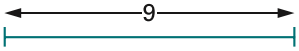
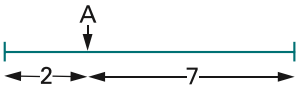
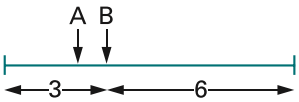
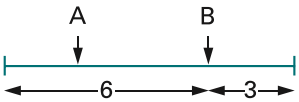
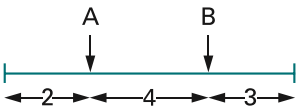
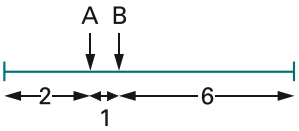
| Treatment | Measured sizes of fragments (kb) | Interpretation |
|---|---|---|
| No digestion | 9 |   |
| Enzyme A | 2 + 7 |   |
| Enzyme B | 3 + 6 | EITHER   |
| | | OR   |
| Enzymes A + B | 2, 3 + 4 |   |
| | alternative result1, 2 + 6 |   |

Fig. 5.25 Restriction mapping of DNA. Note that each experimental result and its interpretation should be considered in sequence, thus building up an increasingly unambiguous map.

as simple as this, and bioinformatic analysis of the restriction fragment lengths is usually needed to construct a map.

### 5.9.2 Nucleic acid blotting methods

Electrophoresis of DNA restriction fragments allows separation based on size to be carried out, however it provides no indication as to the presence of a specific, desired fragment among the complex sample. This can be achieved by transferring the DNA from the intact gel onto a piece of nitrocellulose or nylon membrane placed in contact with it. This provides a more permanent record of the sample since DNA begins to diffuse out of a gel that is left for a few hours. First the gel is soaked in alkali to render the DNA single stranded. It is then transferred to the membrane so that the DNA becomes bound to it in exactly the same pattern as that originally on the gel. This transfer, named a Southern blot after its inventor Ed Southern, can be performed electrophoretically or by drawing large volumes of buffer through both gel and membrane, thus transferring DNA from one to the other by capillary action (Fig. 5.26). The point of this operation is that the membrane can now be treated with a labelled DNA molecule, for example a gene probe (Section 5.9.4). This single-stranded DNA probe will hybridise under the right conditions to complementary fragments immobilised onto the membrane. The conditions of hybridisation, including the temperature and salt concentration, are critical for this process to take place effectively. This is usually referred to as

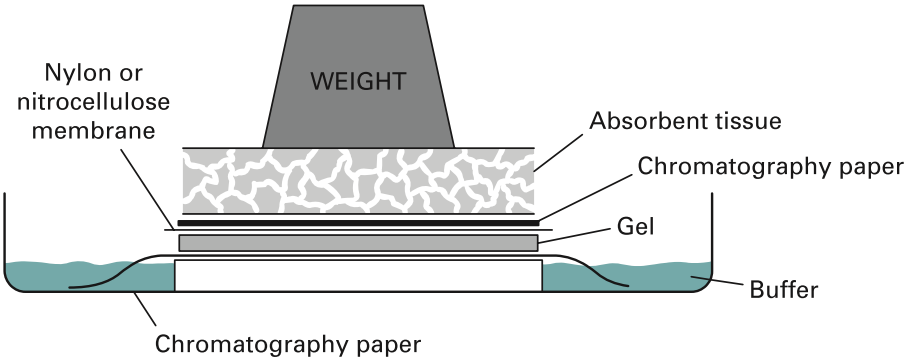



Fig. 5.26 Southern blot apparatus.

the stringency of the hybridisation and it is particular for each individual gene probe and for each sample of DNA. A series of washing steps with buffer is then carried out to remove any unbound probe and the membrane is developed after which the precise location of the probe and its target may be visualised. It is also possible to analyse DNA from different species or organisms by blotting the DNA and then using a gene probe representing a protein or enzyme from one of the organisms. In this way it is possible to search for related genes in different species. This technique is generally termed zoo blotting.

