to stimulate transcription of the cloned gene.

In *E. coli* there are a wide range of suitable promoters that can be used in this way. Amongst naturally occurring promoters, the *lac* and *trp* promoters that normally drive expression of the *lac* (lactose utilization) and *trp* (tryptophan synthesis) operons respectively, and the P_L promoter from bacteriophage lambda are examples of commonly used promoters, the P_L promoter being one of the strongest natural promoters in *E. coli* (or its associated genetic elements).

However, none of these three natural promoters actually represents a perfect match with the consensus sequence obtained by comparing the sequence of a large number of *E. coli* promoters (see Box 15.1)

The –10 and –35 regions are not the sole determinants of the strength of a promoter; a region of about 70 base pairs makes contact with the RNA polymerase and influences the strength of that contact. Nevertheless, these two regions (and the distance separating them) are the most highly conserved and play the major role. Since none of these sequences is a perfect match with the consensus, we can infer that further manipulation of the sequence could produce even stronger promoters, and this is in fact the case. One artificial promoter that is commonly used is known as the *tac* promoter, since it represents a hybrid between the *trp* and *lac* promoters, and it is capable of higher levels of transcription than either of the original natural promoters.

Not all promoters conform to the consensus shown. In particular, some bacteriophage RNA polymerases (notable those from T7, T3 and SP6) have a virtually absolute requirement for a sequence of bases, specific to that enzyme. These polymerases will not initiate transcription from the usual promoters, nor will *E. coli* RNA polymerase recognize one of these special promoters. We met these enzymes in the discussion of cloning vectors (Chapter 6); they can be very useful in ensuring that we only get expression of our cloned gene when we need it, and not before. The ways in which we can control the expression of our cloned gene are considered later.

In addition to the choice of promoter in our expression vector, we need also to consider the plasmid copy number. Most routine cloning vectors for use in *E. coli* are referred to as 'multi-copy' plasmids, but this can conceal a wide variation in the actual copy number. The early cloning vectors (such as pBR322) are normally present in 15–20 copies per cell (although under special conditions they can be amplified to up to 1000 copies per cell). Subsequent development of these vectors removed some control elements, so that many of the currently used vectors (such as the pUC series) have hundreds of copies per cell. More copies of the plasmid means more copies of the cloned gene, and hence more product formation. However, the relationship may not be linear; you will expect to get more product from a 200-copy plasmid than from one with only 20 copies, but not necessarily ten times as much (and indeed excessive amounts of product may kill the cells that produce it).

So if you want to maximize gene expression, not only should you optimize the promoter, but you should use a high copy number plasmid. Under these conditions (assuming translation works perfectly), you may get a bacterial clone in which your product represents up to 50 per cent of the total protein of the cell. For commercial production, such high yields not only mean more product per litre of culture (which is of course important) but in addition the proportion of contaminating protein (and other material) that has to be removed is lower, thus reducing the costs of downstream processing

15.2.2 Stability: conditional expression

There is a downside to such high levels of product formation. Producing vast quantities of a protein that is of no use to the cell is inevitably going to result in a reduction in growth rate, because of the amount of resources that are being diverted in a manner that is non-productive, from the cell's perspective. This applies even if the protein itself has no damaging effects . Slower growth rates will reduce the efficiency of the process. If the protein is directly damaging, the problem becomes much more acute.

It is, however, much worse than that. The slower growth of the producing cells means that there is a very strong selective pressure in favour of any of a wide range of potential mutants that are non-productive. This includes cells that have lost the plasmid altogether as well as any mutation to the plasmid that reduces or prevents product formation. This can be a problem whether you are growing a few millilitres in the laboratory or many thousands of litres in an industrial fermenter. However, on a laboratory scale it is possible to include antibiotic selection in your culture, to ensure that any mutants that have lost the plasmid will be unable to grow. On a large scale this is not only an expensive solution, but also the disposal of large volumes of antibiotic-containing waste is a problem.

One way of ameliorating this situation is to use controllable promoters, i.e. promoters whose activity can be altered by changes in the culture conditions. The promoters listed above provide examples of such control. For example the *lac* promoter is naturally expressed only if *E. coli* is growing on lactose as a carbon and energy source. (Normally bacterial geneticists use IPTG instead of lactose, as it is both more convenient and is not broken down by the cell's β-galactosidase; it is known as a *gratuitous inducer*). In the absence of an inducer, a repressor protein binds to a DNA sequence known as the *operator* (which in this case overlaps with the *lac* promoter) and prevents transcription from the *lac* promoter. The inducing agent binds to the repressor protein, altering its conformation so that it no longer binds to the operator (Figure 15.4). We can grow the culture to an appropriate density in the absence of an inducer, so that we will not get high levels of gene expression and hence the selective pressure will not exist. Then, when we have enough cells, we add the inducing agent and switch on gene expression.

However, we have to remember that we are using a multi-copy vector. *E. coli* produces enough of the repressor protein to switch off the single copy of the promoter that it has in the chromosome, but this is not enough to switch off several hundred copies of this promoter. We refer to the repressor being *titrated out* by the presence of so many copies of the promoter. So we have also to increase production of the repressor protein. The gene that codes for the repressor (the *lacI* gene) is not actually part of the *lac* operon; it has its own promoter. So one way of increasing production of the LacI repressor protein is by using a mutated version of the *lacI* gene which has a more active promoter (an *up-promoter* mutant). This altered *lacI* gene is known as *lacI*^{q}. Alternatively, we can put the *lacI* gene onto the plasmid itself, so subjecting it to the same gene dosage effect and therefore increasing the production of LacI. Commonly, we would do both, i.e. put a *lacI*^{q} gene onto the plasmid.

Adding IPTG to a laboratory culture is fine, but on a commercial scale it is not an ideal solution. Adding IPTG to an industrial scale fermenter would be

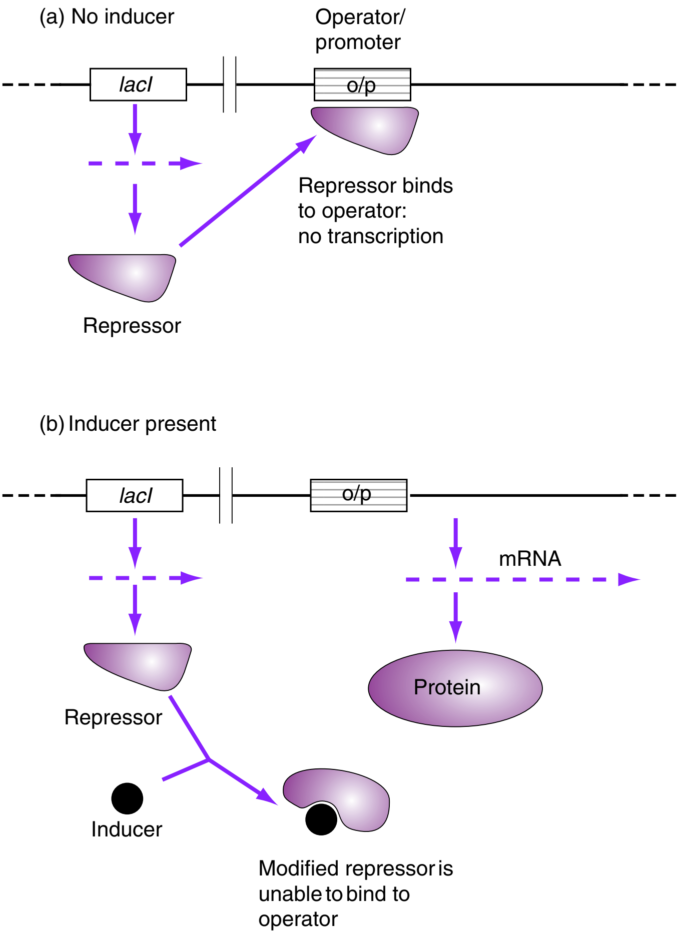

Figure 15.4 Regulation of the *lac* promoter

very expensive as well as creating other problems (including the disposal of the waste material).

An alternative strategy would involve the use of a promoter such as the *trp* promoter, which controls transcription of the tryptophan operon. This is subject to repression by tryptophan; *E. coli* switches off expression of the tryptophan operon when the enzymes encoded by it are not needed, i.e. if there is a plentiful supply of tryptophan. It is possible to monitor and control the availability of tryptophan so that there is an adequate supply during the growth phase, and then limit the supply of tryptophan when expression is required. You may be puzzled by this. If we stop supplying tryptophan, how is the cell going to make the protein that we want, which will probably contain some tryptophan residues? However, it is possible to supply a low level of tryptophan which is not enough to switch off the *trp* promoter but will enable production of the required protein. And we can then feed the culture continuously with a low level of tryptophan so that protein production will continue.

This may solve the problem in part, by removing the selective pressure imposed by excessive product formation; but there is still some element of selection imposed by the presence of so many copies of the plasmid, which may also slow growth rates. We can counter this by using a plasmid with a different replication origin, so that replication is tightly controlled at only one or two copies per cell (see Chapter 6). However, the level of expression achieved with a low copy vector will be less than that achievable with a multi-copy plasmid, other things being equal. We can adopt a similar strategy to that described above for programming gene expression, using a so-called *runaway plasmid*. If the control of plasmid copy number is temperature sensitive, then growing the culture initially at say 30°C will produce cells with only a few copies of the plasmid. Then, once sufficient growth has been achieved, the culture can be shifted to a higher temperature, say 37°C; control of plasmid replication is lost and the copy number increases dramatically, until it represents perhaps 50 per cent of the DNA of the cell. If we switch on gene expression at the same time, we will get a very substantial amount of product. Eventually the cells will die, but by that time we have enough of our product.

An alternative way of achieving a similar effect is by providing the vector with two origins of replication: one that results in many copies of the plasmid, and a second that will produce only one or two copies per cell. If we can control which of the two origins is used, we can again switch conditions at an appropriate stage, so that the culture starts off with only a few copies of the plasmid per cell and then when we want to switch on gene expression we can, as well as inducing the promoter, switch to the other replication origin and increase the copy number of the plasmid. (We do not need to consider here the details of how that is done.)

15.2.3 Expression of lethal genes

Some genes code for products that are very damaging or even lethal to a bacterium such as *E. coli*. Expression of such genes in *E. coli* poses obvious problems. You might consider that the above approach would be equally applicable in such a situation, but it is not quite that simple. The regulation of many promoters is not tight enough. The *lac* promoter, for example, although only showing full activity in the presence of an inducing agent, is nevertheless active at a lower level even in the absence of induction. If the gene product is *very* damaging, the cell will not be able to tolerate even this low level of expression. Other promoters with tighter control, such as the T7 promoter referred to earlier (see also Chapter 6), have to be used.

Even in the absence of a known promoter, there may be enough transcription to cause a lethal effect, since a wide variety of DNA sequences have an artefactual low level of promoter activity. This is especially a problem with multi-copy vectors because of the gene dosage effect, and can be reduced by using tightly controlled low copy vectors. Alternatively, obtaining high levels of production of a protein that is highly damaging to *E. coli* may require the use of an alternative host.

15.2.4 Translational fusions

If we want to provide our gene with translational signals (ribosome binding site and start codon) as well as a promoter, we can use a similar approach. For this purpose we need a different type of expression vector: one that will give rise to a *translational fusion*. In this case, part of the translation product (the protein or polypeptide) is derived from the insert and part from the vector (Figure 15.5). When using a translational fusion vector, we have to be much more careful in the design of our construct.

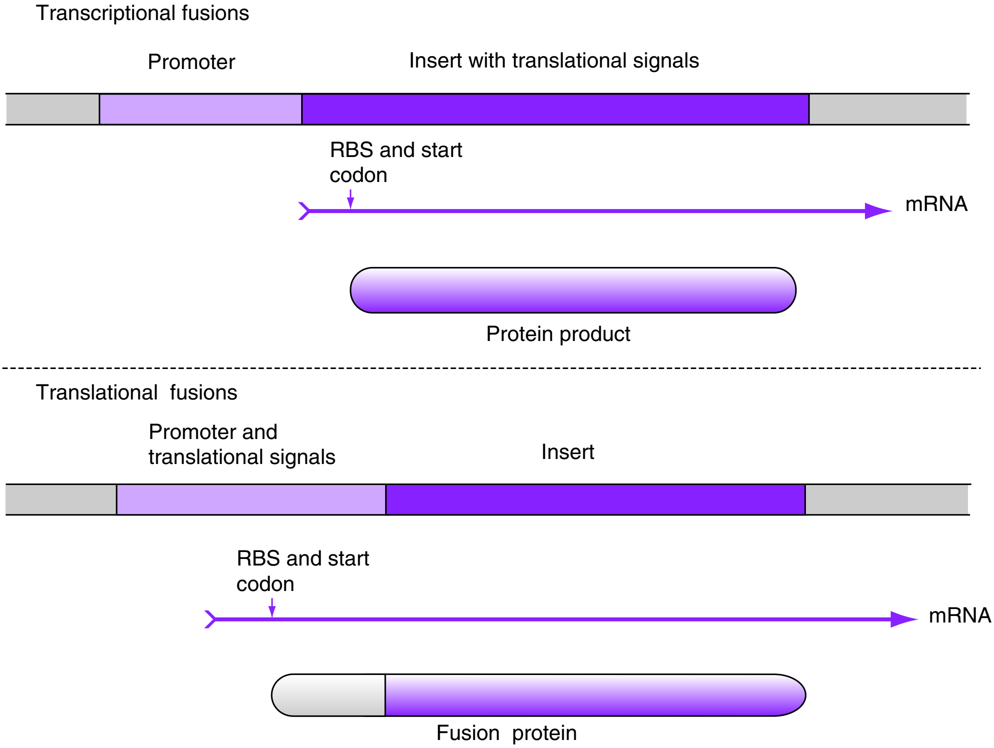

Figure 15.5 Transcriptional and translational fusions

With a transcriptional fusion vector, as described above, we simply have to make sure the insert is the right way round. Within reason, it does not matter too much where it is – some untranslated leader mRNA can be tolerated, so it does not have to be precisely located with respect to the promoter. However, with a translational fusion, the location is all important. Since translation starts at the initiation codon in the vector sequence and the ribosomes then read the sequence in triplets, you have to make sure that the correct reading frame is maintained at the junction. One or two bases out in either direction, and your insert will be read in the wrong frame, giving rise to a completely different amino acid sequence (and probably resulting in premature termination as the ribosomes will soon come across a stop codon in this frame – see Figure 15.6).

You can only achieve this if you know the sequence of your insert, and the sequence of the vector, or at least of that region of the vector between the cloning site and the start codon. (Contrast this with a transcriptional fusion vector, where it is quite possible to insert an uncharacterized DNA fragment into the vector and be successful in obtaining expression.) Furthermore you have to work out exactly what will happen at the cloning site when you cut the insert and the vector and then join them together. If you are relying on a restriction site that is naturally present in the insert, there is only a one in three chance that it will be in the right frame when joined to the vector, even assuming the insert goes in the right way round. You might need to alter your choice of vector, or to modify either the vector or the insert (e.g. by using linkers or adaptors). Alternatively, you can use PCR to generate the insert fragment,

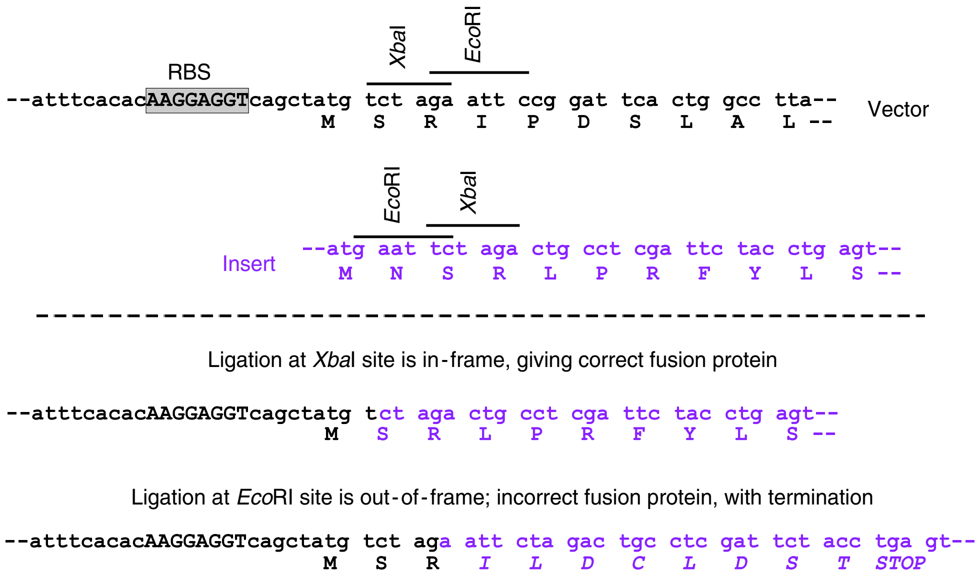

Figure 15.6 Translational fusion: in-frame and out-of-frame fusions

in which case you must design the primer to contain a restriction site that will produce the correct reading frame (see Chapter 9).

Finally, having done all this, it is essential to check, as always, by sequencing the recombinant product, that ligation has indeed resulted in the construct that you have designed, and that the reading frame is actually correct. Loss of a single base during ligation will result in an incorrect reading frame.

In theory, it is possible to use a vector which just has the ribosome binding site and no start codon, and to use the natural start codon of the cloned gene. However, in *E. coli* the optimum distance between the Shine–Dalgarno sequence and the start codon is only five to seven bases, so there is not a lot of room for manoeuvre. More commonly the vector will have the start codon as well, and the insert will not have a start codon. In this case, the translational fusion product will have a few amino acids derived from the vector sequence, and will lack some of the N-terminal amino acids that are normally found in this polypeptide. For many purposes, this is of little significance, as proteins can often tolerate a considerable amount of variation in the N-terminal sequence. In some cases it may be important to have a product that is precisely the same as the naturally occurring one – for example if you are making a protein for human therapeutic purposes. You will then need to carry out more precise manipulations rather than using off the shelf expression vectors; for example, you can ensure that the vector-derived amino acids replace exactly those that are lost from the insert.

Sometimes, it is useful to be able to add a short sequence of amino acids to the N-terminus of your product. We will consider the applications of such *tagged proteins* later in this chapter.

15.3 Expression in Eukaryotic Host Cells

Although bacteria are convenient hosts for many purposes, and *E. coli* is usually the host of choice for initial gene cloning (including the production of primary gene libraries), bacterial systems have many limitations for the *expression* of cloned genes, especially for large-scale production of proteins of eukaryotic origin. In particular, the post-translational modifications needed for conversion of the primary product of translation into the mature, folded, biologically active product are more likely to occur in a eukaryotic host. There is a wide variety of systems available; in this chapter we will consider just a few examples. Many of the concepts discussed in relation to bacterial gene expression are also relevant to expression in eukaryotic cells, even if the details are different.

