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Southern blotting

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There have been many significant technical advances in molecular biology in the last 20 years but one of the most important is the technique known as Southern blotting. This simple and powerful procedure has had an enormous impact on the study of DNA and is in widespread use in molecular genetics laboratories throughout the world. The techniques on which Southern blotting is based are discussed and the use of the process is illustrated by reference to a specific genetic disease.

In the course of research and diagnostic procedures in molecular genetics, many questions can present themselves, for example:
Is DNA present in a particular sample?
Is there a mutation present in a particular important region of DNA such as a gene?
How big is a specific fragment of DNA?
Has rearrangement or deletion of a particular DNA region or gene occurred?
Has a cloning procedure proved successful?

The answers to questions such as these could, in a diagnostic situation, indicate whether or not a child is likely to be born affected by a serious genetic disease. Southern blotting has proved to be a key process in answering basic questions on the nature of DNA molecules. The technique was invented by Dr E. Southern of Edinburgh and published in 1975 (Southern, 1975), but like so many other advances depended absolutely on earlier discoveries. Southern blotting involves such important developments as:
the discovery of restriction enzymes;
the application of gel electrophoresis to the study of DNA;
the use of radioactive DNA probes to detect DNA sequences in a highly specific manner.

In order to understand the Southern technique, there follows a brief description of the developments essential to the Southern process. All these developments depend on the particular properties of DNA structure.

DNA STRUCTURE

DNA exists as a double-stranded structure in which the fundamental units of each strand are known as nucleotides. Nucleotides are made up of four bases: adenine (A), thymine (T), guanine (G) and cytosine (C) which are themselves linked to a sugar (ribose). The sugars are phosphorylated and it is through the phosphate groups that the bases are linked to form the strands of DNA. The two strands are held together principally by the hydrogen bonding between the A on one strand and the T on the other and the G on one strand and the C on the other. This phenomenon, known as base pairing, has significant implications, some of which will become apparent later. An immediate implication is, however, that if the order of bases on one strand is known then the order on the other strand can be deduced, since for every A on one strand there must be a T on the other and for every G a corresponding C:

Strand 1 AGCTTGCTAATGCCG
Strand 2 TCGAACGATTACGGC

The strands are said to be complementary and together form the famous Watson and Crick 'double helix' from the pattern of winding which was first observed by X-ray crystallography. The DNA molecules contained in the cells of an organism hold the information required to produce that organism and the DNA molecule can be enormously large. The haploid human genome contains about 3×10^9 base pairs (bp) which is the common unit of measurement of DNA. The **AT** pair shown previously is a single bp. In the human cell there is about 2 m DNA arranged in structures called chromosomes. Chromosomes contain about 6×10^7 bp and each chromosome has sufficient DNA to code for thousands of genes each with an average length of about 3000 bp (Alberts *et al.* 1983).

When we wish to study a particular gene a number of problems become apparent. DNA molecules can be extremely big, while the gene of interest is extremely small; a gene could be 1 000 000 times smaller than the chromosome from which it originates. In clinical situations, DNA is often extracted from blood samples so that total genomic DNA is obtained, that is, DNA representing all the chromosomes. The amounts of DNA extracted in clinical situations are usually small, 500 millionths of 1 g (500 μg) would be a reasonable yield from 10 ml blood. The target gene is present in extremely small amounts, so the problems faced in the study of the gene become clear. Methods are needed to break down the DNA into fragments of manageable size, the fragments have to be separated in some way and finally, the gene of interest detected. As the values quoted previously may indicate, it is a formidable task to study a single gene against the background of the total human genome. Fortunately, however, powerful tools became available to assist in the complex analyses required to study gene structure.