The same basic process of nucleic acid blotting can be used to transfer RNA from gels onto similar membranes. This allows the identification of specific mRNA sequences of a defined length by hybridisation to a labelled gene probe and is known as Northern blotting. It is possible with this technique to not only detect specific mRNA molecules but it may also be used to quantify the relative amounts of the specific mRNA. It is usual to separate the mRNA transcripts by gel electrophoresis under denaturing conditions since this improves resolution and allows a more accurate estimation of the sizes of the transcripts (Section 5.7.2). The format of the blotting may be altered from transfer from a gel to direct application to slots on a specific blotting apparatus containing the nylon membrane. This is termed slot or dot blotting and provides a convenient means of measuring the abundance of specific mRNA transcripts without the need for gel electrophoresis; it does not, however, provide information regarding the size of the fragments.

### 5.9.3 Design and production of gene probes

The availability of a gene probe is essential in many molecular biology techniques yet in many cases is one of the most difficult steps. The information needed to produce a gene probe may come from many sources; however, the availability of bioinformatics resources and genetic databases has ensured that this is the usual starting point for gene probe design.

In some cases it is possible to use related genes, that is from the same gene family, to gain information on the most useful DNA sequence to use as a probe. Similar proteins or DNA sequences but from different species may also provide a starting

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Polypeptide | | Phe | Met | Pro | Trp | His | |
| Corresponding nucleotide sequences | 5′ | T<br>TTC | ATC | T<br>CCC<br>A<br>G | TGG | T<br>CAC | 3′ |

Fig. 5.27 Oligonucleotide probes. Note that only methionine and tryptophan have unique codons. It is impossible to predict which of the indicated codons for phenylalanine, proline and histidine will be present in the gene to be probed, so all possible combinations must be synthesised (16 in the example shown).

point with which to produce a so-called heterologous gene probe. Although in some cases probes are already produced and cloned it is possible, armed with a DNA sequence from a DNA database, to chemically synthesise a single-stranded oligonucleotide probe. This is usually undertaken by computer-controlled gene synthesisers which link dNTPs (deoxyribonucleoside triphosphates) together based on a desired sequence. It is essential to carry out certain checks before probe production to determine that the probe is unique, is not able to self-anneal or that it is self-complementary, all of which may compromise its use.

Where little DNA information is available to prepare a gene probe it is possible in some cases to use the knowledge gained from analysis of the corresponding protein. Thus it is possible to isolate and purify proteins and sequence part of the N-terminal end or an internal region of the protein. From our knowledge of the genetic code, it is possible to predict the various DNA sequences that could code for the protein, and then synthesise appropriate oligonucleotide sequences chemically. Due to the degeneracy of the genetic code most amino acids are coded for by more than one codon, therefore there will be more than one possible nucleotide sequence that could code for a given polypeptide (Fig. 5.27). The longer the polypeptide, the greater the number of possible oligonucleotides that must be synthesised. Fortunately, there is no need to synthesise a sequence longer than about 20 bases, since this should hybridise efficiently with any complementary sequences, and should be specific for one gene. Ideally, a section of the protein should be chosen which contains as many tryptophan and methionine residues as possible, since these have unique codons, and there will therefore be fewer possible base sequences that could code for that part of the protein. The synthetic oligonucleotides can then be used as probes in a number of molecular biology methods.

### 5.9.4 Labelling DNA gene probe molecules

An essential feature of a gene probe is that it can be visualised or labelled by some means. This allows any complementary sequence that the probe binds to be flagged up or identified.

There are two main types of label used for gene probes: traditionally this has been carried out using radioactive labels, but gaining in popularity are non-radioactive labels.

Perhaps the most common radioactive label is 32-phosphorus ($^{32}P$), although for certain techniques 35-sulphur ($^{35}S$) and tritium ($^{3}H$) are used. These may be detected by the process of autoradiography where the labelled probe molecule, bound to sample DNA, located for example on a nylon membrane, is placed in contact with an X-ray-sensitive film. Following exposure the film is developed and fixed just as a black-and-white negative. The exposed film reveals the precise location of the labelled probe and therefore the DNA to which it has hybridised.