15.3.1 Yeast expression systems

Yeasts have many advantages for expression of cloned genes. They grow rapidly, in simple defined media, and as unicellular organisms they are relatively easy to manipulate and enumerate. *Saccharomyces cerevisiae* has been used as an experimental organism in microbial genetics for many years and there is now a wealth of biochemical and genetic information available to support its use for gene cloning – including the genome sequence. Powerful and versatile systems are also available for several other yeast species, notably *Pichia pastoris*.

Saccharomyces cerevisiae

Vectors for cloning and expression of genes in the yeast *S. cerevisiae* were introduced in Chapter 6. In many respects, especially if using episomally replicating vectors, the concepts are similar to those involved in the design of plasmid-based expression vectors in bacteria. There is a choice between vectors carrying the 2μm origin of replication, which are maintained episomally at high copy number (up to 40 copies per cell) , and centromere vectors which are maintained at low copy number (one to two copies per cell) (see Chapter 6 for more detail). Both types of vectors are designed as shuttle vectors, that is they also carry an *E. coli* replication origin, which enables the initial construction and verification of the recombinant plasmid to be carried out in *E. coli* before transferring the finished construct into yeast cells. As with bacterial expression vectors, these *S. cerevisiae* vectors are designed with a controllable promoter adjacent to the cloning site to enable expression of the cloned gene to be switched on or off. Most commonly this involves the promoter and enhancer sequences from the *GAL1* (galactokinase) gene, which is strongly induced by the addition of galactose.

Pichia pastoris

Pichia pastoris is able to use methanol as a carbon source, the first step in the pathway being due to the enzyme alcohol oxidase (the product of the *AOX1* gene). This gene is tightly controlled, so that in the absence of methanol no alcohol oxidase is detectable. On addition of methanol to the culture, the *AOX1* gene is expressed at a very high level. The use of the *AOX1* promoter in the expression vectors, adjacent to the cloning site, therefore provides vectors that are capable of generating substantial levels (up to several grams per litre) of the required product.

Unlike the bacterial systems, the vectors in *P. pastoris* are not maintained episomally, but are designed to be integrated into the yeast chromosome. They do, however, contain an *E. coli* replication origin, so that they are maintained as plasmids in *E. coli*, enabling formation and verification of the recombinant plasmids in *E. coli* before transformation of *Pichia*.

Not only are the expression levels in *Pichia* usually higher than those obtainable in other systems (prokaryotic or eukaryotic), but scaling up to industrial levels of production is relatively straightforward, and the organism grows readily in simple defined media, leading to lower costs than those involved in insect or mammalian systems (as described below).

15.3.2 Expression in insect cells: baculovirus systems

Baculoviruses, such as the *Autographa california* nuclear polyhedrosis virus (AcMNPV), infect insect cells and are exploited as the basis of systems for gene expression in such cells. During normal infection, large amounts of a virus-encoded protein called polyhedrin are produced which forms a matrix within which the virus particles are embedded. The high level of production of polyhedrin is due to a very strong promoter, which can be used to drive expression of the cloned gene, by inserting it downstream from the polyhedrin promoter. Production of polyhedrin itself is not completely essential for virus production, but in its absence the virus does not produce inclusions; the resultant plaques can be distinguished from those produced by wild-type virus. However, the viral DNA is itself too large (> 100kb) for direct manipulation to be carried out easily. The gene to be expressed is therefore first inserted into the polyhedrin gene in a smaller *transfer vector*. Once again, the transfer vector is a shuttle vector, i.e. it contains an *E. coli* origin of replication and other functions that allow manipulation in an *E. coli* host. For production of recombinant virus particles, the transfer vector must recombine with viral DNA. For this to happen, insect cells are cotransfected with the recombinant transfer vector and with viral DNA. Recombination within the insect cells leads to the production of recombinant viruses. These are separated from the wild-type, non-recombinant, viruses by picking the plaques that are characteristic of polyhedrin-deficient viruses.

More efficient versions of this system are now available. In the example shown in Figure 15.7, the transfer vector contains a multiple cloning site adjacent to (and downstream from) the polyhedrin promoter so that the cloned gene will be transcribed from that promoter. Flanking this region, on either side, are two DNA regions derived from the virus that allow homologous recombination with viral DNA. For the other component of the system, instead of intact viral DNA, a linearized DNA is used. The linearized viral DNA lacks a portion of an essential gene, thus eliminating the production of

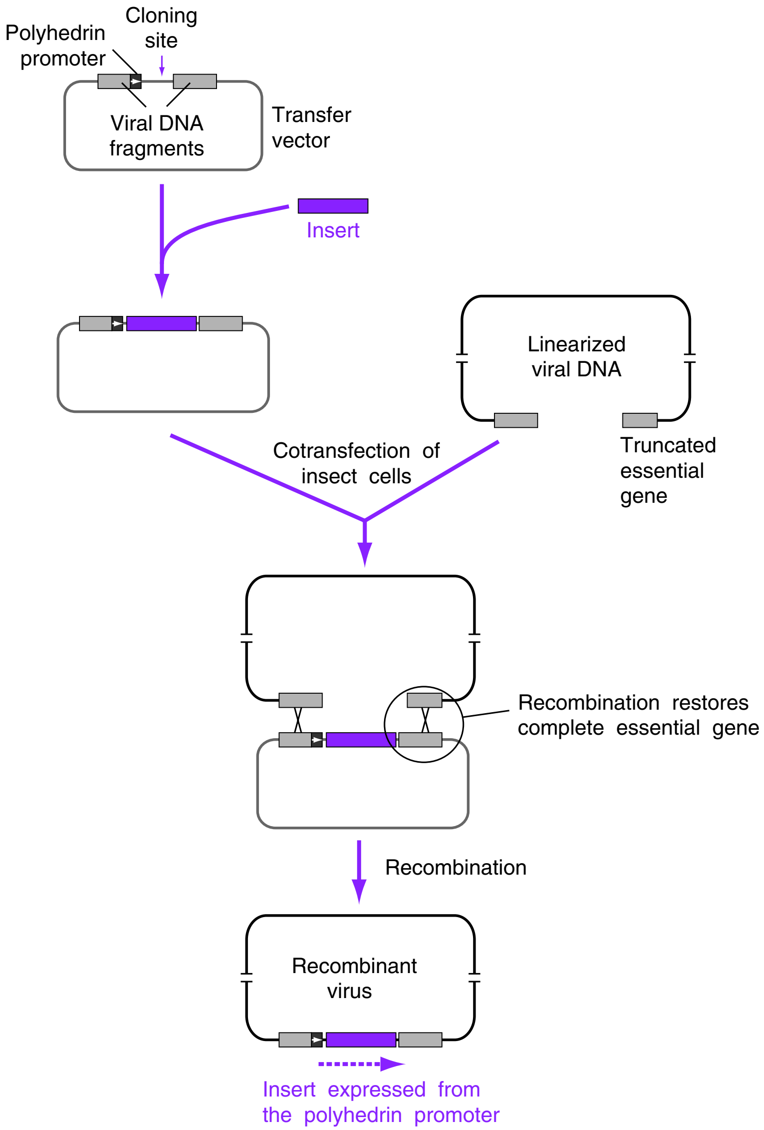

Figure 15.7 Cloning using a baculovirus vector

non-recombinant viruses. Recombination with the transfer vector, after cotransfection of insect cells, restores this essential gene. Additional features can be built in to this system, including the incorporation of a marker such as β-galactosidase into the transfer vector so that blue recombinant plaques can be readily identified.

Following transfection, viral plaques can be picked and the virus characterized to verify the presence of the cloned gene. The characterized recombinant virus can then be used to infect a large-scale culture of insect cells; high levels of expressed protein are usually obtained before the cells lyse. The levels of production are generally lower than those obtainable with *Pichia* expression systems, but insect cells are claimed to provide post-translational modification that is more closely similar to that in mammalian cells. On the other hand, the yeast cells are much easier and cheaper to grow.

15.3.3 Expression in mammalian cells

There is an enormous variety of expression vectors and systems for use with mammalian cells, and a full treatment of this field is way beyond the scope of this book. Although the details are more complex than the systems described so far, the general principles remain familiar.

Most vectors use the enhancer/promoters from the human cytomegalovirus (CMV), the SV40 virus, or the herpes simplex virus thymidine kinase (HS-TK) to drive transcription. These give high level, constitutive, expression. As in prokaryotic systems, it is sometimes desirable to control the onset of expression. One way of achieving such regulation is by interposing the operator sequence (*tetO*) from the bacterial tetracycline resistance operon between the promoter and the cloned gene. If the mammalian cells are cotransfected with a second plasmid containing the tetracycline repressor gene (*tetR*), also expressed using the CMV promoter, the TetR protein will bind to the *tetO* site, thus preventing transcription. When tetracycline is added to the culture medium it will bind to the TetR protein, altering its conformation and releasing it from the DNA, thus derepressing transcription of the cloned gene. The advantage of this is that, because this system is of prokaryotic origin, its activation does not affect the induction of native mammalian genes. Similarly, an insect ecdysone-responsive element is sometimes used to induce gene expression in mammalian cells. Like the tetracycline system, ecdysone does not have any effect on native mammalian genes, and the induction is therefore specific to the genes placed under its control.

Additional sequences can be added to enable targeting of the product to specific cellular locations such as the nucleus, mitochondria, endoplasmic reticulum or cytoplasm, or secretion into the culture medium.

In contrast to bacterial cells, the introduction of DNA into mammalian cells does not depend on the independent replication of the vector; the introduced DNA can be stably integrated into the nuclear DNA. However, some expression vectors can be stably maintained at high copy extrachromosomally, such as those containing the origin of replication from the Epstein Barr Virus (EBV); with a suitable promoter system these are capable of allowing high levels of protein expression. The use of retroviral vectors for integrating foreign genes in mammalian genomes, and for obtaining gene expression, was considered in Chapter 6; for the application of these techniques in whole animals, see Chapter 17.

The advantage of using mammalian cells for expression of eukaryotic genes, especially those from mammalian sources, rather than the other systems described, lies in the greater likelihood of a functional product being obtained. This is especially relevant for studies of structure–function relationships and the physiological effect of the protein on cell function. However, the relative difficulty, and cost, of scaling up production, compared with either *Pichia* or baculovirus systems, makes mammalian cells less attractive if the objective is the large-scale production of recombinant proteins for other uses.

15.4 Adding Tags and Signals

15.4.1 Tagged proteins

The fact that a translational fusion vector (see above) can add a short stretch of amino acids to the N-terminal end of your polypeptide product can be turned to advantage. Purification of the recombinant product by conventional means can often be tedious and inefficient. However, if the vector contains not just a start codon but also a short sequence coding for a few amino acids (known as a *tag*), the resulting fusion protein will carry this tag at the N-terminus. For example, the tag may constitute a recognition site (an *epitope*) that can be recognized by a monoclonal antibody (see Chapter 8, Box 8.2). You can then recover the protein from the cell extract in a single step by affinity purification, using the ability of the monoclonal antibody to bind to the epitope tag (Figure 15.8). A further application of this type of vector is that you can use this specific monoclonal antibody to detect the expression of your product, if you have no other reasonably convenient assay. There is a variety of such tags (and corresponding monoclonal antibodies) available, and they can be added to either end of your insert (N- or C-termini), using modified PCR primers, rather than by incorporation into the vector.

An alternative to epitope tagging is provided by designing the expression vector (or manipulating the insert) so that a sequence coding for a number of histidine residues is added to one end of the sequence to be expressed. This will produce a His-tagged protein which can be purified using a Ni^{2+} resin. The affinity of the histidine residues for nickel will result in the tagged protein being retained by the resin, while other proteins will be washed through. The His-tag can also be used as an epitope tag, since there are antibodies available that will specifically recognize this sequence of histidine residues, which facilitates detection of the tagged product.

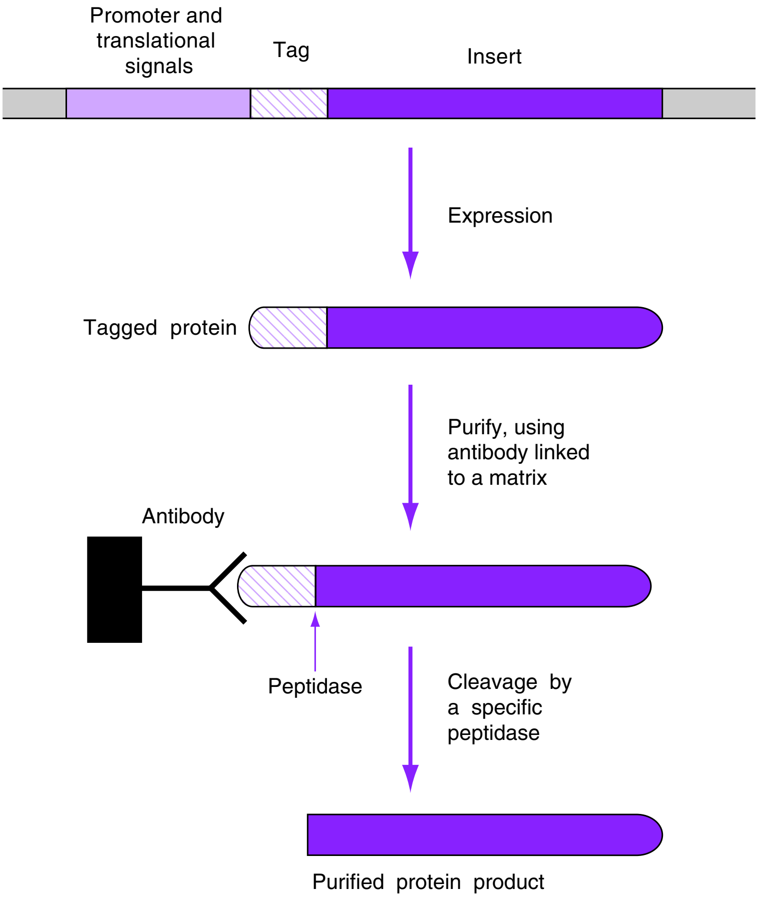

Figure 15.8 Tagged proteins

Of course you may not want to have a tag permanently attached to your product. For this reason, vectors can be designed to contain not only the tag but also a site at which the product can be cleaved by highly specific peptidases. If the peptidase is sufficiently specific it will cleave the product only at this site and not elsewhere. Therefore you can affinity purify your product, cleave it with the peptidase, and separate the protein from the cleaved tag (see Figure 15.8).

15.4.2 Secretion signals

In a similar way to the incorporation of tags into the product, we can add secretion signals. *E. coli* does not secrete many proteins into the culture supernatant – most of the proteins secreted across the cytoplasmic membrane remain in the periplasm, trapped by the outer membrane. In some other bacteria, particularly Gram positive bacteria such as *Bacillus subtilis*, many enzymes are secreted into the culture supernatant. This can be advantageous for several reasons. First, if a protein is synthesized at high levels and accumulated within the cytoplasm, it will result in a very high concentration of that protein. This can cause aggregation of the protein into insoluble *inclusion bodies*. These may be damaging to the cell, and are often very difficult to resolubilize. Secretion of the protein into the culture medium will prevent this, since the volume of the supernatant is very much greater than that of the total volume of the cytoplasm of all the cells. Even with a very dense culture, the space occupied by the cells is only a small proportion of the culture volume. Furthermore, although *Bacillus* secretes a number of enzymes, the culture supernatant will contain a much simpler mixture of proteins than the cell cytoplasm. The task of purifying the required product is therefore much simpler.

Secretion of proteins by bacteria depends normally on the presence of a *signal peptide* at the N-terminus. This labels it as a secreted protein, so it is recognized by the secretion machinery and transported across the cytoplasmic membrane. Therefore if we incorporate a sequence coding for a signal peptide into our vector (or into the insert) the final product may be secreted. It is not inevitable, as it also depends on the overall structure of the protein. If the protein is naturally secreted in its original host, then we may be successful. If it is normally a cytoplasmic protein, the chances of getting it secreted successfully are very much lower.

As described above, in mammalian systems, signals can be added to direct the product to specific cellular locations, or to obtain a secreted product.

15.5 *In vitro* Mutagenesis

In the early part of this chapter we described how codon usage may affect optimization of gene expression in *E. coli*, and in other hosts may prevent expression altogether. What can we do about it?

If we can identify a limited number of rarely-used codons in our cloned gene, which are likely to cause a reduction in gene expression, then we can use site-directed mutagenesis (see below) to alter the sequence at these specific positions, so as to change a rare codon into a more appropriate one – still maintaining the same coding properties so we are not altering the nature of the final product.

If there are a large number of such rare codons, then changing them one by one would be extremely laborious. Similarly, if we decide that the base composition of the sequence is causing problems, it would be excessively tedious to use site-directed mutagenesis to change the whole thing. In these situations we could use synthetic techniques to remake the complete gene – either by direct DNA synthesis or by PCR-based methods such as assembly PCR. All these techniques are described in the subsequent sections. Their potential applications extend far beyond adjusting codon usage, into a field known as *protein engineering*.

15.5.1 Site-directed mutagenesis

This refers to the specific alteration of either a single base or a short sequence of bases in a cloned gene. If there is a unique restriction site within your cloned DNA fragment, at or near the point where you want to make a change, then there are several simple possibilities, including the use of exonucleases to make a short deletion, and/or the insertion of a short synthetic oligonucleotide (Figure 15.9). Although these methods are limited in their scope they can be useful, for example in the removal or addition of restriction sites within a DNA fragment. In addition, the deletion procedure will remove various lengths of DNA, which enables you to identify the limits of regions that have specific functions, such as the ability to bind regulatory proteins (see Chapter 13). Those deletions which extend into the regulatory region will produce clones showing altered regulation of the gene concerned.