RESTRICTION ENZYMES

About 25 years ago, a type of enzyme was discovered which had a very specific property. This enzyme when added to a DNA sample introduces cuts in the DNA but only at particular sequences. For example, an enzyme called EcoRI isolated from the common bacteria *Escherichia coli* cuts DNA every time it encounters the sequence GAATTC, while the enzyme Sau3A isolated from *Staphylococcus aureus* will cut DNA every time it encounters the sequence GATC. Some enzymes recognize six bases and some four bases and on average a four-base enzyme will find a target every 256 bases in a random DNA sequence, while a six-base enzyme will find a target in similar DNA every 4096 bases. There are now hundreds of such enzymes available commercially and any one or combination of enzymes can be used to break down DNA samples into very small fragments (Roberts, 1982). Thus, the problem of how to break down DNA into manageable pieces can be easily overcome in the laboratory. The next step is to separate the vast number of fragments generated by restriction enzymes in some way, since by ordering the fragments detection is made easier.

GEL ELECTROPHORESIS

Gel electrophoresis is a common technique in biological science. Gels can be used to separate all kinds of molecules and DNA is no exception. The most commonly used gel

in the Southern analysis is the agarose gel. Agarose is a polysaccharide which can form a loose matrix when heated with water. To separate DNA fragments produced in a restriction digest, a slab of agarose is prepared by heating about 1 g agarose powder in 100 ml salt solution (40 mM-Tris acetate, 1 mM-EDTA, pH 8·0). The hot mixture is poured into a rectangular mould 110×140×10 mm in size and allowed to set. A well-former is placed at one end which produces small slots in the gel. When set, the gel is placed in a special tank and completely immersed in salt solution. The tank has electrodes at either end. DNA which has been treated with restriction enzyme is placed in the slots and the tank connected to a powerpack which provides a controlled electricity supply to drive the electrophoresis. DNA fragments which carry a negative charge begin to migrate through the gel towards the positive terminal. The smallest fragments move most quickly. At the end of a given period, the DNA fragments are distributed in a lane in the gel with the largest fragments at one end and the smallest at the other (Sambrook *et al.* 1989). Somewhere in this collection of millions of DNA fragments may be one of particular interest, containing perhaps a mutation which could seriously affect the life of an unborn child.

SOUTHERN BLOTTING

Agarose gels are very fragile and a gel comprising 10 g agarose/l as described previously must be handled very gently. The gel is, after all, 990 ml water/l. Somewhere in the matrix of the gel are the gene fragments of interest but which are virtually impossible to

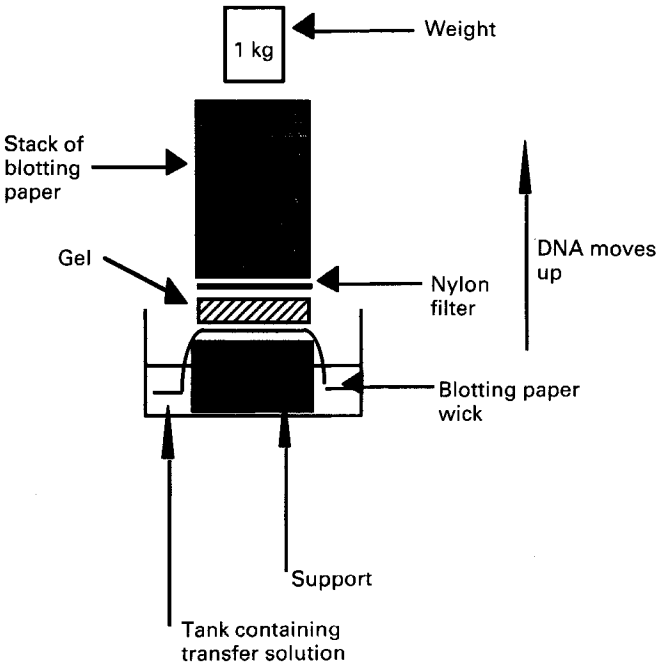

Fig. 1. The original Southern blotting method requires no special equipment to carry out the DNA transfer. As the process continues, the blotting paper in the stack becomes wet and can be replaced to ensure complete transfer of the DNA to the nylon filter.