Non-radioactive labels are increasingly being used to label DNA gene probes. Until recently radioactive labels were more sensitive than their non-radioactive counterparts. However, recent developments have led to similar sensitivities which, when combined with their improved safety, have led to their greater acceptance.

The labelling systems are either termed direct or indirect. Direct labelling allows an enzyme reporter such as alkaline phosphatase to be coupled directly to the DNA. Although this may alter the characteristics of the DNA gene probe it offers the advantage of rapid analysis since no intermediate steps are needed. However indirect labelling is at present more popular. This relies on the incorporation of a nucleotide which has a label attached. At present three of the main labels in use are biotin, fluorescein and digoxygenin. These molecules are covalently linked to nucleotides using a carbon spacer arm of 7, 14 or 21 atoms. Specific binding proteins may then be used as a bridge between the nucleotide and a reporter protein such as an enzyme. For example, biotin incorporated into a DNA fragment is recognised with a very high affinity by the protein streptavidin. This may either be coupled or conjugated to a reporter enzyme molecule such as alkaline phosphatase. This is able to convert a colourless substrate *p*-nitrophenol phosphate (PNPP) into a yellow-coloured compound *p*-nitrophenol (PNP) and also offers a means of signal amplification. Alternatively labels such as digoxygenin incorporated into DNA sequences may be detected by monoclonal antibodies, again conjugated to reporter molecules such as alkaline phosphatase. Thus rather than the detection system relying on **autoradiography** which is necessary for radiolabels, a series of reactions resulting in the products of either a colour, light or the product of a **chemiluminescence** reaction take place. This has important practical implications since autoradiography may take 1–3 days whereas colour and chemiluminescent reactions take minutes.

### 5.9.5 End labelling of DNA molecules

The simplest form of labelling DNA is by 5′ or 3′ **end-labelling**. 5′ end labelling involves a phosphate transfer or exchange reaction where the 5′ phosphate of the DNA to be used as the probe is removed and in its place a labelled phosphate, usually $^{32}P$, is added. This is usually carried out by using two enzymes; the first, alkaline phosphatase, is used to remove the existing phosphate group from the DNA. Following removal of the released phosphate from the DNA, a second enzyme, polynucleotide kinase, is added which catalyses the transfer of a phosphate group ($^{32}P$-labelled) to the 5′ end of the DNA. The newly labelled probe is then purified, usually by chromatography through a Sephadex column, and may be used directly (Fig. 5.28).

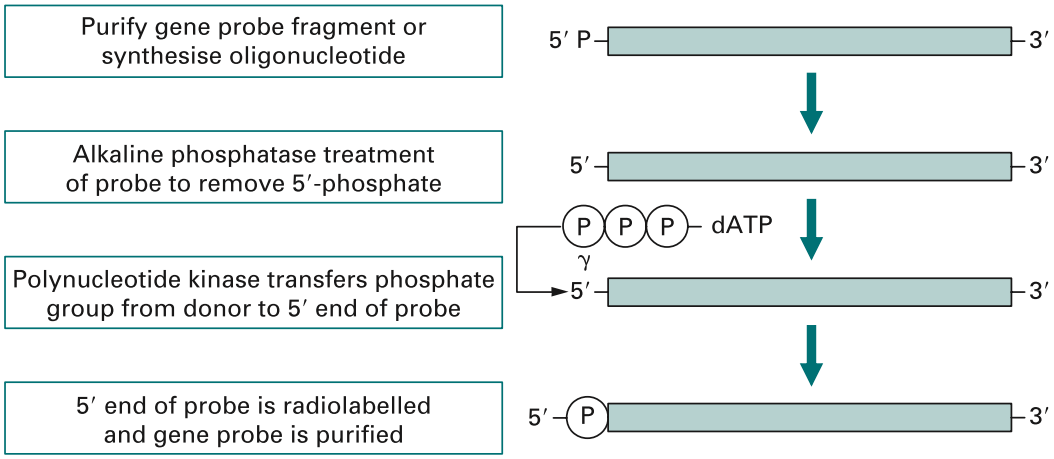



Fig. 5.28 End-labelling of a gene probe at the 5′ end with alkaline phosphatase and polynucleotide kinase.