The fundamental limitation is the requirement for a unique restriction site at the appropriate place. More generally applicable techniques involve the use of

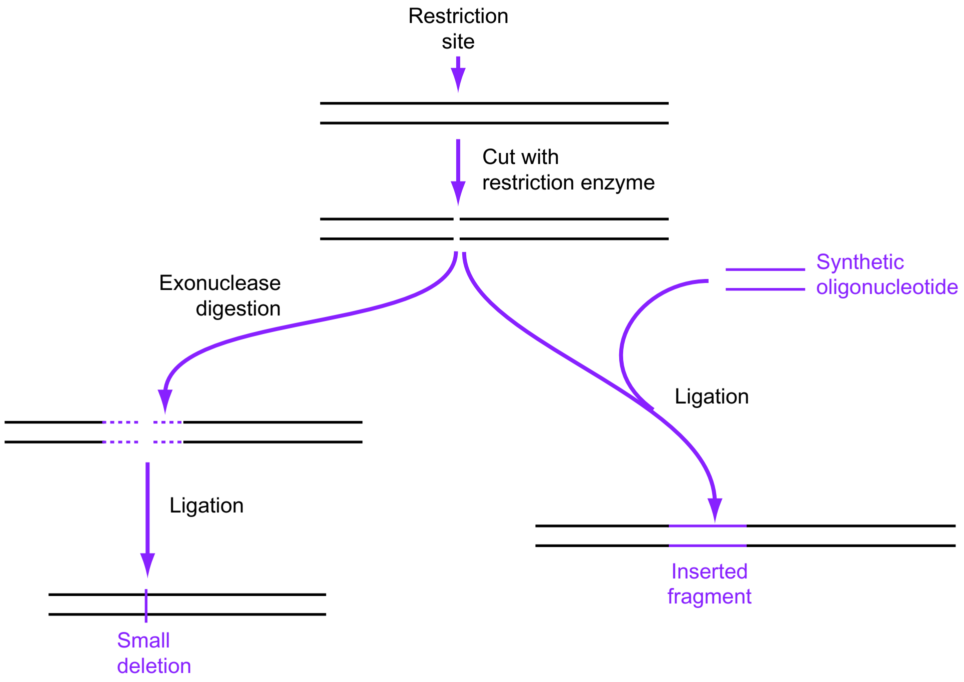

Figure 15.9 *In vitro* mutagenesis: examples of some simple procedures

oligonucleotide primers to introduce changes in the DNA sequence. There are a number of variations in the technique, so we will just describe the principle of the method.

Fundamentally, it relies on the ability of single-strand DNA molecules to anneal to one another even if their sequences do not match perfectly. Therefore, if we design an oligonucleotide that contains the sequence we require, which is only slightly different from the natural sequence, it will anneal to the complementary strand at the corresponding position. This provides a primer that can be used by DNA polymerase. If we start with a single-stranded circular DNA molecule (such as can be readily obtained using an M13 vector, see Chapter 6), then DNA polymerase will synthesize the complementary strand right round the circle, producing a double-stranded molecule (Figure 15.10). DNA ligase

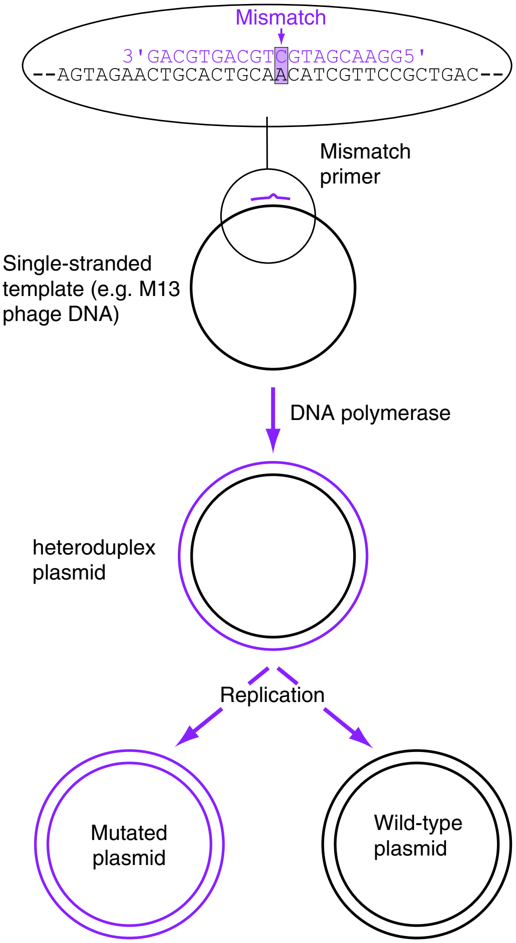

Figure 15.10 Site-directed (*in vitro*) mutagenesis

can then seal the nick to produce a completely closed, double-stranded, circular molecule in which one strand contains the natural sequence and the second strand carries the required mutation (a heteroduplex DNA). When you transform *E. coli* with this, subsequent replication will produce some double-stranded circular DNA molecules with the wild-type sequence, and some with the mutation. If this is done with an M13 vector, the result will be a mixture of mutant and non-mutant phages.

This is not a very efficient system. Even if everything goes perfectly, the best you can expect is that 50 per cent of the clones will carry the mutation. In practice the proportion of mutant clones is usually considerably lower than this, for a variety of reasons. Predominantly, we have to take account of the mismatch repair system in the host cells. The repair of mismatched DNA occurs preferentially on the new (*in vitro* generated) strand, which is unmethylated, rather than on the old, (*in vivo* generated) strand, which is methylated. There are ways around some of these problems, including the use of host strains that are defective in mismatch repair, and other procedures that selectively eliminate the parental DNA strand.

There are also a variety of PCR-based methods, which are more efficient. A simple PCR reaction can be used if the site of the desired mutation is near enough to the end of the gene so that a primer containing the mismatch can be used. Usually, however, the site for the mutation will be too far from the end for this simple approach to be useful. In that case, you have to use a more complex method. The simplest, conceptually if not practically, is to start with two PCR reactions that produce products which overlap in the region of the mutation; in Figure 15.11, primer B, used in PCR reaction 1, is complementary to primer C used in the second PCR. Both primers contain the required alteration in the sequence (but on opposite strands). If you mix the products of the two reactions, denature and re-anneal, some of the single strands from reaction 1 will anneal to strands from reaction 2 in the region where they overlap, corresponding to the sequences of primers B and C. (Of course, there will also be re-annealing of the products of PCR 1, and similarly of the products of PCR 2; these are not shown in the figure, and will not be amplified by the subsequent PCR.) One of the two possible hybrid molecules contains 3′ ends which can act as a primer for extension by DNA polymerase to produce a complete double-stranded molecule containing the mutation (on both strands). The other type of hybrid DNA cannot be so extended, as the overlap region has 5′ ends. A subsequent PCR, using the outermost primers (A and D) will amplify only the full-length product as shown. This can then be cloned in order to test the consequences of the mutation.

The early applications of PCR for site-directed mutagenesis suffered from the risk of introducing unwanted mutations into the product, due to the absence of proof-reading ability of *Taq* polymerase. The introduction of alternative heat-stable polymerases which do have proof-reading ability, and hence

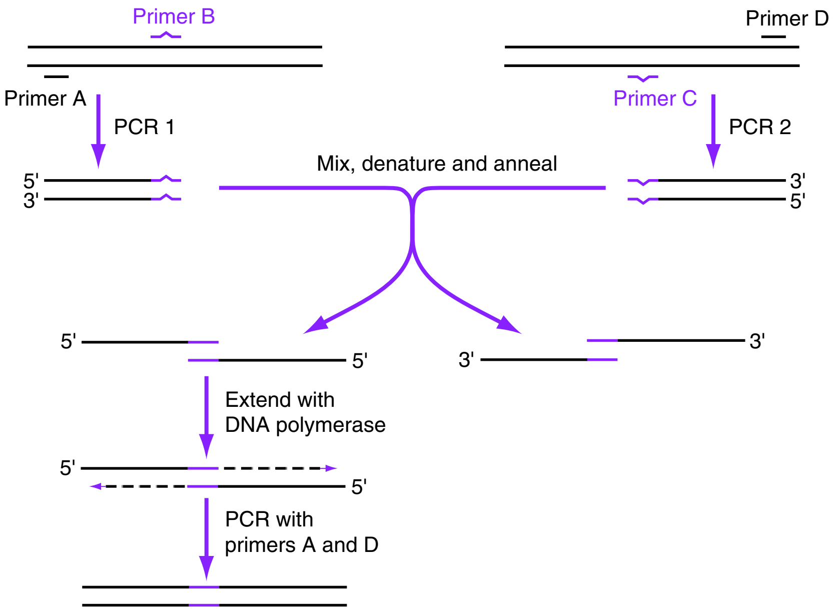

Figure 15.11 PCR-based mutagenesis

show greater fidelity, has greatly enhanced the application of PCR for this purpose. Nevertheless, as always, it is important to sequence the product to ensure first that the required mutation has indeed been achieved, and second that no other alterations have been introduced into the sequence.

15.5.2 Synthetic genes

As indicated previously, although it is possible to use site-directed mutagenesis to introduce a number of changes into the sequence of a gene, it becomes a rather laborious procedure if we want to make extensive changes – such as altering the codon usage throughout. One possibility then is to abandon the natural gene and simply synthesize a fresh one from scratch. We would take the amino acid sequence of the protein and use an optimal set of codons to *back translate* it (in a computer, not on the bench) into a nucleic acid sequence with optimized codon usage (and/or optimized base composition). It is actually rather more complicated than that, as you would want to check that you are not introducing other undesirable features such as secondary structures that might interfere with transcription; you also would want to consider the introduction (or omission) of restriction endonuclease sites at strategic points.

In principle, you would program a DNA synthesizer to make the DNA sequence which could then be cloned. Here again, it is not quite that simple.

DNA synthesizers work by adding bases sequentially, one at a time, to the growing oligonucleotide through a series of chemical reactions and washing steps. With each base added, there is a very small but finite possibility of the failure of the reaction, the main consequence of which is a gradual reduction in the yield of the product. Synthesis of oligonucleotides of 30–50 bases long is very reliable, and longer sequences can be made, but the practical limit is not much more than 100 bases. One way round this is to make the gene in shorter fragments and ligate them, bearing in mind that the synthesizer produces single-stranded DNA, so you need to make two complementary strands for each fragment. This would clearly involve a lot of work, but if you really need to do it, it is quite possible.

15.5.3 Assembly PCR

An alternative procedure, which is a lot simpler in practice than that described above, is simply to mix together a complete set of synthetic fragments (each of which overlaps, and is complementary to, its neighbours on either side). As before, these fragments are designed to optimize codon usage and base composition, at the same time avoiding sequences that would lead to structures such as hairpin formation. This complex mixture is then subjected to a number of rounds of PCR, which results in, amongst other products, some full-length DNA. This is further amplified, specifically, by addition of primers directed at the ends of the full-length gene, and further rounds of PCR. Although the synthesis of a complete gene of say 1000 base pairs involves a highly complex mixture of starting oligonucleotides, which might be expected to produce an impossible number of different products, the procedure can be surprisingly successful. We have successfully used it to produce a 300 bp fragment with radically different codon usage and base composition for expressing a gene from the malaria parasite *Plasmodium* (30 per cent G+C) in a mycobacterial host (65 per cent G+C).

15.5.4 Protein engineering

The above discussion refers to the use of site-directed mutagenesis and synthetic DNA as a means of altering the base composition and/or codon usage of a naturally occurring gene, as an aid to expressing that gene. The product is still the same as the naturally occurring one. However, there is no reason why we should limit these techniques to the production of naturally occurring proteins. We can just as easily synthesize a gene which codes for an altered protein. We could introduce specific changes into the sequence and test their effects on, for example, the substrate specificity of the enzyme. Or we could introduce cysteine residues into the sequence at strategic points so that the protein produced would contain additional disulphide bridges. This would be expected to increase the thermal stability of the enzyme, which could be advantageous. (Unfortunately such a change often results in the loss of enzyme activity as well.)

This concept, often referred to as *protein engineering*, would in principle culminate in the production of totally novel enzymes, tailor-made to carry out specific enzyme reactions. The limiting factor now is not the techniques for producing such engineered proteins, but the inadequacy of our knowledge of how a specific sequence of amino acids will fold into a three-dimensional structure, and how we can predict the enzymic activity of such a novel protein.

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16

Medical Applications, Present and Future

Genetic technology has many applications relevant to human medicine. Some of these have already been considered to some extent: detection and differentiation of microbial pathogens, and research and diagnosis of human genetic diseases, for example. In this chapter, we want to consider a few topics in more detail.

16.1 Vaccines

One example of the application of gene technology is in the development of new vaccines. Until recently, all vaccines were one of three types.

(1) *Killed vaccines* (such as the Salk polio vaccine), in which a suspension of the pathogen was inactivated, for example by heating it, so as to retain its immunogenicity but rendering it incapable of causing disease. Influenza vaccines are also of this type.

(2) *Live attenuated vaccines*, which use a mutant strain of the pathogen which is attenuated (i.e. it has lost the ability to cause disease) but is still immunogenic. Many familiar vaccines are of this type, including vaccinia (smallpox vaccine), oral polio vaccine (Sabin), and BCG (an attenuated strain of *Mycobacterium bovis*, used for protection against tuberculosis).

(3) *Toxoids*. Some bacterial diseases are due to the ability of the bacterium to produce a specific toxin; immunity to the toxin gives protection against the disease. If you can inactivate the toxin (e.g. by chemical treatment) while retaining its immunogenicity, the resulting *toxoid* will be an effective vaccine. The principal examples here are diphtheria and tetanus vaccines.

To these three types we can add a fourth, even without resorting to gene technology. Some killed vaccines, although giving good protection, also have undesirable side effects. If we can separate the components of the vaccine that are needed for protection from those that cause the side effects, we will have an equally effective, but much safer, vaccine. For example, the old pertussis vaccine consisted of a killed suspension of *Bordetella pertussis*, the bacterium that causes whooping cough. From this complex mixture, it is possible to purify the components that are needed for the protective effect, producing a safer vaccine. A vaccine of this type is known as a *subunit vaccine*.

Gene technology can contribute to the development of new vaccines by introducing more convenient and more versatile ways of producing vaccines of the above types, e.g. by new ways of attenuating virulent strains, or by producing subunit vaccines using cloned genes. In addition, genetic manipulation allows the development of totally new types of vaccines, for example by expressing relevant genes from one or more pathogens in an avirulent host strain, or in a more radical departure, by constructing recombinant DNA molecules that can be used as a vaccine in their own right, without using a host organism to carry them. This chapter will consider these developments, as summarized in Figure 16.1, although it should be noted that there are other technologies that also contribute to the development of novel vaccines, such as the use of synthetic peptides, and conjugate vaccines (in which a polysaccharide antigen is linked to a protein carrier); since these technologies do not involve genetic manipulation they are outside the scope of this book.

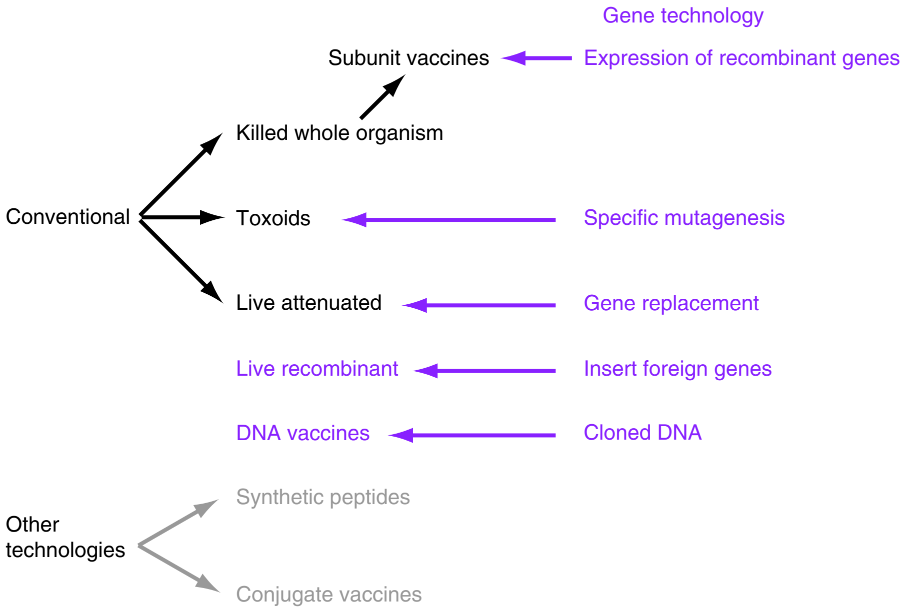

Figure 16.1 Vaccines

16.1.1 Subunit vaccines

The concept behind the use of genetic manipulation to produce a recombinant subunit vaccine is simple. If you can identify the key (protein) antigen from the pathogen that is needed for a protective effect, you can clone the gene responsible using an expression vector and produce large quantities of the protein. This has many advantages over producing the same component from the pathogen itself: it is cheaper and more effective, since you may get much higher levels of expression; it is easier to produce a safe vaccine, as there is no problem with contamination with any other damaging components of the vaccine; and the production process is safer, since you do not have to grow large-scale cultures of a dangerous pathogen.

The best example, illustrating the power of this approach (as well as some of the problems), is the development of a vaccine against the hepatitis B virus, which causes a serious form of hepatitis and is transmitted sexually, as well as by blood products and by direct inoculation (e.g. using contaminated syringe needles). Unfortunately, this virus cannot be grown in cell culture, which makes the development of a conventional vaccine next to impossible. (Vaccines have been produced using viral particles purified from the blood of human carriers of the virus, but supplies are limited and the risk of transmitting other diseases is obvious). However, we know that one virus protein, the surface antigen (HbSAg, see Figure 16.2) is able to confer protection, so the expression of this gene in a suitable host should yield a product that can be used as a vaccine. However, when this gene was expressed in *E. coli*, it was found that the product did not give effective protection. The problem here is that the product from *E. coli*, although having the authentic amino acid sequence, does not adopt the correct conformation. However, it was found that the expression of this gene in the yeast *Saccharomyces cerevisiae* did give rise to HbSAg with a natural conformation, and this product was immunogenic and protective – and this is the basis of the currently used hepatitis B vaccine.

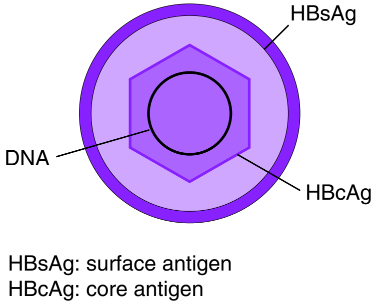

Figure 16.2 Hepatits B virus: diagrammatic structure

We can also use genetic manipulation as an alternative to chemical treatment of toxins as a way of producing toxoids for immunization. For example, the cholera toxin consists of two types of subunit: the A component is responsible for the toxic effects after it gets into the target cells, while the B component is needed for attachment to cell surface receptors. The B component by itself is non toxic. Expression of the B component alone will give a product that can cause an immune response without the toxicity of the intact toxin.

We can also manipulate the product so as to cause an immune response to specific regions of the antigen. For certain viruses, immunization with the whole virus will produce a response that is directed mainly towards certain exposed regions of the major antigens – they are said to be *immunodominant*. However, these are often the very regions that are most variable; evolution has ensured that this happens. Other regions of the antigen may be more conserved, but we do not usually make a good immune response to those regions, because they are hidden. If we use gene cloning to express just the conserved region of the antigen, we may be able to force an immune response against the conserved regions of the antigen rather than the hypervariable ones – thus potentially establishing a vaccine that would protect against a wider range of strains.

16.1.2 Live attenuated vaccines

One of the limitations of non-living vaccines, including both conventional killed vaccines and subunit vaccines, is that the protective effect on initial immunization can be relatively poor and short-lived. Boosters are commonly needed, but even so the protection is usually inferior to that conferred by a live vaccine.

The conventional route for development of a live attenuated vaccine involves repeated laboratory subculture, especially under non-favourable conditions. For example, a virus may be repeatedly cultured using a cell line that is a poor host for the wild virus, or a bacterium may be repeatedly grown using a medium in which the pathogen grows very weakly. The TB vaccine BCG was developed by repeated subculture on potato slices soaked in glycerol and ox bile over some 20 years. The principle is that as the pathogen adapts to these unusual conditions it will at the same time lose the ability to infect human or animal hosts. The only way of testing this is to examine the virulence of the organism at intervals – usually by challenge of an experimental animal model. If there is no suitable animal model, virulence tests are difficult.

Although this empirical procedure has been successful in producing several widely used vaccines, it is rather hit and miss. Many mutations are likely to accumulate, apart from those that give rise to the desired attenuation, so there is a good deal of uncertainty in the nature of the resulting strain. For a virus, it is now relatively straightforward to determine the sequence of the candidate vaccine – and even for the much larger genome of a bacterium, determining the genome sequence of the potential vaccine, and comparing it with the original sequence of the pathogen, is becoming a routine possibility. However, it is still difficult to know which of these mutations is associated with loss of virulence.

It can be important to understand the nature of the loss of virulence. If it is due to just a single mutation, and especially if that is a simple point mutation (i.e. a change in the base sequence at a single position), then it is quite possible for that mutation to revert. In other words, a further mutation at that site may restore the original sequence of the gene, and the strain is no longer attenuated. This can happen, for example, with the live polio vaccine. The vaccine itself is safe, but the attenuated virus persists for some time in the body (which of course contributes to its success in stimulating a good immune response). During that time, there is continued opportunity for it to revert to a virulent form. This does not do any direct damage, as the person 'infected' is protected by the immunity generated by vaccination. However, vaccinated individuals shed, for a time, virus into the environment, and if it has reverted to a virulent form, any non-immunized individuals who come into contact with this revertant are at risk of contracting polio. As the polio eradication campaign becomes increasingly successful, and the natural occurrence of the virus diminishes, reversion of the vaccine strain becomes a potentially significant cause of the continuation of this disease.

It is also necessary to recognize the possibility that the strain will not only have lost virulence, but also may have lost the ability to induce protection. The structure of the key antigens may have changed for example. Furthermore, it is possible for a strain to become too attenuated. The most effective protection is achieved by a virus or bacterium that is able to undergo at least a limited degree of replication in the tissues before being eliminated by the host defences. If the strain is over-attenuated, the body will eliminate it before it has a chance to evoke a full immune response.

With the classical empirical procedure, it is difficult to control these factors. You have to produce many different variants and test each one for both properties: the loss of virulence, and the ability to produce protective immunity. Genetic technology provides rational ways of achieving these goals in a controlled manner. With some of the smaller viruses, it is possible to use site-directed mutagenesis (Chapter 15) to modify selected genes with the goal of attenuating the virus. With larger viruses, this is usually not possible directly, but indirect techniques can be used to introduce defined mutations. However, unless you have a thorough knowledge of the structure of the virus, most of your mutations are likely to destroy the viability of the virus altogether, rather than just removing its virulence.

The larger genome of a bacterium provides more targets, and potentially many that would eliminate virulence while still retaining the ability to grow in the laboratory. However, we cannot alter these genes by direct site-directed mutagenesis. In Chapter 14, however, we described the use of allele replacement, or gene knockouts, for the removal or inactivation of specific genes. We can use this process to develop attenuated strains that may be useful as vaccine candidates.

This requires some knowledge of the genes that are necessary for virulence. If pathogenicity is due to the production of a protein toxin, then you would expect the inactivation of the toxin gene to produce an attenuated derivative. Usually it is not so easy. With most bacteria, pathogenicity is multi-factorial. Much effort is being devoted to attempts to identify genes that are needed for virulence, especially by identifying genes that are selectively expressed during infection (using for example the techniques described in Chapters 13 and 14) – which would identify targets that could be knocked out in order to attenuate the bacterium.

The most successful approach so far has been to target genes that are involved in certain central biochemical pathways rather than the more obvious virulence genes. This takes advantage of knowledge that the bacterium at the site of infection will not have a ready supply of some key substrates, such as some amino acids. It will have to make them for itself. Mutants in which key genes in that pathway have been disrupted will therefore be unable to multiply in the body. Of course we can still grow these strains in the laboratory, simply by adding the required amino acid to the medium. One such pathway is that for aromatic amino acid biosynthesis. *Salmonella* mutants in which the *aroA* gene has been disrupted have reduced virulence, although not sufficiently attenuated for vaccine use.

16.1.3 Live recombinant vaccines

One of the novel advances in vaccine technology that has been made possible by genetic manipulation is the construction of vaccines that confer immunity to several diseases simultaneously. This can be achieved by inserting the genes for key antigens from different pathogens into a single live vaccine. Much of this work has been carried out using the vaccinia virus (the smallpox vaccine) as the carrier. Vaccinia is a rather large and inconvenient virus to use for genetic manipulation (with a genome size of 187 kb), and the insertion of genes requires the use of *in vivo* recombination. This is shown schematically in Figure 16.3. The required gene is inserted into an *E. coli* vector, adjacent to a promoter derived from vaccinia. This plasmid also carries a defective thymidine kinase (TK) gene from vaccinia, interrupted by the promoter and the cloning site. If an animal cell is infected with vaccinia virus, and at the same time transformed with the recombinant plasmid, the homology between

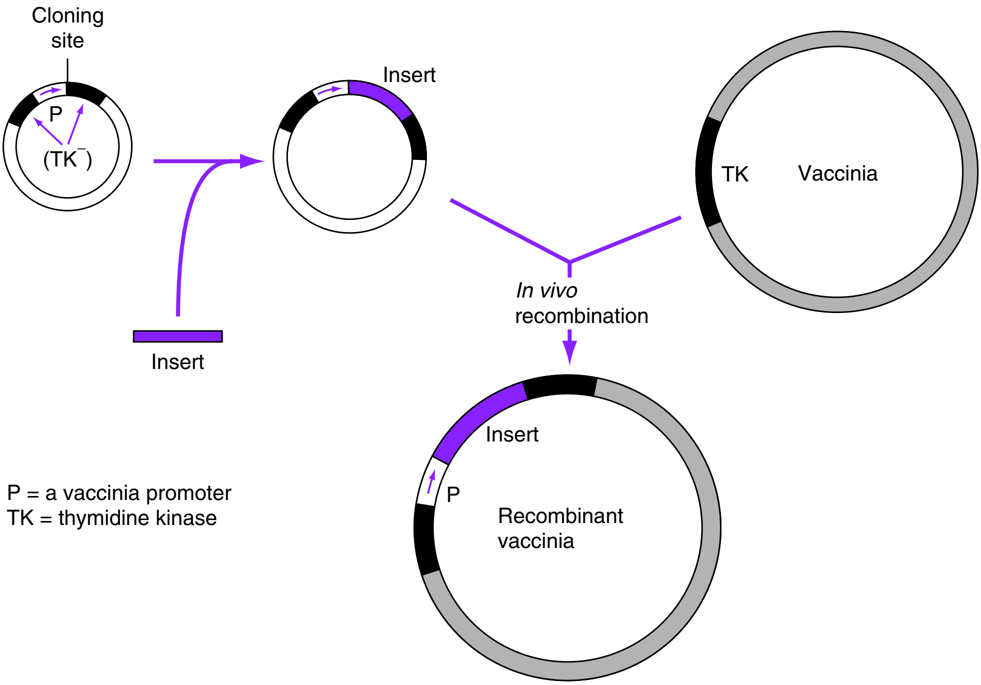

Figure 16.3 Construction of recombinant vaccinia virus: the plasmid carries an interrupted vaccinia TK gene and recombination occurs between the TK sequences on the plasmid and vaccinia DNA after co-infecting animal cells; the resulting recombinant is TK, and expresses the insert gene from the vaccinia promoter

the TK genes on the plasmid and the virus will allow specific homologous recombination, resulting in the incorporation of the insert from the recombinant plasmid, together with the adjacent promoter, into the viral DNA. The recombinant virus now lacks a functional TK gene, which provides a way of selecting the recombinants.

Antigens from a wide variety of viruses, and other pathogens, have been expressed in this way as recombinant vaccinia viruses. In one example, a recombinant vaccinia virus expressing a gene from the rabies virus was formulated into a bait for oral immunization of animals in the wild against rabies in parts of Europe. The applications of vaccinia recombinants are not limited to single antigens. It is possible to insert several genes, from different sources, into vaccinia, producing candidate vaccines that are capable of immunizing simultaneously against several different diseases.

However, vaccinia is not an ideal candidate vector for human vaccines. Despite its widespread use in the smallpox eradication campaign, it is not a very safe vaccine: for example, it is not suitable for people with skin conditions such as eczema, nor for people who are immunodeficient. However, there are other candidate carriers, notably attenuated strains of *Salmonella* and also the TB vaccine BCG. These are very safe vaccines, and the manipulations involved are relatively straightforward. At a basic level, you simply clone the genes you want on a shuttle vector, including appropriate expression signals, and transform the chosen host. Ultimately there are further considerations: we usually use antibiotic resistance genes as a selectable marker for this; for practical use as a vaccine, the inclusion of resistance genes is unlikely to be acceptable, and alternative strategies have to be adopted. Furthermore, plasmids are generally not completely stable, and insertion into the chromosome is preferred. One way of achieving this is to use a suicide plasmid with a bacteriophage integration system. The expression of the phage integrase then recombines the plasmid with the attachment site on the chromosome. There is a wide range of such potential vaccines at different stages of development.

16.1.4 DNA vaccines

The most radical of the new approaches stems from the surprising observation that injection of DNA, usually as a recombinant plasmid containing the relevant genes, is able to stimulate an immune response to the product of those genes, and in some cases this leads to protective immunity. This presumably occurs because the plasmid is taken up by some of the cells in the body, resulting in expression of the genes carried by the plasmid. One advantage of this approach is the ease of administering the vaccine. The plasmid DNA can be coated onto tiny inert particles which are projected at high velocity into the skin, penetrating a very short distance below the surface of the skin (see also the discussion of *biolistics* in Chapter 17). The particles are small enough, and the velocity is high enough, for the particles to penetrate into cells within the skin. The plasmid is constructed so that the genes to be expressed are located downstream from a strong constitutive promoter (such as the CMV promoter, see Chapter 6). Although the plasmid is not replicated within these cells, there is sufficient transient expression of the cloned genes to generate an immune response. DNA vaccines represent an exciting new development in vaccine technology. However, we have to recognize that there are many questions to be answered before they are accepted for widespread use in humans.

At the same time, this technique has achieved extensive use as a means of raising antibodies to recombinant proteins in experimental animals. Instead of the time-consuming procedure of purifying the protein and immunizing animals in the conventional way (see Box 8.2), a recombinant plasmid, carrying the cloned gene together with appropriate expression signals, can be used directly as an immunogen.

16.2 Detection and Identification of Pathogens

The standard procedure for detecting a pathogen causing an infectious disease is to grow it in the laboratory from a suitable clinical specimen (such as sputum or blood). However, many viruses, and some bacteria, are difficult or even impossible to grow in the laboratory. In these cases, molecular methods – especially PCR – provide an important tool for the laboratory diagnosis of infection, and are now in common use in clinical virology laboratories.

In addition, PCR tests and gene probes are both available for the identification and speciation of pathogens that have been cultured. Molecular techniques such as RFLP analysis (see Chapter 12) are widely used for the differentiation of bacterial strains (*typing* or *fingerprinting*) for epidemiological purposes. For example, the standard method for typing strains of *Mycobacterium tuberculosis*, the bacterium that causes tuberculosis (TB), is shown in Figure 16.4. Each track shows a different isolate of *M. tuberculosis*. Total DNA preparations were cut with *PvuII* and electrophoresed through an agarose gel. Following Southern blotting, the membrane was hybridized with a labelled probe derived from the insertion sequence IS*6110*. If two patients have caught TB from the same source, the IS*6110* patterns will be identical (see the arrowed tracks in Figure 16.4). If they are different, we can conclude that they are not part of the same outbreak.

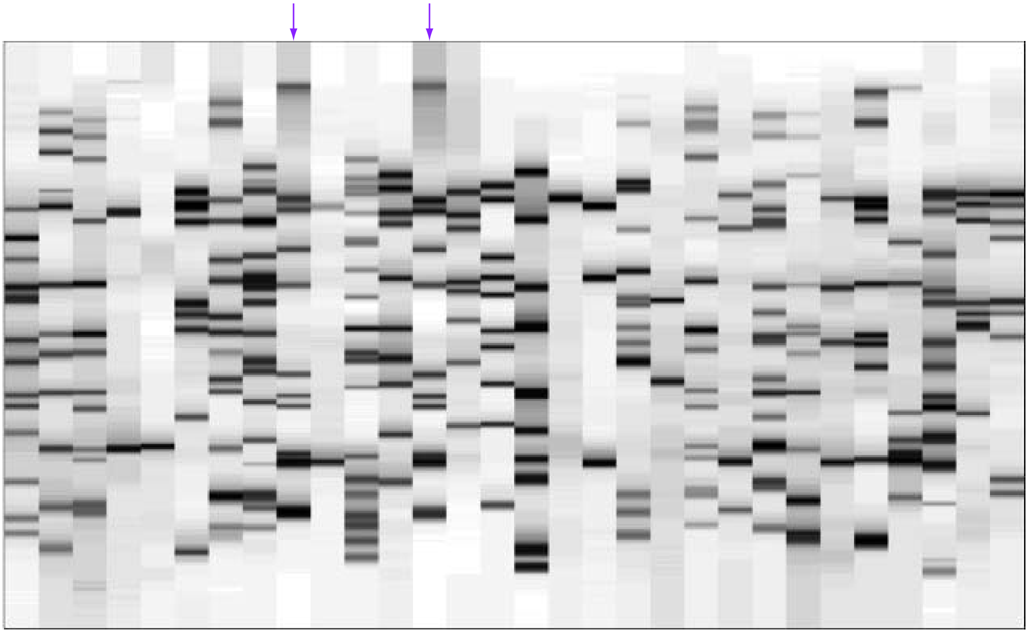

Figure 16.4 Fingerprinting of *Mycobacterium tuberculosis* using IS*6110*

16.3 Human Genetic Diseases

16.3.1 *Identifying disease genes*

One of the most important applications of gene technology lies in the ability to detect genetic mutations that result in specific diseases, or in a predisposition to the development of such a disease. We have already discussed various methods employed for identifying mutations that cause disease (or other distinguishing traits) in different chapters of this book. Here, we will take the opportunity to look back at the general strategy and integrate the different parts we have touched upon earlier.

The classical approach towards identifying a disease gene is based on the concept of linkage analysis, as described in Chapter 14, which enables the position of genetic markers to be mapped to specific regions of one of the chromosomes. Before the advent of molecular methods, the construction of a genetic map in model organisms (especially *Drosophila* and mice) was carried out by painstaking hard work, involving large numbers of controlled crosses between strains with different genetic markers. The extent to which two different genetic markers are inherited together (co-segregate), or in other words the degree of linkage between the markers, provides an estimate of their relative position. Genes that are close to one another will tend to be inherited together. In this way, a genetic map (or linkage map) can be gradually built up, showing the relative position of the available genetic markers. The position of specific disease-associated genes can be located by the degree of linkage to other markers. Of course, linkage analysis in humans is much more difficult, as controlled crossing is not an option.

In some cases, it is possible to supplement the linkage analysis, with another form of mapping, known as *cytogenetic mapping*. Staining of eukaryotic chromosomes with a dye such as Giemsa stain produces a characteristic banding pattern, as some parts of the chromosome take up the stain much more heavily than others (see Figure 16.5). If the mutation causes a change in the banding pattern, then its position can be detected microscopically. This is especially useful in organisms such as *Drosophila*, where the salivary gland cells contain bundles of sister chromatids lying side by side. This *polytene* structure makes the banding pattern much more obvious. However, cytogenetic mapping is also useful in other organisms, as an initial stage in gene mapping; for example, the human gene coding for the enzyme phenylalanine hydroxylase (which is defective in people with phenylketonuria) can be located to the band designated 12q24 on the long arm of chromosome 12. However, this is a relatively imprecise method of mapping, with a resolution of several million base pairs. If the gene concerned has already been cloned, *in situ* hybridization can be used to determine its position on the chromosome more precisely. This

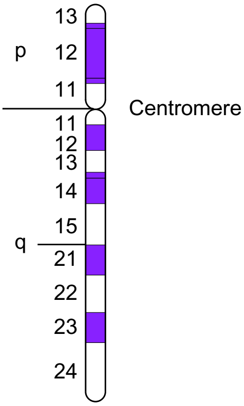

Figure 16.5 Chromosome banding patterns: this shows a simplified representation of the banding pattern of human chromosome 12

usually involves fluorescently-labelled probes, and hence is known as *fluorescent* in situ *hybridization* or *FISH*.

One limitation of genetic linkage analysis is the need for markers that have an identifiable phenotype. Since there is a limited number of such markers, even in well-studied organisms, the linkage map will be relatively crude. However, we can use molecular markers, such as RFLPs and microsatellites (see Chapter 12), to locate a disease-associated gene more precisely, by mapping its linkage to a specific polymorphism. This approach can be applied to human genetics by examining very large pedigrees from which DNA samples are available, rather than by making controlled crosses.

These forms of gene mapping simply show you the chromosomal location of a gene that determines susceptibility to a specific disease (or some other trait). They do not identify the gene, its product, or its function. However, they do provide a route for cloning such a gene. It is important to realize that we may not have any information about the function of this gene, beyond knowing that there is a gene in which mutations result in a certain disease, so none of the approaches outlined in Chapter 8 for identifying a gene in a library is available to us. However, if we have mapped its position well enough, so that we know it is close to a defined marker (whether this is another gene, a polymorphism or a microsatellite), then we can use a probe for that locus to screen a large insert gene library (using for example YAC vectors) to obtain a clone that carries the defined marker and a substantial amount of adjacent DNA, which we hope contains the disease gene. Since this form of cloning relies solely on the position of the gene, it is known as *positional cloning* (Figure 16.6).