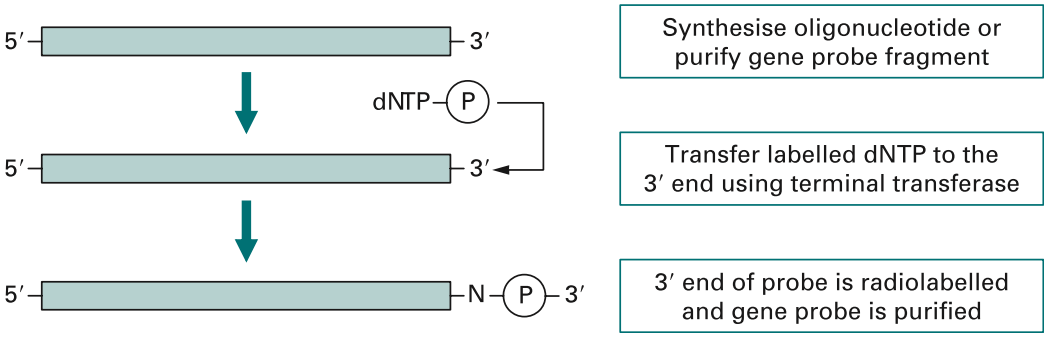



Fig. 5.29 End-labelling of a gene probe at the 3′ end using terminal transferase. Note that the addition of a labelled dNTP at the 3′ end alters the sequence of the gene probe.

Using the other end of the DNA molecule, the 3′ end, is slightly less complex. Here a new dNTP which is labelled (e.g. $^{32}$P-αdATP or biotin-labelled dNTP) is added to the 3′ end of the DNA by the enzyme terminal transferase. Although this is a simpler reaction a potential problem exists because a new nucleotide is added to the existing sequence and so the complete sequence of the DNA is altered which may affect its hybridisation to its target sequence. End-labelling methods also suffer from the fact that only one label is added to the DNA so they are of a lower activity in comparison to methods which incorporate label along the length of the DNA (Fig. 5.29).

### 5.9.6 Random primer labelling and nick translation

The DNA to be labelled is first denatured and then placed under renaturing conditions in the presence of a mixture of many different random sequences of hexamers or hexanucleotides. These hexamers will, by chance, bind to the DNA sample wherever they encounter a complementary sequence and so the DNA will rapidly acquire an approximately random sprinkling of hexanucleotides annealed to it. Each of the hexamers can act as a primer for the synthesis of a fresh strand of DNA catalysed by DNA polymerase since it has an exposed 3′ hydroxyl group. The Klenow fragment of DNA polymerase is used for random primer labelling because it lacks a 5′ to

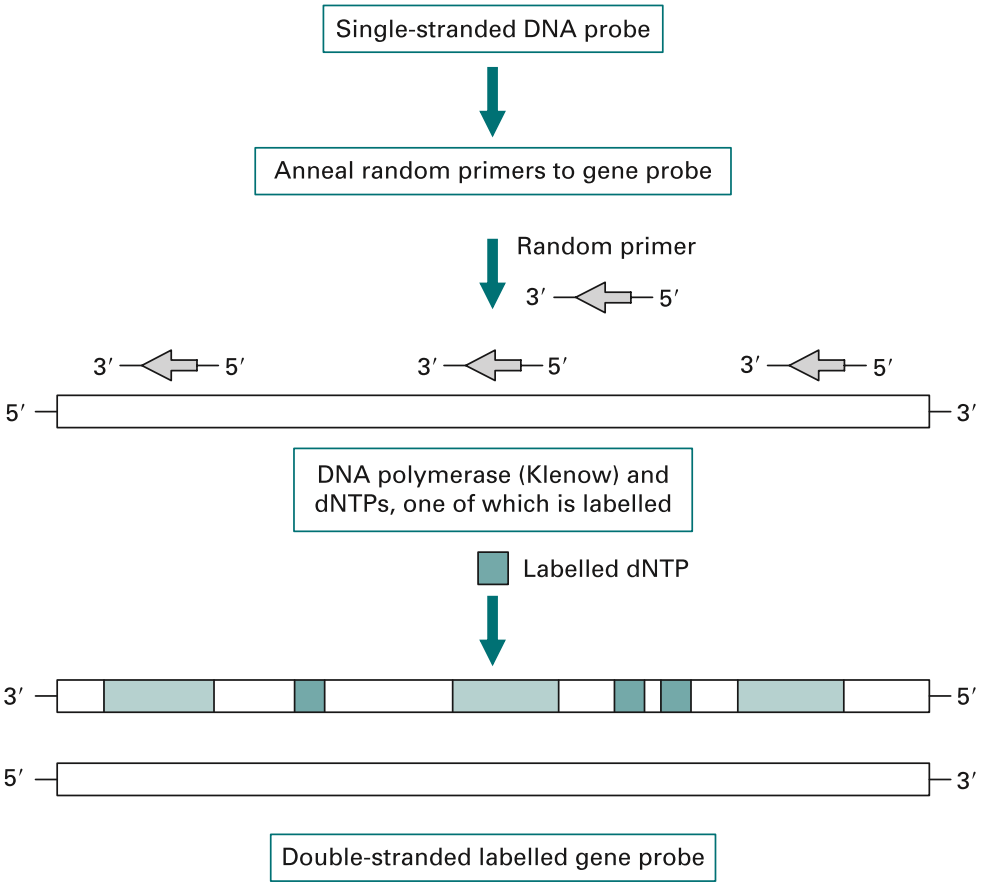



Fig. 5.30 Random primer gene probe labelling. Random primers are incorporated and used as a start point for Klenow DNA polymerase to synthesise a complementary strand of DNA whilst incorporating a labelled dNTP at complementary sites.

3′ exonuclease activity. This is prepared by cleavage of DNA polymerase with subtilisin, giving a large enzyme fragment which has no 5′ to 3′ exonuclease activity, but which still acts as a 5′ to 3′ polymerase. Thus when the Klenow enzyme is mixed with the annealed DNA sample in the presence of dNTPs, including at least one which is labelled, many short stretches of labelled DNA will be generated (Fig. 5.30). In a similar way to random primer labelling the polymerase chain reaction may also be used to incorporate radioactive or non-radioactive labels (Section 5.11.4).

A further traditional method of labelling DNA is by the process of **nick translation**. Low concentrations of DNase I are used to make occasional single-strand nicks in the double-stranded DNA that is to be used as the gene probe. DNA polymerase then fills in the nicks, using an appropriate dNTP, at the same time making a new nick to the 3′ side of the previous one (Fig. 5.31). In this way the nick is translated along the DNA. If labelled dNTPs are added to the reaction mixture, they will be used to fill in the nicks, and so the DNA can be labelled to a very high specific activity.

### 5.9.7 Molecular-beacon-based probes

A more recent development in the design of labelled oligonucleotide hybridisation probes is that of **molecular beacons**. These probes contain a fluorophore at one end of the probe