The distance that can be covered by this approach can be extended by *chromosome walking* (see Chapter 14). In this technique, the first clone, carrying

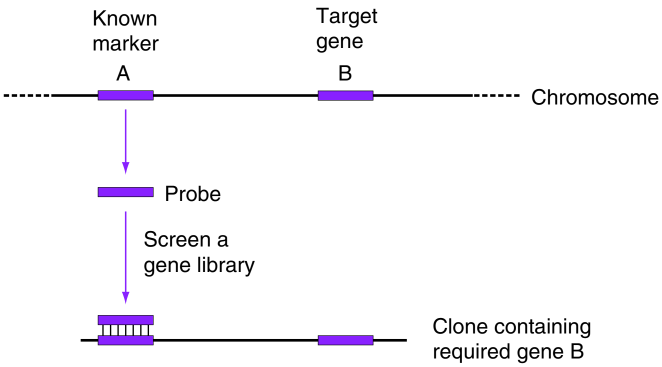

Figure 16.6 Positional cloning

the known marker, is used to probe the gene library again, so as to identify clones carrying adjacent sequences; these are in turn used as probes to obtain the next region of the chromosome, and so on. When your 'walk' reaches the region where you expect the gene to be, analysis of the sequence by the methods described in Chapter 11 will enable the identification of genes and their putative products. This will lead to a candidate gene that may be responsible for the disease in question, and the structure of this gene can be tested in individuals with and without the disease to confirm the association.

The processes of positional cloning and chromosome walking are simpler to describe than to carry out. However, the availability of the sequence of the human genome, and of genomes of other organisms, makes it a lot simpler. It is now possible to screen putative genes in the area of interest in a matter of hours on a computer, rather than in months or years in a laboratory.

An alternative approach is to base the search on gene products (mRNA or proteins) rather than on the DNA itself. This is based on the assumption that expression of the relevant gene will be altered in the diseased state, or that the structure of the product will be different. Both of these assumptions can be used – either separately or in combination – to devise protocols for the identification of candidate genes. The most important of these protocols was described in Chapter 13.

The final identification of the relevant gene will usually rely on a combination of techniques. The putative genes identified by positional cloning and chromosome walking can be tested for their association with the disease state by specifically testing the expression of those genes in the diseased tissue, or by analysing the structure of the protein. In experimental animals, final confirmation will come from a combination of gene knockout and complementation experiments (Chapter 17).

The power of these techniques therefore relies on a combination of approaches. Classical genetics (often termed *forward* genetics) starts with a phenotype (whether a diseased state or yellow peas) and works towards an identification of the genes responsible. On the other hand, the molecular approach offers the possibility of starting with differences in a defined DNA sequence, and investigating the phenotypic effects of such a change. This is often called *reverse* genetics.

Sometimes one or more candidates are obvious without any further experiments being necessary. For example, no reverse genetics was needed to conclude that mutations in the red and green cone photopigment genes were candidates for the cause of red/green colour blindness. Nor was any forward genetic analysis needed – the linkage of these conditions to the X chromosome is well established from observations of their pattern of inheritance. Thus, the mapping of these two *a priori* candidate genes to the X chromosome constituted strong supporting evidence for the initial hypothesis. In this way, forward and reverse genetics interact – the chromosomal location and the expression pattern are both important parts of the jigsaw puzzle.

The central piece of evidence, however, is quite naturally the sequencing of the allele from affected individuals and the confirmation that it is different from the wild type. This is not, however, sufficient on its own. The mutation must first be analysed, using methods that we have described in Chapter 11. First of all, is it located where it could be predicted to have a biological effect? This would often involve the alteration of the encoded amino acid sequence in a subtle or more drastic way. However, even if the coding region is completely unaltered from the wild type, mutations in the promoter region that affect the expression of the gene, mutations in splice sites or insertions that otherwise affect mRNA processing could still cause disease. However, even if you did show the cause to be one of these, the scientific community would normally demand even more evidence before your discovery could be published. Some polymorphisms, such as amino acid substitutions, occur completely normally in the population without causing any abnormality. One way to show that the mutation causes disease is to express the mutated protein and compare its biological properties to its wild-type counterpart. Another way, preferably used in combination, is of course to show that the mutation that you have discovered co-segregates with the disease to a significant degree in a genetic pedigree.

16.3.2 Genetic diagnosis

The identification of mutations that give rise to human genetic diseases not only leads to significant insights into the nature of those diseases but also provides a mechanism for molecular diagnosis. This is of particular benefit in prenatal diagnosis, which involves obtaining samples containing foetal cells by amniocentesis or chorionic villus sampling – the latter technique having the advantage of being able to be carried out at an earlier stage of pregnancy, which is important if abortion is considered as an option. The gene(s) to be tested can be amplified by PCR and specific point mutations detected by the use of a pair of labelled oligonucleotides, one of which will hybridize to the normal sequence and the other being specific for the mutant (see Chapter 12). Alternatively, real-time PCR procedures (see Chapter 13) can be devised that will differentiate the two versions of the sequence directly, producing much quicker results.

A further advantage of molecular techniques for detecting mutations is the ability to detect the presence of the defective gene in heterozygous carriers. If the mutation is recessive, the heterozygotes will show no symptoms of the condition, but a proportion of the offspring of two heterozygous parents will have the disease. In the past, genetic counselling of prospective parents from families with a history of a genetic disease has relied on statistical probabilities to assess the likelihood that both parents carry the affected gene. With molecular techniques, it is possible to be certain, one way or the other.

Similar considerations apply to genes which only show their effects later in life, and to those genes that only cause a predisposition to a specific disease. For example, in some families there is a particularly high incidence of breast cancer, indicating an inherited predisposition to this disease. In many cases this has been traced to a specific gene, *BRCA1*. Individuals carrying a mutated gene have a higher risk of developing breast cancer, although the presence of the affected gene does *not* mean that it is certain that they will develop the disease.

There are substantial ethical issues raised by the availability of these techniques. Even if one accepts abortion as an option under some circumstances, its use as a method to prevent the birth of a ‘defective’ child raises the uncomfortable spectre of eugenics. This is more of a concern for the future, whereas the ability to test people for the presence of genes that may affect their subsequent health or lifespan already has serious implications for their ability to obtain life or medical insurance, subject to legal restrictions that may limit the powers of insurers in this respect.

16.3.3 Gene therapy

Knowing which mutation, in which gene, causes a disease is not a prerequisite for treating it. Many genetic diseases have been successfully treated for decades or even centuries thanks to insights in the pathophysiology of the conditions, or indeed because of the discovery of treatments by trial and error or even sheer coincidence. Nonetheless, although it is neither the beginning nor the end, discovering the exact genetic nature of a condition is a very important step. First, as described above, it makes it possible to devise precise diagnostic methods to confirm who has, or is predisposed to, a specific condition. It may then be possible to alleviate and sometimes even prevent the disease. Second, knowing the primary structure of the mutated gene enables us to make predictions of the impact of the mutation with the help of computer programs (Chapter 11). It also allows us to express the resulting protein (if any) and characterize it in the laboratory (Chapter 13). Furthermore, it puts us in the position of being able to produce a mouse engineered to carry the same mutation, so that we have an animal model that closely approximates the human condition we wish to study (Chapter 17). All of this will be of great help in the development of improved treatments for the condition. However, there will probably always be conditions where treatment is not enough to alleviate the symptoms. This is the rationale for developing treatments involving gene transfer (*gene therapy*).

Gene therapy treatments could potentially take place by targeting the specifically diseased organs in an affected individual (*somatic gene therapy*), or by correcting the gene defect in gametes or fertilised eggs (*germline gene therapy*). There are strong reasons why most gene therapy research focuses on the former. First, the genetic manipulation of embryos is inherently a very contentious issue, and the controversy is exacerbated by the fact that germline therapy would lead to an inheritable alteration in the genetic material. Even the prenatal diagnosis and specific abortion of diseased embryos, which a significant percentage of the population would object to, has a far greater acceptance in society as a whole.

Second, there is an obvious need to treat people who are alive and suffering now, and indeed there will always be people born suffering from genetic disease. However, somatic gene therapy would only lead to a cure for that patient, and would not remove the underlying genetic cause – so there would still be the risk of the individual concerned passing the defective gene on to his or her offspring.

Viral vectors for gene therapy

The principle of delivering genes to the cells of a human being is not different from that of delivering genes to any other cells. Gene expression can be either *transient* or *permanent*. Delivery can be either directly into the affected tissues – *in vivo* – or into cells transiently removed from the patient's body – *ex vivo*. For *ex vivo* delivery, the methodology is virtually the same as for transfecting mammalian cell lines in culture – genes can be delivered using liposomes, calcium phosphate precipitation, or electroporation. However, the methodology that is attracting the greatest interest by far, both for the development of *in vivo* and *ex vivo* methods, is the use of viral vectors.

Amongst these are the *adenoviruses*. These are common causes of respiratory tract infections, especially pharyngitis (sore throat), but it is possible to produce defective viruses that do not cause disease, and to replace the deleted regions with a cloned gene. The recombinant DNA will persist for some time within the infected cell, but not indefinitely. This limitation has both advantages and disadvantages. The advantage is the fact that any negative effects are likely to be reversible. The disadvantage is that re-infection is needed to sustain the effect. Nonetheless, trials with adenovirus vectors are already in progress. One case where they are particularly promising is in the therapy of cystic fibrosis, a recessive disorder caused by a mutation in a chloride transporter, which manifests itself most strongly in respiratory problems. Naturally, a viral vector based on a respiratory virus is potentially a very promising vehicle for reintroducing the correct form of the gene into these tissues.

Another DNA virus that can be used for gene therapy is herpes simplex virus I (HSV-I). Unlike adenovirus infections, HSV-I infections persist throughout the lifetime of the patient, although they remain latent through most of it. The preference of this virus for a particular cell type – nerve cells – makes it especially interesting for correcting mutations affecting these cells.

Retroviruses are the natural choice of vector for stable expression. The biology of these viruses, and their use as cloning vectors, was described in Chapter 6. The main feature that is important here is that after infection the RNA genome of the virus is copied into DNA by reverse transcriptase and integrated efficiently into the host genome. By replacing the genes needed to form new viral particles, two birds are killed with one stone – space is created for the introduction of the gene that is to be transferred, and the recombinant virus is prevented from re-infecting other cells.

Integration into the host genome is an effective way of ensuring reliable and lasting expression, that is also transferred to the progeny of the infected cell, and we shall therefore encounter retroviruses once again in the following chapter as a vector for the production of germline transgenics. Disadvantages include the limited space available for the gene that is to be inserted, and the fact that retroviruses are not very efficient in cells that do not divide, such as nerve cells. In addition, we have to remember that this class of viruses includes some notable pathogens, not only HIV but also other viruses that cause some forms of cancer in humans and animals. Although the vectors described are defective, their widespread use for gene therapy would require extensive checks to ensure that untoward effects do not occur by interaction with other naturally occurring retroviruses.

The potential of gene therapy is not limited to inherited diseases. In particular, there is considerable potential for novel cancer treatments by suppressing the activity of oncogenes, for example by using *antisense technology* (see Chapter 17), or by targeting toxic genes to cancerous tissue. The serious nature of these diseases provides a powerful argument for employing an approach that has such potential, but there are concerns. Partly these rest on a sort of feeling that altering DNA in this way is somehow unnatural. On the other hand, there is a feeling that in some way we are in danger of letting the genie out of the bottle. Could the techniques developed for the praiseworthy objective of eliminating the suffering caused by diseases such as cystic fibrosis be exploited for other ends? These are serious questions that demand a wider rational debate, and scientists have a role in trying to ensure that a wider audience is sufficiently educated about the issues to achieve this level of rationality.

From Genes to Genomes: Concepts and Applications of DNA Technology.
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17 Transgenics

17.1 Transgenesis and Cloning

Much of this book deals with the genetic manipulation of individual cells in culture, by introducing genes from other sources or in other ways specifically altering the genetic composition of those cells. A major aspect of this is the expression of a gene from one source in *cells* of a different type, in culture. In this chapter, we will deal with the stable introduction of a gene into another *organism*, which is referred to as *transgenics*. For a bacterial geneticist, there is no distinction between manipulating individual cells and manipulating an organism. In effect, all the previous discussion of genetic modification of bacteria (and of unicellular eukaryotes such as *Saccharomyces*) could be labelled as transgenics. On the other hand, for somebody who works with truly multicellular organisms, such as plants or animals, the distinction between manipulating a cell line in culture and genetic modification of a whole plant or animal is very real and important. A gene can be stably transfected into a cell line quite cheaply, and this can be used to provide fast and reasonably useful answers to many questions – but is inevitably limited in scope. In particular, it cannot provide definitive answers to questions about the differentiation of cells in the normal animal, nor about the interaction between different cells. The creation of a genetically manipulated whole organism is much more difficult and much more expensive, but often much more informative. Systems such as *Drosophila* (Chapter 14), and the nematode *Caenorhabditis elegans*, provide a useful compromise, in that they are reasonably easy to work with but at the same time are capable of addressing some of these difficult questions – assuming that the lessons learned from fruit flies are also applicable to higher animals. More controversially than these research-oriented applications, a transgenic organism may be created not to answer a question, but for a specific, usually commercial, purpose. We will discuss both of these uses in some detail later.

It is important to distinguish the production of transgenic plants and animals from cloning of the plants or animals themselves. The former involves the introduction of foreign DNA, in the form of cloned genes. In contrast, the latter means obtaining progeny that are genetically identical to the original plant or animal, which includes methods ranging from the widely publicized procedure that led to the birth of Dolly the sheep to techniques for asexual propagation of plants (such as taking cuttings) that have been in use for hundreds of years.

When you introduce a *transgene* into an organism, you insert into the genome a foreign gene, be it from the same species or from a different one. Owing to the virtual universality of the coding properties of DNA, huge evolutionary distances can be bridged in transgenesis – the example of a fish gene inserted into tomato plants for cold hardiness achieved notoriety. In order to be of any use in a multicellular organism, the transgene must be inserted into germline DNA, so that it can be propagated in subsequent generations. Other applications where genes are inserted into *somatic cells* (cells that are not involved in reproduction of the organism), for example for gene therapy, were considered in Chapter 16.

In principle, the technology is available to insert transgenes into any species (the first transgenic monkey was recently created). In some species, it is also technically possible to do what is conceptually the opposite – to *knock out* a gene (which may involve disrupting the gene rather than deleting it entirely). The use of gene knock-outs in bacteria was considered in Chapter 14; as we will see later on, obtaining gene knock-outs in whole animals follows a similar procedure.

17.2 Animal Transgenesis and its Applications

In order to ensure expression of the transgene in the entire animal, the transgenic construct must first be introduced into the germline. The techniques for doing this are most advanced, and most commonly used, in mice. The most obvious, and most common, method is to physically inject the transgenic construct into the nucleus of a fertilized egg, which is then cultured *in vitro* for several cell generations before being implanted in a foster mother (Figure 17.1). This is conceptually simple, but by no means a trivial affair. Not all eggs survive the injection without damage, not all of them will develop, and not all of them will contain the transgene. Only a proportion of the resulting embryos will be transgenic, so it is necessary to screen the embryos (using gene probes and/or PCR) for the presence of the foreign gene. The result for each embryo is all or nothing – if the gene is present in one cell it is usually present in all cells. *Mosaics* (animals in which the gene is present in some cells or tissues and not in others, also known as *chimaeras*) do not usually occur with this procedure (although mosaics are sometimes created intentionally in other ways) and so the transgene will be present in the germline cells and transmitted to the progeny. The chromosomal location of the integrated DNA differs from one animal to another, as does the number of copies of the integrated DNA, which may range from a single copy to several hundred. The level of expression of the inserted gene may therefore vary from one animal to another.

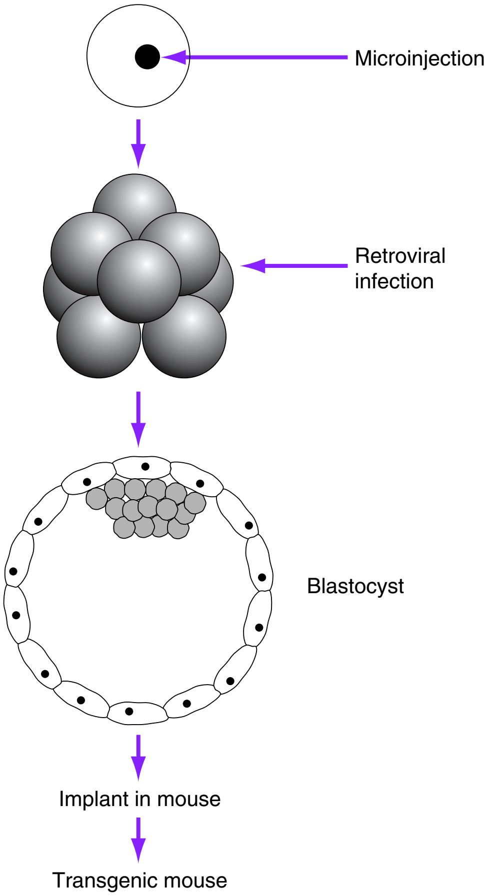

Figure 17.1 Two methods of producing transgenic mice

One alternative method is to use retroviral vectors, as described in Chapter 6. Technically, this is much simpler. The fertilized egg can be allowed to develop to the eight-cell stage before infecting it with the (defective) recombinant virus (Figure 17.1). This method has distinct limitations, however. First, these vectors can only accommodate approximately 8 kb of DNA, which may not be sufficient for the whole coding region and the necessary promoter elements (see below). Second, the modified retrovirus does not contain the genes necessary for replication as a virus. It needs assistance from a *helper virus* to be infective. This helper virus could lodge itself into the genome as well and, like a prophage, become infective again at some stage in the future. If the helper virus is also present in the embryo, it can result in the unwanted spread of the recombinant construct to other animals. The method described in Chapter 6 uses *helper cells* as an intermediate stage, to produce infective virus particles that do not need a helper virus, and thus circumvents this problem. However, there is still the possibility that a subsequent retrovirus infection of the transgenic animals might result in mobilization of the recombinant DNA. A further limitation is that, although infection of eight-cell embryos is technically simpler than micro-injection, the fact that cell division has occurred before infection takes place means that there is a possibility that not all the cells in the embryo will be infected. Mosaicism (see above) is therefore a potential problem, but non-chimaeric animals, containing the cloned gene in all their cells, can be obtained by allowing the animals to reproduce and selecting the progeny that carry the gene concerned. Retroviruses are thus limited in their application for transgenic animals, but do play a significant role in the development of potential techniques for gene therapy (see Chapter 16).

A third method is the transfection of *embryonic stem cells*, and their subsequent incorporation into mouse embryos. This method allows selection and enrichment of transgenic cells, which permits an even richer range of potential manipulations. These are discussed in a separate section below.

17.2.1 Expression of transgenes

Even when successfully integrated in the genome, the positioning of the transgene is more or less completely random. The objective is obviously that the gene lodges itself into a region where it does not disrupt another gene. Because most of the DNA in plants and animals does not actually form part of a gene, the odds are actually stacked against any such problems occurring. Nonetheless, occasionally they do. In some instances, this is lethal. In others, there is an obvious risk that the disruption of a different gene creates a phenotype which has nothing to do with the transgene itself.

The random localization of the transgene will also affect its level of expression. This is because there are large-scale regional differences on the chromosomes – the environment surrounding the transgene may not be conducive to its expression.

We also need to consider the regulation of the expression of the inserted transgene. In Chapter 15, we discussed the expression of cloned genes in mammalian cells in culture. In that context, the emphasis was on promoters that were constitutively active, or could be turned on and off by altering the culture conditions. Neither approach is really suitable for a transgenic animal, where we would normally want to target gene expression to specific tissues.

For some purposes, the ideal objective would be to introduce the native transcription unit into a genotype that lacks it. This would require a cloned sequence covering the whole gene and its associated regulators, including activator sequences and promoter elements as well as the introns and exons. The conceptually simplest, and often preferable, method of constructing such a transgene exploits the large coding capacity of yeast artificial chromosome

(YAC) vectors, as described in Chapter 6. Such an approach was not available when these methods were first used, but now constitutes an attractive option in this situation.

One alternative is to mix and match promoter and coding sequences. The closest to a YAC transgene would be to combine a genomic clone of the promoter region of the native gene with a cDNA clone of the same gene. The absence of introns may have some effect, but they are certainly not in any way *necessary* for mammalian gene expression – some mammalian genes do not have them in the first place. What may make more of a difference, however, is the promoter. You would certainly have pinpointed it using reporter gene constructs before spending a minor fortune on producing a transgenic organism. However, the conditions in the cell line where this was done do not necessarily apply to the conditions in the native cells. Moreover, there may be important elements – positive or negative – that are located some considerable distance away from the transcription start site.

Apart from combining a cDNA with its own promoter, there are many inventive and informative combinations that can be – and have been – made, either to obtain tissue-specific expression of the transgene or to identify the tissues or cell types in which that gene would naturally be expressed. Many of these examples are related to topics that have been discussed in earlier chapters in relation to the manipulation of unicellular microorganisms or of cell lines in culture. Here are a few examples.

(1) If the promoter region of the gene you want has not been characterized, a cDNA copy of your gene may be combined with a different promoter that has the same cell or tissue specificity.

(2) In order to investigate the exact cell/tissue specificity of a promoter, or its activity under different conditions, it may be combined with a reporter gene such as *lacZ* (which is easy to detect histologically) or luciferase (which is easy to detect *in vivo*). Similar applications of reporter genes for individual cells in culture were described in Chapter 13.

(3) A promoter may be combined with a gene, such as the gene for the A subunit of diphtheria toxin (DTA) which will specifically damage any cell type in which it is expressed. (The inserted gene does not include that part of the toxin, the B subunit, that is needed for cell entry, so it will only affect the cells in which it is expressed.)

(4) A promoter may be combined with a gene that adds a particular functionality to a particular cell type. A common variant of this is the rescue of a recessive mutated phenotype by introducing the wild-type gene, which will provide conclusive proof of the identity of the gene. This can obviously be

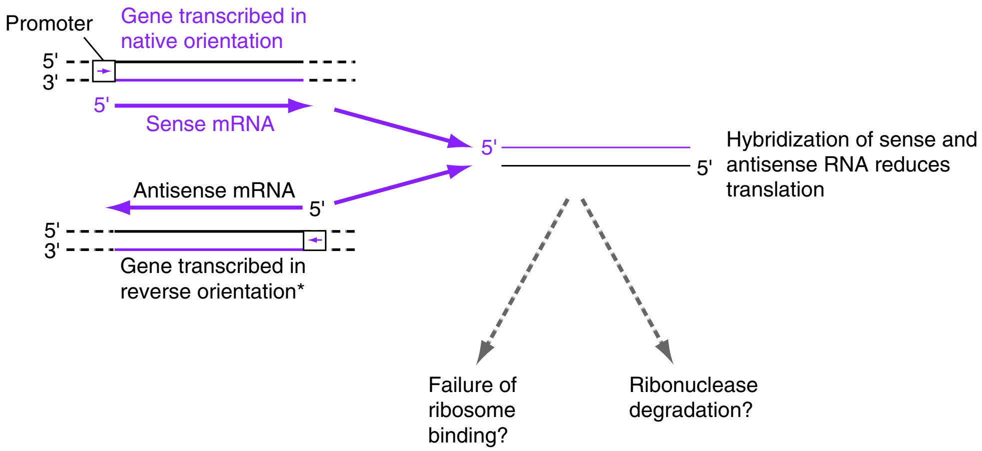

Figure 17.2 Antisense RNA

accomplished with a YAC clone as well (see also the discussion of complementation in Chapter 14).

(5) Alternatively, you may produce a construct that *removes* functionality from the cell where the promoter is expressed. This may be done in a number of ways. You may place the cDNA in the reversed direction, so that it produces an *antisense* mRNA, which will bind to the native species and reduce its translation (Figure 17.2). The reasons for this effect are not fully understood, but it is thought that either ribosomes are unable to bind to the hybrid RNA, or that the double-stranded RNA is degraded by ribonucleases. We will consider an application of this approach in the section on transgenic plants, later in this chapter.

17.2.2 Embryonic stem-cell technology

This technology, currently only available in mice, is based not on the manipulation of individual fertilized eggs or embryos, but on cells that can be grown in culture. Some types of cells taken from an adult animal can be grown in culture, for a short time. In some cases, these can give rise to established *cell lines* which can be maintained indefinitely through serial subculture. Manipulation of animal cells in culture (as considered in previous chapters) is considerably easier than introducing genes into a fertilized egg. However, the differentiation of such cells is not usually reversible; they cannot be used to produce a whole animal. This is a marked contrast to the situation in many plants, where cells from various parts of the plant, although differentiated, are still capable of producing whole plants; such cells are said to be *totipotent*. This is why it is so easy to propagate many plants asexually, e.g. by taking cuttings, or even by starting from *in vitro* cell cultures. It is also why there was so much excitement over Dolly the sheep, which was a demonstration that it *is* actually possible, through some intricate techniques, to reverse the differentiation of some cells from an adult animal.

Obviously, cells at an early stage in the development of an embryo still have the potential to develop into many types of cell in the adult mouse; these are known as *embryonic stem cells* (ES cells). If cells are collected at the blastocyst stage, they can be grown in culture for a limited period of time; if established ES cell lines are used it is not necessary to harvest cells from embryos. The availability of cells in culture, whether they are fresh ES cells or a cell line, means that many of the techniques described in earlier chapters can be employed – in particular, the investigator can include selectable markers in the construct. These convey resistance to toxic compounds that are then added to the growth medium. This is similar to antibiotic selection for plasmids in bacteria: only cells that have successfully integrated the transgene will survive. These selected cells are then injected into blastocysts and implanted into a surrogate mother (Figure 17.3).

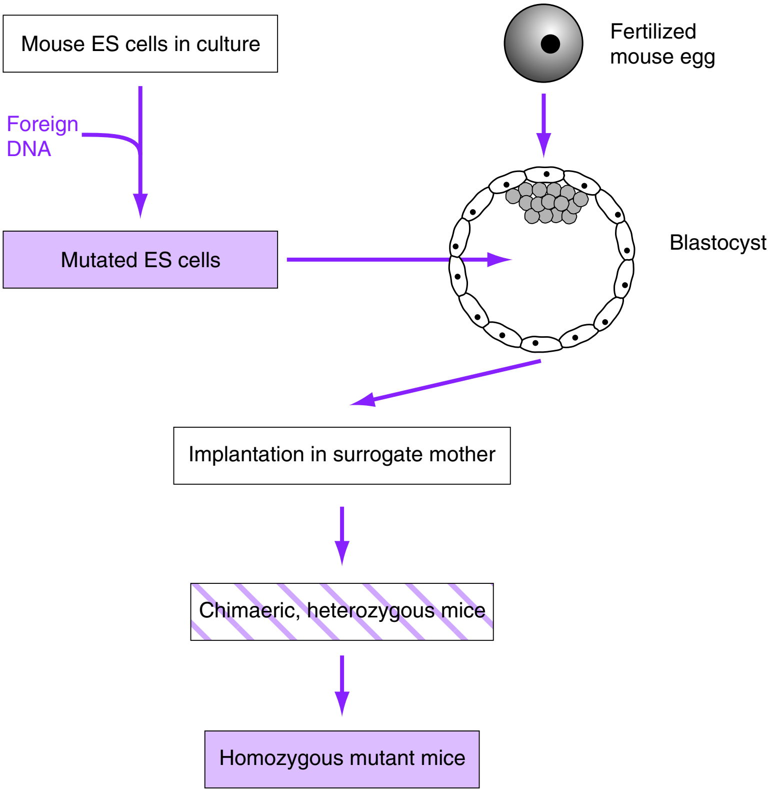

Figure 17.3 Embryonic stem-cell technology

The progeny will be chimaeric, since the blastocyst will still contain some of the non-manipulated cells, and heterozygous, since normally only one of the chromosomes will be altered. Subsequent breeding and selection of the progeny will give rise to stable homozygous mutant mice.

A further advantage of ES technology is that it allows the introduction of the construct in an exactly specified site in the host genome. This is done by including two sequences that are identical to two particular adjacent regions in a host chromosome. Just as in normal meiosis, recombination at these sites will occur with a certain frequency, leading to the specific insertion of the transgene exactly in the desired place – a process known as *homologous recombination* (Figure 17.4). The insert gene would normally be accompanied by a selectable marker gene, so that we can select the cells in which the insert has become integrated into the chromosome. However, it has to be remembered that in animal cells random integration of transfecting DNA occurs quite frequently, so it may be necessary to screen a number of clones in order to find one that has the insert at the right place. This can be made easier by including a second marker gene on the construct, outside the region flanked by the homologous sequences. Random integration of the transfecting DNA would lead to integration of the whole construct, while integration by homologous recombination will only insert the region between the two recombination sites (see Figure 17.4). The cells we are looking for will therefore lack the second marker from the construct, and can thus be easily identified.

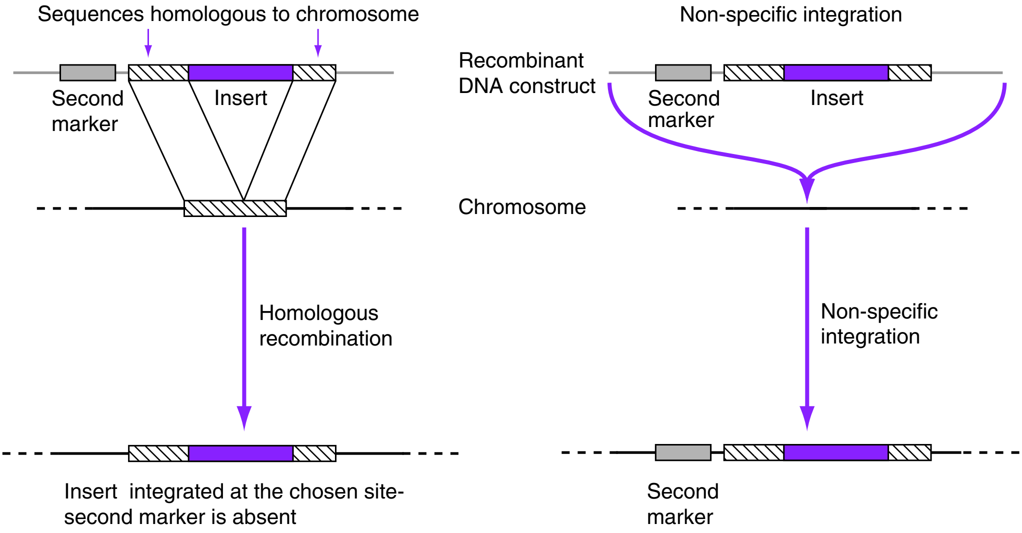

Figure 17.4 Gene insertion at a specific site

17.2.3 Gene knock-outs

ES cell technology with homologous recombination also makes it possible to not only *add* a gene, but also to *remove* (or *modify*) an existing one. In the above description of introducing a transgene into a specific site, our choice of homologous sequences would be designed so that integration will take place in a part of the genome that allows the gene to be freely expressed without disrupting any other genes. However, disrupting the gene may be exactly what we want to do, and we can achieve it in a very specific way. A transgenic construct is produced that contains resistance markers, and a part of the gene we wish to manipulate with a specific manipulation made. This could be the disruption of the promoter, the introduction of a stop codon early on in the coding region, or the deletion of part of the gene. In the version shown in Figure 17.5, the target gene is disrupted by the incorporation of a *neo* gene. (This gene gets its name because it makes bacteria resistant to neomycin, but in animal cells another aminoglycoside antibiotic, known as G418 or Geneticin, is used.) The resulting offspring will therefore not express the target gene – we have created a *knock-out mouse*. This procedure is basically the same as that described in Chapter 14 for allelic replacement in bacteria. Knock-out mice have been absolutely crucial in elucidating the exact function of many genes. The method is not without its pitfalls, however. The absence of the gene may be lethal to the embryo. Alternatively, overexpression of other genes may compensate for the absence of the knocked-out gene, thus obscuring the phenotype.

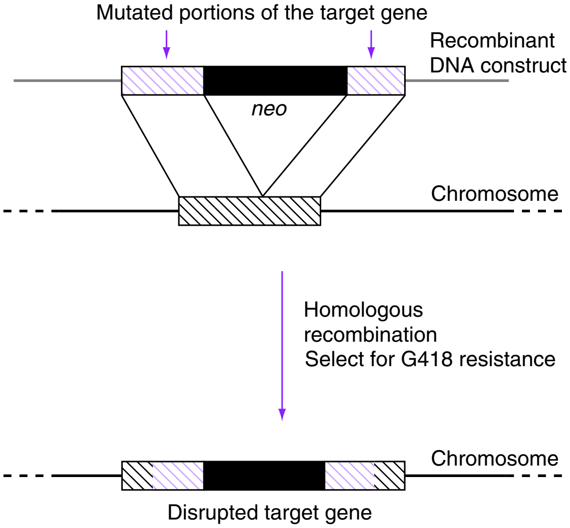

Figure 17.5 Gene knock-outs: *neo* codes for an aminoglycoside phosphotransferase that gives resistance to enomycin and to G418 (Geneticin)

As an alternative strategy, we can employ *antisense technology*, in which expression of the reverse complement of the mRNA interferes with normal translation. This usually reduces, rather than eliminates, the synthesis of the relevant protein, and has a potential role in gene therapy (see Chapter 16).

From this description of the procedures that can be used, it should be clear that transgenic technology is extremely valuable for research into the role and regulation of specific gene products in the development and characteristics of a variety of animals, including predominantly mice, rats, *Xenopus*, and zebrafish. We now want to turn to the practical applications, real and potential, of these techniques.

17.2.4 Gene knock-in technology

Most diseases are not caused by the complete disruption of gene function, but by mutations that alter it only in part. This is not only because a smaller mutation is statistically more likely to happen than a bigger one, but also because its effects are more likely to be subtle and less likely to be lethal. Such mutations can be introduced by *knock-in* technology, where one or more exons are replaced with an altered one. Indeed, producing animal models of disease is not the only important application of this technology, or even necessarily the most important one. The concept is important in any situation where we do not want to lose a gene completely, so knock-out technology is not appropriate, but rather we want to introduce specific changes into that gene. One important example of the application of knock-in technology is the production of mouse strains in which critical portions of significant genes have been replaced with, or engineered to be more similar to, their human equivalents. The potential value of such strains, for example in the testing of the toxicity and/or action of new drugs, is obvious.

The procedure for producing knock-in mice is very similar to that described above for gene knock-outs, or to the procedure in Chapter 14 for allelic replacement in bacteria. The desirable mutation(s) is introduced into the exon(s) in question, using site-directed mutagenesis or other procedures described in Chapter 15, and a selectable marker such as the *neo* gene (see above) is inserted into an intervening intron. Homologous recombination will then result in the replacement of the chromosomal gene with the modified sequence.

17.2.5 Applications of transgenic animals

Another application that is at least as controversial as gene therapy is the use of transgenic technology for commercially important animals such as cattle and sheep. This should be put in perspective. Conventional properties of farm animals, such as their rate of growth or milk production, have been manipulated by selected breeding for thousands of years, resulting in the specialized breeds that we see today that bear little resemblance to their original ancestors. Against this background, the novel aspect of transgenic technology is the ability to introduce additional genes, from other sources. This means that the animals can be used as a source of a variety of useful products.

One approach that is being studied in particular involves attaching the foreign genes to a promoter that will be active in mammary tissue, such as the casein or beta-lactoglobulin promoters. Consequently, the protein concerned will be produced in large quantities in milk. This opens the field not only for enriching the milk itself with various proteins, but also for the use of cattle or sheep as bioreactors for producing great amounts of proteins with various uses. The production of pharmaceutically useful proteins by farm animals has been termed ‘pharming’. This can offer substantial advantages over the use of recombinant bacterial cultures. In the first place, it avoids the need to build and run expensive industrial-scale fermenters. In addition, using animal hosts means that the product is likely to have the appropriate post-translational modifications, which is often not the case for products obtained from bacteria.

Recombinant plants also offer considerable potential in this way, as described in the following section.

17.3 Transgenic Plants and their Applications

Producing transgenic plants is a much simpler affair, as plant cells, unlike animal cells, are *pluripotent*. In many cases, plant cells can be grown in culture, and manipulated, and whole plants regenerated from these individual cells – although this is not as easy for monocotyledons (cereals and grasses), which is unfortunate as this includes many of the major food crops. There is a variety of ways of introducing DNA into plant cells, including protoplast transformation/electroporation, microinjection, biolistics, and transfer from *Agrobacterium tumefaciens*.

For protoplast transformation, the plant cell wall is digested with suitable enzymes to obtain protoplasts. These will take up DNA quite readily, and the efficiency can be improved by the use of electroporation (see Chapter 6), or by fusion with DNA-containing liposomes. Microinjection involves the direct injection of DNA into the nucleus of the plant cell, in much the same way as was described above for animal cells.

Biolistics involves coating tiny beads of gold with DNA and bombarding the plant cells at high velocity; this technique has also been used with other types of cell (see also the discussion of DNA vaccines in Chapter 16). For plants it has the advantage of being also applicable to embryonic plants, and so avoids the difficulty of regenerating some types of plants from individual cells.

Each of the above methods has been encountered elsewhere in this book, in relation to different types of cells. The most distinct way of getting foreign DNA into a plant cell involves the bacterium *Agrobacterium tumefaciens*. In nature, this causes a type of plant tumour known as a *crown gall*. The pathogenic ability of these bacteria is associated with the presence of a type of plasmid, known as a *tumour-inducing* (Ti) plasmid. The tumour is produced by the transfer of plasmid DNA (or more specifically a 23 kb fragment of the plasmid, known as T-DNA) from the bacterium to the plant cells, where it becomes integrated into the plant chromosomal DNA. This can be exploited by hooking foreign genes up to the plasmid in such a way that the genes concerned are also transferred into the plant cells.