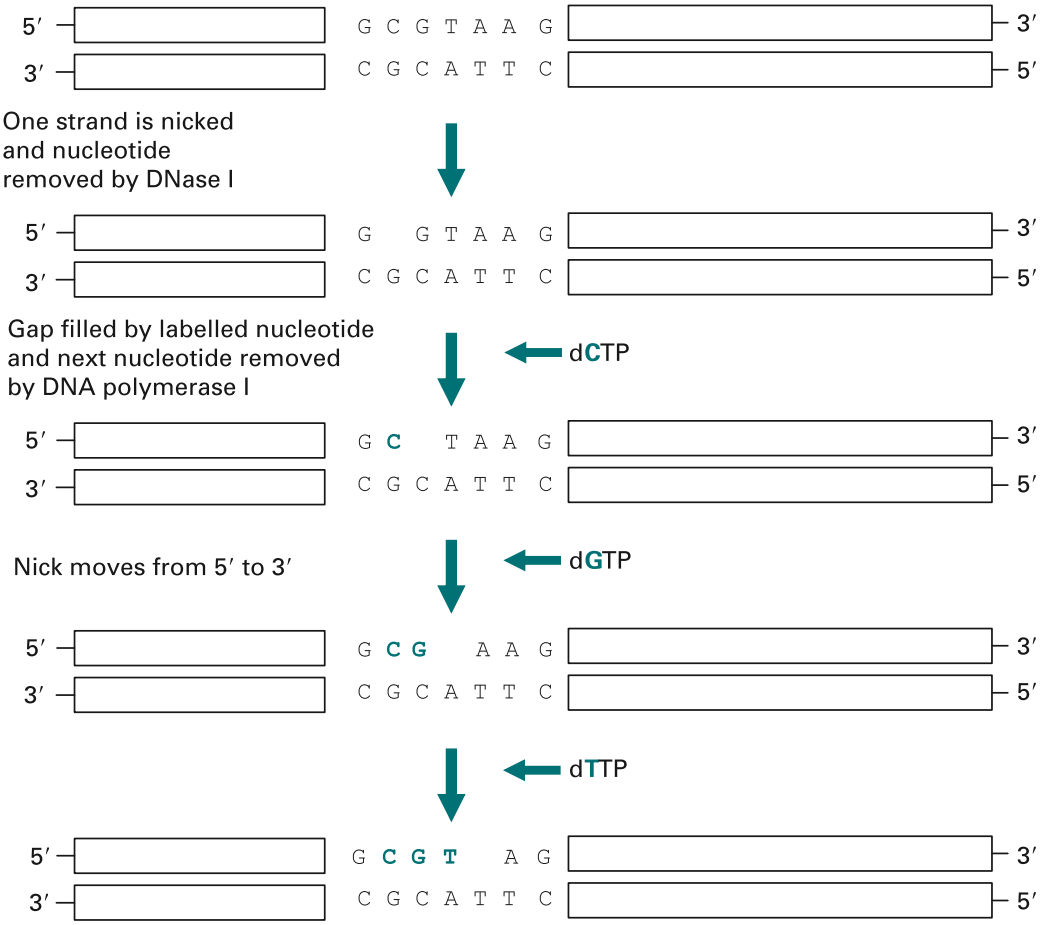



Fig. 5.31 Nick translation. The removal of nucleotides and their subsequent replacement with labelled nucleotides by DNA polymerase I increase the label in the gene probe as nick translation proceeds.

and a quencher molecule at the other. The oligonucleotide has a stem–loop structure where the stems place the fluorophore and quencher in close proximity. The loop structure is designed to be complementary to the target sequence. When the stem–loop structure is formed the fluorophore is quenched by Förster or fluorescence resonance energy transfer (FRET), i.e. the energy is transferred from the fluorophore to the quencher and given off as heat. The elegance of these types of probe lies in the fact that upon hybridisation to a target sequence the stem and loop move apart, the quenching is then lost and emission of light occurs from the fluorophore upon excitation. These types of probe have also been used to detect nucleic acid amplification system products such as the polymerase chain reaction (PCR) and have the advantage that it is unnecessary to remove the unhybridised probes.

## 5.10 THE POLYMERASE CHAIN REACTION (PCR)

### 5.10.1 Basic concept of the PCR

The polymerase chain reaction or PCR is one of the mainstays of molecular biology. One of the reasons for the wide adoption of the PCR is the elegant simplicity of the