This is actually more difficult than it sounds. The natural Ti plasmids are very large (over 200 kb), and so it is not possible to clone genes directly into them. One strategy for overcoming this is illustrated in Figure 17.6 . In this procedure, the initial construct in *E. coli* is made in an *intermediate vector*, which is a small plasmid containing a part of the T-DNA fragment from a Ti plasmid. The recombinant intermediate vector can be transferred to *A. tumefaciens* by conjugation. Within the *A. tumefaciens* cell, a single recombination event between the homologous T-DNA sequences on the intermediate vector

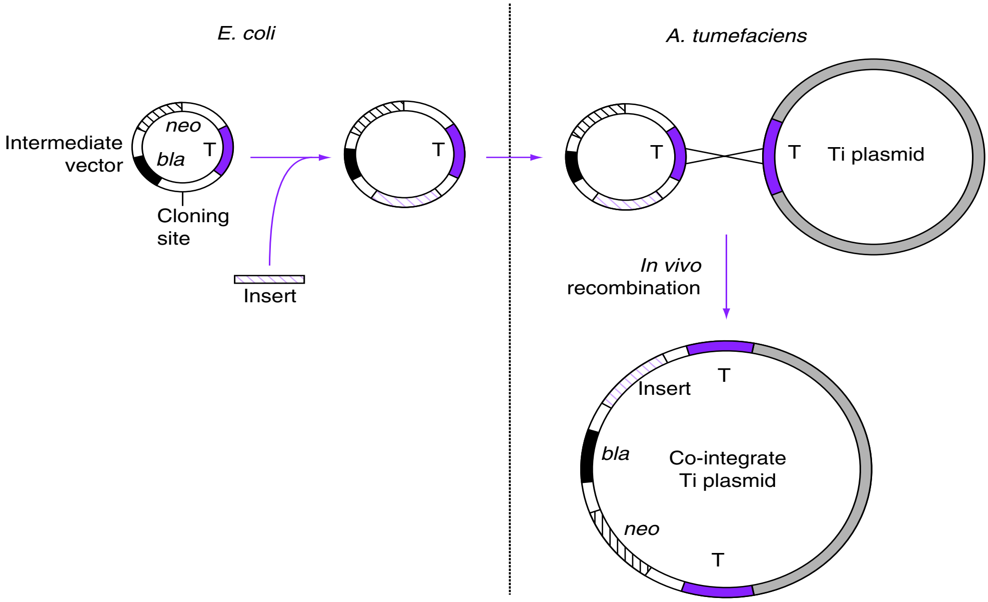

Figure 17.6 *neo* = neomycin phosphotransferase, selection for resistance to G418 in plant cells; *bla* = beta-lactamase, selection for ampicillin resistance in *E. coli*; T = T-DNA fragment transmitted to plant cells

and the resident Ti plasmid will result in the incorporation of the entire intermediate vector plasmid into the T-DNA region of the Ti plasmid. The intermediate plasmid is not able to replicate in *A. tumefaciens*, so selection for antibiotic resistance will enable recovery of the bacterial cells carrying the co-integrate plasmid. Infection of a plant will now result in the transmission of the genes from the intermediate vector (including the cloned insert) into the plant cells along with the T-DNA.

Rather than infecting an intact plant, *A. tumefaciens* can be used to infect plant cells in culture and transformants can be isolated on a selective medium. All cells in a plant regenerated from a transformed cell will contain the cloned gene, and so the gene will be inherited in the subsequent progeny of that plant. However, plant cells infected with a wild-type Ti plasmid will not regenerate properly, because of the oncogenic effects of the T-DNA; but only short sequences at the ends of the T-DNA region are necessary for transmission to the plant cells. The oncogenic genes between these sequences can be removed, resulting in *disarmed* Ti plasmids. Any DNA that is inserted between these two sequences, in place of the normal oncogenic genes, will be transmitted to the plant cells and, in the absence of the oncogenes, the plant cells can be regenerated into mature plants carrying the foreign DNA.

Just as in animal transgenesis, a suitable combination of promoter and gene is chosen to express the gene in the desired location and quantity. One promoter that is used for this purpose is derived from the cauliflower mosaic virus. Clearly, producing genetically modified (GM) plants is much simpler than GM animals and, unsurprisingly, the commercial exploitation of this fact has gone much further, and is already a reality.

One of the applications that has received a lot of publicity is the production of plants that are resistant to insect attack. This most commonly involves the insertion of a gene from the bacterium *Bacillus thuringiensis* coding for an insecticidal toxin which is highly poisonous to certain groups of insects. Expression of this gene by a plant makes it resistant to insect attack, and thus substantially reduces the need for spraying the crop with insecticides.

The second widely publicized application is that of resistance to herbicides (principally glyphosate). This is often confused with insecticide resistance, but the cases are quite different. Expression of a herbicide-resistance gene in a crop plant enables the farmer to spray the crop with a broad-spectrum herbicide to eliminate weeds.

17.3.1 Gene subtraction

In addition to adding genes to plants, it is possible to inactivate specific plant genes. In general, this involves the use of anti-sense RNA (see Figure 17.1) rather than gene knock-outs. The principle of antisense technology, as described earlier in this chapter, is that the production of the reverse complement of the mRNA interferes with the production of the corresponding protein.

One application of this is in delaying the ripening, and spoilage, of tomatoes. In the later stages of ripening, an enzyme (polygalacturonase) is produced. This enzyme breaks down polygalacturonic acid in the cell walls of the tomato and thus softens the tomato. Softening is desirable, but if it goes too far, you get a spoilt, squishy tomato. Introduction of part of the polygalacturonase gene, under the control of a cauliflower mosaic virus vector but in the 'wrong' orientation, produces an antisense RNA which binds to the normal polygalacturonase mRNA and greatly reduces the enzyme levels in the fruit. Enough enzyme is made to achieve a gradual softening, but the fruit can be stored for a much longer period before it spoils.

There is an extremely wide variety of other modifications that have been made, and the list will grow very rapidly. This includes genes that convey tolerance to environmental factors such as cold or salt, or its texture, taste, or colour (the latter important both in agricultural and horticultural crops); we have mentioned previously the use of plants for the production of pharmaceutically important proteins. Of particular importance are modifications that improve the nutritional content of the product. Many people in poorer countries rely extensively on a single food crop, such as rice, which often does not provide an adequate level of all the essential nutrients and vitamins in their diet.

17.4 Summary

In this chapter, as in the rest of the book, we have introduced the techniques and some examples of their applications. Although this only scratches the surface of a rapidly expanding subject, we hope it will have given you some understanding of the underlying concepts involved, of the advances in knowledge that have already been attained, and of their future possibilities. Some of these possibilities may be undesirable, but in many ways these techniques will play a major role in improvements in health, to use just one example. From your study of this book, you should be better placed to understand, and hopefully contribute to, the debate over the use and control of genetic modification.

From Genes to Genomes: Concepts and Applications of DNA Technology.
Jeremy W Dale and Malcom von Schantz

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Web sites

Note that this is only a small selection of the available sites. Many other sites not mentioned are also valuable resources. Note also that web site addresses may change, although the ones selected are likely to be reasonably stable, at least in their home pages. Within each site, the structure is likely to be more fluid, so only the home pages are listed. You will need to explore the site from that point to find the facilities that you want.

Address	Organization	Facilities include
http://www.ebi.ac.uk/	European Bioinformatics Institute	Databases: EMBL, Swiss-Prot, TrEMBL Tools: BLAST, FASTA, MPsrch, CLUSTAL
http://www.ncbi.nlm.nih.gov/	National Center for Biotechnology Information (NCBI)	GenBank database plus search and comparison tools
http://www.ddbj.nig.ac.jp/	DNA Data Bank of Japan	DDBJ database plus search and comparison tools
http://www.isb-sib.ch/ http://www.expasy.org/	Swiss Institute of Bioinformatics (SIB)	SwissProt database Prosite SWISS-2DPAGE ExPASy (Expert Protein Analysis System) proteomics server
http://www.sanger.ac.uk/ http://www.ensembl.org/	Sanger Centre	Genome sequence data Ensembl genome data resources Software (Artemis, Pfam, genomic analysis tools)
http://www.tigr.org/	The Institute for Genomic Research	Genome sequencing projects
http://www.mpiib-berlin.mpg.de/	Max Planck Institute for Infection Biology	2D-protein gel databases
http://www.hgmp.mrc.ac.uk	UK Human Genome Mapping Project Resource Centre	Bioinformatics resources, clones, genotyping service
http://www.rzpd.de	RZPD Deutsches Ressourcenzentrum für Genomforschung	Clones, libraries, ESTs,

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Glossary

Adaptor a short double-stranded oligonucleotide used to add sticky ends to a blunt-ended fragment, or to change one sticky end to a different one (see *linker*).

Affinity chromatography use of a specific *ligand*, attached to an insoluble matrix, to bind the required protein and enable its purification.

Agrobacterium tumefaciens a bacterium that produces tumour-like growths (crown galls) on certain plants; used for transfer of DNA into plant cells.

Alkaline phosphatase enzyme that removes the terminal 5′ phosphate from DNA, and thus can be used to prevent ligation.

Allelic replacement see *gene replacement*.

Allosteric effect alteration of the conformation of a protein, through the binding of a *ligand*, resulting in a change of the activity of a different site on the protein.

Annealing formation of double-stranded nucleic acid from two single-stranded nucleic acid molecules; see *hybridization*.

Anticodon The region of tRNA that pairs with the codon.

Antisense RNA RNA that is complementary to a specific mRNA, and which can interfere with translation.

Autoradiography detecting radioactively labelled material using X-ray film or a phosphoimager.

Auxotroph a mutant that requires the addition of one or more special supplements to its growth medium (c.f. *prototroph*).

Avidin a protein with high affinity for biotin; used for detecting biotinylated probes.

Back translation (reverse translation) predicting all possible nucleic acid sequences coding for a specific amino acid sequence.

Bacterial artificial chromosome (BAC) a vector based on the *F plasmid*, used to clone very large DNA fragments.

Bacteriophage a virus that infects bacteria (often shortened to ‘phage’).

Baculovirus an insect virus used as a vector for eukaryotic gene expression.

Bioinformatics computer-based analysis of biomolecular data, especially large-scale data sets derived from genome sequencing.

Biolistics the use of high-velocity microprojectiles for introducing DNA into cells.

Biotin a small molecule that can be attached to dUTP and incorporated into a nucleic acid as a non-radioactive label; detected with avidin.

BLAST basic local alignment sequencing tool; a computer program for searching for similar sequences.

Blunt end the end of a double-stranded DNA molecule in which both strands terminate at the same position, without any single-strand extension (as opposed to a *sticky* end); also known as a *flush* end.

Box a short sequence of bases in DNA conforming (more or less) to a consensus for that particular type of box; usually has a regulatory function.

Caenorhabditis elegans a nematode species widely used for studies of genetics and cell differentiation.

cDNA complementary (or copy) DNA, synthesized using mRNA as a template, a reaction carried out by reverse transcriptase.

cDNA library a collection of cDNA clones which together represent all the mRNA present in a sample at a particular time (c.f. *genomic library*).

Chaperone a protein that affects the folding of other proteins or the assembly of complex structures.

Chimaera an animal or plant containing a mixture of cells with different genotypes.

Chromosome walking a technique for identification of DNA regions adjacent to a known marker by sequential hybridization of clones.

***cis*-acting** a control region that influences genes on a more or less adjacent region of the same DNA molecule only, and has no effect on other DNA molecules (c.f. *trans-acting*).

Cloning obtaining a homogeneous population of cells by repeated single colony isolation; also used to refer to obtaining copies of recombinant DNA carried by such a cell ('gene cloning') and, by further extension, to the reactions used to make such recombinants.

CLUSTAL a computer program for multiple alignment of protein or DNA sequences.

Codon a group of three bases in mRNA that codes for a single amino acid.

Codon bias difference in the frequency of occurrence of *synonymous codons*.

Codon usage a measure of the relative use of *synonymous codons*.

Cohesive end see *sticky end*.

Colony/plaque lift transfer of colonies or plaques from a plate onto a membrane for hybridization.

Competent a bacterial cell that is able to take up added DNA.

Complementary strand a nucleic acid strand that will pair with a given single-strand of DNA (or RNA).

Complementation restoration of the wild-type phenotype by the introduction of a second DNA molecule, without recombination.

Conjugation transfer of genetic material from one bacterial cell to another by means of cell to cell contact.

Consensus sequence a sequence derived from a number of related, non-identical sequences, representing the nucleotides that are most commonly present at each position.

Constitutive the gene product is always formed irrespective of the presence of inducers or repressors (see *induction*, *repression*).

Contig composite DNA sequence built up from a number of smaller overlapping sequences during a sequencing project.

Copy number the number of molecules of a plasmid present in a cell.

***cos* site** the sequence of bases of bacteriophage lambda that is cut asymmetrically during packaging, generating an unpaired sequence of 12 bases at each end of the phage DNA.

Cosmid a plasmid that contains the *cos* site of bacteriophage lambda; after introducing a large insert, the recombinant cosmid forms a substrate for *in vitro* packaging.

CpG island a chromosomal region rich in CG dinucleotides.

ddNTP any one of the dideoxynucleotides ddATP, ddCTP, ddGTP, ddTTP.

Denaturation (a) reversible separation of the two strands of DNA by disruption of the hydrogen bonds (usually by heat or high pH); (b) disruption of the secondary and tertiary structure of proteins.

Dideoxynucleotide a nucleotide lacking an OH at both 2′ and 3′ positions, which therefore terminates DNA synthesis; used in DNA sequencing.

Direct repeat two identical or very similar DNA sequences, reading in the same direction (see also *inverted repeat*).

DNA ligase enzyme for joining DNA strands by formation of phosphodiester bonds.

dNTP any one of dATP, dCTP, dGTP, dTTP.

Domain a region of a protein that folds into a semi- autonomous structure.

Domain shuffling a natural process in which various domains of a protein appear to have originated, evolutionarily, from different sources (see *mosaic genes*).

Dot (or slot) blot a hybridization technique where the samples containing the target nucleic acid are applied directly to the membrane in a regular pattern, usually defined by a manifold.

Electrophoresis separation of macromolecules (DNA, RNA, or protein) by application of an electric field, usually across an agarose or acrylamide gel.

Electroporation inducing cells to take up DNA by subjecting them to brief electric pulses.

Embryonic stem cell a *totipotent* cell derived from the embryo of an animal; used in transgenics.

End filling converting a sticky end to a blunt one by enzymatic synthesis of the complementary strand (c.f. *trimming*).

Endonuclease an enzyme that cuts a DNA molecule at internal sites (c.f. *exonuclease*).

Enhancer a *cis*-acting sequence that increases the utilization of a promoter.

Episome a plasmid that is able to integrate into the chromosome; in eukaryotic systems, often used to emphasize the extrachromosomal state.

Epitope portion of a protein that is recognized by a specific antibody.

EST expressed sequence tag; a partial cDNA molecule, picked at random from a library and sequenced.

Eukaryote a cell that has a discrete nucleus, bounded by a membrane; e.g. fungal, protozoan, plant, and animal cells (c.f. *prokaryote*).

Exon coding sequence, part of a single gene and flanked by *introns*.

Exonuclease an enzyme that removes nucleotides from the ends of a DNA molecule (c.f. *endonuclease*).

Expression vector a cloning vector designed for expression of the cloned insert using regulatory sequences present on the vector.

Fingerprint polymorphic pattern of bands on a gel for differentiating individual genomes.

FISH fluorescent *in situ* hybridization; using a probe labelled with a fluorescent dye to locate specific genes on a chromosome, or within a cell or tissue.

Flush end see *blunt end*.

Footprinting identification of sequences that bind a specific protein, by visualization of the protection of positions that are protected from attack by DNase I.

F plasmid a natural, low-copy, plasmid of *E. coli*. (see *Bacterial artificial chromosome*).

Frameshift insertion or deletion of bases, other than in multiples of three; this changes the reading frame of protein synthesis beyond that point.

Gel retardation reduction in the electrophoretic mobility of a DNA fragment due to protein binding (also known as gel shift or band shift).

Gene knock-out inactivation of a gene by homologous recombination (see also *gene replacement*).

Gene replacement (gene knock-in) replacement of a chromosomal gene by recombination with a homologous sequence, inactivated or otherwise modified *in vitro*.

Gene subtraction use of antisense RNA for partial or controllable reduction in the activity of a specific gene.

Gene therapy use of specific DNA to treat a disease, by correcting the genetic lesion responsible or alleviating its effects.

Genetic map a map of the genetic structure of a genome, showing the relative position of known genes (c.f. *physical map*).

Genome the entire genetic material of an organism.

Genomic library a collection of recombinant clones which together represent the entire genome of an organism (see also *cDNA library*).

Genomics analysis of data derived from the complete DNA sequence of an organism.

Genotype the genetic make-up of an organism (c.f. *phenotype*).

Guessmer a short synthetic oligonucleotide (oligomer) based on the most likely DNA sequence deduced from amino acid sequence data.

Hairpin a region of DNA or RNA that contains a short inverted repeat, which can form a base-paired structure resembling a hairpin (similar to a *stem-loop structure*).

Heteroduplex a double-stranded nucleic acid molecule formed by base- pairing between two similar but not identical strands. Since the two strands are not identical, some regions will remain single-stranded.

Heterozygote a diploid organism that carries two different versions of a specific gene (c.f. *homozygote*).

Homologous recombination a natural process involving the pairing of similar DNA molecules followed by breakage, crossing over, and rejoining of DNA strands.

Homology similarity in the sequence of two genes, from different organisms, that share a common evolutionary origin. Often used, more loosely (as in *homologous recombination*), to describe DNA molecules with a sequence that is sufficiently similar for complementary strands to hybridize, without evidence of common evolutionary origin or function.

Homopolymer tailing see *tailing*.

Homozygous a diploid organism in which the two copies of a specific gene are the same (c.f. *heterozygote*).

Hybridization the formation of double-stranded nucleic acid molecules by the production of hydrogen bonds between wholly or partially complementary sequences.

Hydrophilic ‘water-loving’; substances, or parts of a structure, that are polar, and interact with water, and therefore tend to be exposed to water.

Hydrophobic 'water-hating'; substances, or parts of a structure, that are non-polar, and do not interact with water, and therefore tend to remove themselves from an aqueous environment.

***In situ* hybridization** a form of hybridization where the immobilized target is part of a whole chromosome, or in some cases a whole cell.

***In vitro* packaging** the assembly of mature bacteriophage particles *in vitro* by mixing suitable DNA with cell extracts that contain bacteriophage heads and tails and the enzymes needed for packaging.

***In vitro* mutagenesis** see *site-directed mutagenesis*.

Indel insertion/deletion; indicates, in a comparison of two genome sequences, that there has been either an insertion in one or a deletion in the other.

Induction (a) increasing the synthesis of a gene product through a specific environmental change; (b) applying a treatment to a lysogenic bacterium that results in the bacteriophage entering the lytic cycle.

Insertion sequence a DNA sequence that is able to insert itself, or a copy of itself, into another DNA molecule; carries no information other than that required for transposition (see also *transposon*).

Insertion vector a lambda cloning vector into which DNA can be inserted at a single site (c.f. *replacement vector*).

Insertional inactivation destruction of the function of a gene by insertion of a foreign DNA fragment, either by transposition or by gene cloning.

Intermolecular interaction between two different molecules.

Intramolecular interaction between different parts of the same molecule.

Intron intervening sequence in a eukaryotic gene; removed by splicing (see also *exon*).

Inverted repeat two identical or very similar DNA sequences, reading in opposite directions (see also *direct repeat*).

IPTG iso-propylthiogalactoside; an inducer of beta-galactosidase that is not hydrolysed by the enzyme.

Island a region of the genome with different base composition from that of the overall genome (see also *CpG island, pathogenicity island)*.

Isoelectric focussing separation of proteins by electrophoresis in a stable pH gradient, so that each protein will move to its *isoelectric point*.

Isoelectric point the pH at which a specific protein has no net overall charge.

Isogenic strains that are identical in their genetic composition; normally used to mean identical in all genes except the one being studied.

Isoschizomers restriction endonucleases that recognize the same nucleotide sequence (but do not necessarily cut in the same fashion).

IVET *in vivo* expression technology; a procedure for identifying bacterial genes that are expressed during infection rather than during growth in the laboratory.

kilobase a nucleic acid region that is 1000 bases long (abbreviated to kb).

Lambda temperate bacteriophage of *E. coli*, used as a cloning vector.

Leader nucleotide sequence at the 5′ end of mRNA, before the start point for translation of the first structural gene; often involved in regulating gene expression.

Ligand a (usually small) molecule that binds non- covalently to a specific site(s) on a protein.

Ligation joining two DNA molecules using DNA ligase.

Linkage the degree to which two genes are inherited together.

Linkage analysis mapping the relative position of two genes (or other markers) by determining the extent to which they are co-inherited.

Linker a short double-stranded oligonucleotide with blunt ends and an internal restriction site, used to add sticky ends to a blunt-ended fragment (see *adaptor*).

Lysogeny a (more or less) stable relationship between a bacteriophage (*prophage*) and a host bacterium (lysogen).

Lytic cycle multiplication of a bacteriophage within a host cell, leading to lysis of the cell and infection of other sensitive bacteria.

M13 a filamentous bacteriophage of *E. coli*, with a single-stranded DNA genome; used as a cloning vector.

Macroarray a large set of DNA spots immobilized on a membrane; used for comparative and differential studies of genomes and transcriptomes (c.f. *microarray*).

Mapping determination of the position of genes (*genetic map*), or of physical features such as restriction endonuclease sites (*physical map*).

Melting separation of double-stranded DNA into single strands (see *denaturation*).

Melting temperature (T_m) the temperature at which the two strands of a DNA or a DNA/RNA molecule separate (denature).

Messenger RNA (mRNA) RNA molecule used by ribosomes for translation into a protein.

Microarray a large set of DNA spots immobilized on a glass slide; used for comparative and differential studies of genomes and transcriptomes (c.f. *macroarray*).

Microinjection direct injection of DNA into the nucleus of a cell.

Microsatellite tandem repeats of a short sequence of nucleotides; variation in the number of repeats causes *polymorphism*.

Mobilization transfer by conjugation of a non- conjugative plasmid in the presence of a conjugative plasmid.

Modification alteration of the structure of DNA (usually by methylation of specific residues) so that it is no longer a substrate for the corresponding *restriction endonuclease*.

Monoclonal antibody a homogeneous population of identical antibody molecules produced by an immortalized lymphocyte cell line.

Mosaic genes genes composed of domains from different sources (see *domain shuffling*).

Mosaic a transgenic animal in which the cloned gene is present in only a proportion of the cells or tissues (c.f. *chimaera*).

Motif conserved sequence within a family of proteins indicating a specific function.

Multiple cloning site a short region of a vector containing a number of unique restriction sites into which DNA can be inserted.

Mutagenesis treatment of an organism with chemical or physical agents so as to induce alterations in the genetic material (see also *site-directed mutagenesis*).

Mutant a cell (or virus) with a change in its genetic material (c.f. *mutation*).

Mutation an alteration in the genetic material (c.f. *mutant*).

Nested PCR a technique for increasing the sensitivity and/or specificity of PCR, by using a second set of primers internal to the first pair.

Nick a break in one strand of a double-stranded DNA molecule.

Nonsense mutation base substitution creating a *stop codon* within the coding sequence, causing premature termination of translation.

Northern blot a membrane with RNA molecules transferred from an electrophoresis gel for hybridization.

Oligonucleotide a short nucleic acid sequence (usually synthetic).

Open reading frame (ORF) a nucleic acid sequence with a reading frame that contains no stop codons; it therefore defines a potentially translated polypeptide.

Operator a region of DNA to which a repressor protein binds to switch off expression of the associated gene. Usually found adjacent to, or overlapping with, the promoter.

Operon a group of contiguous genes (in bacteria) that are transcribed into a single mRNA, and hence are subject to co-ordinated induction/repression.

Ordered library a collection of clones containing overlapping fragments, in which the order of the fragments has been determined.

Origin of replication position on a DNA molecule at which replication starts; most commonly used to mean that part of a plasmid that is necessary for replication.

P1 a bacteriophage that infects *E. coli*; used as the basis for some cloning vectors.

Packaging the process of incorporating DNA into a bacteriophage particle (see also *in vitro packaging)*.

Packaging limits the range of DNA sizes that can be packaged into a specific bacteriophage particle.

Palindrome a sequence that reads the same in both directions (on the complementary strands).

Partial digest cutting DNA with a restriction endonuclease using conditions under which only a fraction of available sites are cleaved.

Partitioning distribution of copies of a plasmid between daughter cells at cell division.

Pathogenicity island a DNA region (in bacteria) carrying virulence determinants; often with a different base composition from the remainder of the chromosome.

Phage see *bacteriophage*.

Phage display a technique for expressing cloned proteins on the surface of a bacteriophage.

Pharming producing a recombinant protein from a genetically modified farm animal.

Phenotype the observable characteristics of an organism (c.f. *genotype*).

Physical map a map of the physical structure of a genome, e.g. showing restriction sites, position of specific clones, or ultimately the complete sequence (c.f. *genetic map*).

Plaque a region of clearing, or reduced growth, in a bacterial lawn, as a result of phage infection.

Plasmid an extrachromosomal genetic element, capable of autonomous replication.

Plus and minus strands mRNA is defined as the plus (sense) strand, and the complementary sequence as the minus (antisense) strand; DNA sequences maintain the same convention, so it is the minus strand of DNA that is transcribed to yield the mRNA (plus strand).

Point mutation an alteration (or deletion/insertion) of a single base in the DNA.

Polar mutation a mutation in one gene that affects the expression of others (e.g. genes downstream in an operon); the phenotypic effect may not be directly caused by the original mutation.

Polyadenylation a natural process (mainly in eukaryotes) which produces a long string of adenyl residues at the 3′ end of the mRNA (should strictly be ‘polyadenylylation’ but ‘polyadenylation’ is commonly used).

Polycistronic mRNA messenger RNA coding for several proteins (see *operon*).

Polymerase chain reaction (PCR) enzymatic amplification of a specific DNA fragment, using repeated cycles of denaturation, primer annealing and chain extension.

Polymorphism detectable variation in genome structure between individuals in a population.

Positional cloning using the mapped position of a gene to obtain a clone carrying it.

Post-translational modification modification of the structure of a polypeptide after synthesis, e.g. by phosphorylation, glycosylation, proteolytic cleavage.

Primary structure the base sequence of a nucleic acid, or the amino acid sequence of a protein.

Primer a specific oligonucleotide, complementary to a defined region of the template strand, from which new DNA synthesis will occur.

Primer walking a technique in DNA sequencing whereby information from one sequence run is used to design another primer to extend the sequence determined.

Probe a nucleic acid molecule that will hybridize to a specific target sequence.

Prokaryote a cell that does not have a discrete nucleus bounded by a membrane (c.f. *eukaryote*); often used as synonymous with bacteria but also includes the *Archaea* (formerly termed *Archaebacteria*, but now recognized as evolutionarily distinct).

Promoter region of DNA to which RNA polymerase binds in order to initiate transcription.

Promoter probe vector a vector carrying a promoterless reporter gene, so that inserts carrying a promoter can be detected.

Proof-reading the ability of DNA polymerase to check, and correct, the accuracy of the newly made sequence.

Prophage the repressed form of bacteriophage DNA in a lysogen; it may be integrated into the chromosome or exist as a plasmid.

Protein engineering altering a gene so as to produce defined changes in the properties of the encoded protein.

Proteome the complete content of different proteins in a cell (c.f. *genome, transcriptome*).

Proteomics global study of protein expression in an organism.

Protoplast formed by complete removal of the cell wall, using osmotically stabilized conditions.

Prototroph a nutritionally wild-type organism that does not need any additional growth supplement (c.f. *auxotroph*).

Pseudogene a gene, usually recognizable as a copy of another gene, that does not produce a protein product.

Pulsed-field gel electrophoresis separation of large DNA molecules by application of an intermittently varying electric field.

Purine one of the two types of bases in nucleic acids (adenine, guanine) (see *pyrimidine*).

Pyrimidine one of the two types of bases in nucleic acids (cytosine and thymine in DNA; cytosine and uracil in RNA) (see *purine*).

RACE (rapid amplification of cDNA ends) a PCR-based method for obtaining the full length of a cDNA.

Random primers synthetic oligomeric nucleotides (usually hexamers) designed to act as primers for DNA synthesis at multiple sites.

Reading frame a nucleic acid sequence is translated in groups of three bases (*codons*); there are three possible ways of reading the sequence (in one direction), depending on where you start-these are the three reading frames.

Real time PCR a PCR technique, using fluorescent dyes, that makes it possible to monitor the progress of the amplification as it occurs.

Recombination (a) the production of new strains by mating two genetically distinct parents; (b) the generation of new DNA molecules by breaking and re-joining the original molecules.

Relaxation conversion of supercoiled circular plasmid DNA to an open circular form.

Replacement vector a lambda cloning vector in which a piece of DNA (the *stuffer fragment*) can be removed and replaced by the cloned fragment (c.f. *insertion vector*).

Replica plating transfer of colonies from one plate to others, in the same position, for differential screening.

Replication synthesis of a copy of a DNA molecule.

Replicon a DNA molecule that can be replicated; also used to refer to the replication control region of a plasmid (c.f. *origin of replication*).

Reporter gene a gene that codes for a readily detected protein for study of the regulation of gene expression.

Repression (a) reduction in transcription of a gene, usually due to the action of a repressor protein; (b) establishment of lysogeny with temperate bacteriophages.

Repressor a protein that binds to a specific DNA site to switch off transcription of the associated gene.

Restriction reduction or prevention of phage infection through the production of *restriction endonucleases* which degrade foreign DNA (see also *modification*).

Restriction endonuclease an enzyme that recognizes specific DNA sequences and cuts the DNA, usually at the recognition site.

Restriction fragment length polymorphism (RFLP) variation between individuals or strains in the size of specific restriction fragments; used for strain typing, and for locating particular genes.

Restriction mapping determination of the position of restriction endonuclease recognition sites on a DNA molecule.

Retrovirus a virus with an RNA genome that is copied, by reverse transcriptase, into DNA after infection.

Reverse genetics making specific changes to the DNA and then examining the phenotype; contrasts with classical genetics in which you select mutants by their phenotype and then study the nature of the mutation.

Reverse transcription production of cDNA from an RNA template, by reverse transcriptase.

Ribonuclease an enzyme that digests RNA.

Ribosome binding site the region on an mRNA molecule to which ribosomes initially attach in bacteria.

RNA polymerase enzyme that synthesizes RNA, generally using a DNA template.

RT-PCR reverse transcription PCR; technique for producing an amplified DNA product from an mRNA template.

SDS-PAGE polyacrylamide gel electrophoresis in which proteins are separated according to molecular weight in the presence of sodium dodecyl sulphate.

Secondary structure the spatial arrangement of amino acids in a protein, or of bases in nucleic acid.

Selectable marker a gene that causes a phenotype (usually antibiotic resistance) that can be readily selected.

Shine–Dalgarno sequence see *ribosome binding site*.

Shotgun cloning insertion of random fragments of DNA into a vector.

Shotgun sequencing genome sequencing strategy involving the sequencing of large numbers of random fragments; the individual sequences are subsequently assembled by a computer.

Shuttle vector a cloning vector that can replicate in two different species, one of which is usually *E. coli*; facilitates cloning genes in *E. coli* initially and subsequently transferring them to an alternative host without needing to re-clone them.

Sigma factor polypeptide that associates with (bacterial) RNA polymerase core enzyme to determine promoter specificity.

Signal peptide amino acid sequence at the amino terminus of a secreted protein; involved in conducting the protein through the membrane, or targeting it to specific cellular locations.

Signal transduction extracellular conditions alter the conformation of a transmembrane protein which in turn alters the regulation of metabolic pathways within the cell.

Silent mutation a change in the DNA structure that has no effect on the phenotype of the cell.

Single nucleotide polymorphism (SNP) a single base difference in the DNA sequence of part of a population.

Site-directed mutagenesis a technique for specifically altering (*in vitro*) the sequence of DNA at a defined point.

Southern blot a membrane with DNA fragments transferred from an electrophoresis gel, preparatory to hybridization.

Splicing removal of introns from RNA and joining together of the exons.

Start codon position at which protein synthesis starts, usually AUG.

Stem-loop structure a nucleic acid strand containing two complementary sequences can fold so that these sequences are paired (stem), with the region between them forming a loop of unpaired bases (c.f. *hairpin*).

Sticky end the end of a DNA molecule where one strand protrudes beyond the other; also known as a c*ohesive end* (see *blunt end*).

Stop codon a codon which has no corresponding tRNA, and which signals the end of a region to be translated.

Stringency conditions affecting the *hybridization* of single-stranded DNA molecules; higher stringency (higher temperature and/or lower salt concentration) demands more accurate pairing between the two molecules.

Structural genes genes coding for enzymes (or sometimes other products), as distinguished from regulatory genes.

Stuffer fragment piece of DNA that is removed from a vector such as a replacement lambda vector, and replaced by the cloned DNA fragment.

Suicide plasmid a vector that is unable to replicate in a specific host; maintenance of the selected marker requires integration into the chromosome.

Supercoiling coiling of a double-stranded DNA helix around itself.

Superinfection immunity resistance of a lysogen to infection by the same (or related) bacteriophage.

Suppression the occurrence of a second mutation which negates the effect of the first without actually reversing it.

SV40 Simian virus 40; small virus isolated from monkeys; used as a vector, and also as a source of expression signals in mammalian expression vectors.

Synonymous codons different codons that code for the same amino acid.

T7 Lytic bacteriophage of *E. coli*; the requirement of T7 RNA polymerase for a highly specific promoter is used in several contexts.

TA cloning a method for cloning PCR products, exploiting the tendency of *Taq* polymerase to add a non- specific adenyl residue to the 3′ end of the new DNA strand.

Tagging constructing a recombinant so that the protein formed has additional amino acids at one end, facilitating purification by affinity chromatography, or targeting the protein to specific destinations.

Tailing adding a not exactly defined number of nucleotides to the 3′ end of DNA, using *terminal transferase*.

Tandem repeat occurrence of the same sequence two or more times, directly following one another.

***Taq* polymerase** a thermostable DNA polymerase, commonly used for PCR.

Telomere end region of a eukaryotic chromosome containing sequences that are replicated by a special process, counteracting the tendency of linear molecules to be shortened during replication.

Temperate describes a bacteriophage that is able to enter *lysogeny*.

Template a single strand of nucleic acid used for directing the synthesis of a complementary strand.

Terminal transferase an enzyme that adds nucleotides, non-specifically, to the 3′ ends of DNA (see *tailing*).

Terminator site at which transcription stops.

Tertiary structure folding of secondary structure components of a protein.

TI plasmid Tumour-inducing plasmid, in *Agrobacterium tumefaciens*; used as a vector in genetic manipulation of plants.

Topoisomerase an enzyme that alters the supercoiling of DNA by breaking and rejoining DNA strands.

Totipotent describes a cell that is capable of giving rise to all types of cell within a whole animal or plant.

***trans*-acting** a gene that influences non-adjacent regulatory DNA sequences, through the production of a diffusible protein (c.f. *cis-acting*).

Transcription synthesis of RNA according to a DNA template.

Transcriptional fusion a recombinant construct in which a promoterless insert is transcribed from a promoter on the vector (c.f. *translational fusion*).

Transcriptome the complete mRNA content of a cell (c.f. *genome, proteome*).

Transduction bacteriophage-mediated transfer of genes from one bacterium to another.

Transfection introduction of viral nucleic acids into a cell.

Transformation introduction of extraneous DNA into a cell; also used to mean the conversion of an animal cell into an immortalized, tumour-like, cell.

Transgenic describes an animal or plant possessing a cloned gene in all its cells, so that the introduced gene is inherited by the progeny of that animal or plant.

Translation synthesis of proteins/polypeptides by ribosomes acting on a mRNA template.

Translational fusion a recombinant construct in which an insert lacking a translation start site is joined (in frame) to a fragment carrying translational signals (c.f. *transcriptional fusion*).

Transposon a DNA element carrying recognizable genes (e.g. antibiotic resistance) that is capable of inserting itself into the bacterial chromosome or a plasmid, independently of the normal host cell recombination machinery (c.f. *insertion sequence*).

Transposon mutagenesis disruption of genes by insertion of a transposon.

Trimming converting a sticky end to a blunt end by removing the unpaired nucleotides (c.f. *end filling*).

Two-dimensional gel electrophoresis separation of a complex mixture of proteins by a combination of *isoelectric focussing* and *SDS-PAGE.*

Universal primers sequencing primers derived from the sequence of the vector (pUC series); any insert can be sequenced with the same primers.

Upstream activator sequence a sequence, upstream from the promoter, which is required for efficient promoter activity.

Vaccinia smallpox vaccine virus, used as a vector for recombinant vaccine construction.

Vector a replicon (plasmid or phage) into which extraneous DNA fragments can be inserted, forming a recombinant molecule that can be replicated in the host cell.

Western blot a membrane with proteins transferred from an electrophoresis gel, usually for detection by means of antibodies.

X-gal 5-bromo-4-chloro-3-indolyl-β-D- galactoside; chromogenic substrate for beta-galactosidase.

Yeast artificial chromosome (YAC) a vector that mimics the structure of a yeast chromosome, used to clone very large DNA fragments.

Yeast centromere plasmid (YCp) a yeast vector containing a centromere; replicates at low copy number.

Yeast episomal plasmid (YEp) an autonomously replicating vector based on a yeast plasmid (the '2 μm circle').

Yeast integrating plasmid (YIp) a yeast vector that relies on integration into the yeast chromosome.

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