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analyse while still contained within the gel. The great achievement of Southern was to devise a simple and reliable method of extracting the DNA fragments from the gel in a manner which then allowed straightforward analysis using radioactive probes. The problem of getting the DNA out of the gel was solved by placing a portion of thin nitrocellulose filter material (this filter looks like thin white paper) on top of the gel which was itself sitting on a wick soaked in salt solution. A pad of blotting paper was placed on top of the filter and a weight placed on top of that. As the weight pressed down on the stack, salt solution began to move up through the gel and into the blotting paper, carrying as it did so, the DNA fragments. When the DNA fragments met the filter they could move no further so they adhered to the filter surface (Fig. 1). The process was usually allowed to continue for a few hours to ensure that all the DNA was carried out of the gel. By this technique a filter was obtained which had bound to it all the DNA originally in the gel and in exactly the same pattern as it had been in the gel. The procedure was likened to blotting an ink signature hence the term 'Southern blotting' was coined (Sambrook *et al.* 1989).

Today, more rapid methods of blotting are available. In vacuum transfer, for example, the DNA is drawn out of the gel under vacuum in 1 or 2 h onto extremely tough filter material made of nylon, which is much less fragile than the nitrocellulose first used. Nylon filter is very efficient at binding DNA and with the DNA fragments bound to such a robust material, it is now possible to carry out many different analyses of the bound DNA. The final question remaining is how to determine whether or not an altered DNA gene is present in the original sample? It is here that the last of the three developments referred to earlier is involved.

HYBRIDIZATION

One of the remarkable properties of DNA is that if a solution of double-stranded DNA fragments is heated to 65–70°, the double strands will separate into single strands, but on cooling the strands will come together exactly as they were before. The reason for this was hinted at earlier. DNA strands existing in the double-helix form are said to be complementary, that is, a strict base-pairing rule exists (A with T and C with G). For every single strand produced when DNA is melted, there is only one single strand which will complement it exactly. This process of two strands coming together by base pairing is known as hybridization and is the basis of extremely sensitive detection used in the Southern blot (Sambrook *et al.* 1989).

Over the years, a vast number of DNA fragments have been isolated from the human genome and the genomes of many other organisms by the process known as cloning. Small fragments of DNA from various sources have been incorporated (cloned) into bacterial or viral DNA and by using the bacterial or viral replication systems, large quantities of the cloned material can be produced. For many of the cloned DNA regions, the exact origin in the genome from which they were derived is known and these cloned fragments can be used in hybridization studies as molecular probes.

SOUTHERN BLOTTING AND DNA PROBES

DNA is isolated from a suitable source, for example, leucocytes and 4–5 μg are digested using a restriction enzyme. The digested DNA is then placed in a well in an agarose gel

and electrophoresis carried out to separate the digested fragments. When electrophoresis is complete, the DNA is transferred to a nylon filter by Southern blotting. The nylon filter is then placed in a container with a few millilitres of a solution called a prehybridization solution and heated to 65°. The heating causes the DNA strands bound to the nylon filter to separate and the solution which contains a mixture of protein, polysaccharides and detergent coats the surface of the filter (this is known as blocking and prevents non-specific binding of the probe). The separated strands become accessible to DNA probe. A cloned DNA fragment (the DNA probe) is then labelled in an enzymic reaction. A common labelling method is to add radioactive nucleotides to the probe DNA, although many non-radioactive labelling methods also exist. The solution containing the labelled probe is then added to the filter in its container at 65° and hybridization allowed to continue for a few hours. In the process of hybridization, the radioactive probe will base pair to the region of the genome bound to the filter which complements it exactly. After a suitable time, the filter is washed and an X-ray film is laid on top. The film is developed 12–24 h later and if the probe has detected anything, bands should be visible on the film (Sambrook *et al.* 1989).

The technique as described is in use for many purposes, and is particularly useful in the diagnosis of certain genetic diseases. A specific example is illustrated below.

MYOTONIC DYSTROPHY

Myotonic dystrophy is an autosomal dominant disorder with an incidence of one in 8000 and is one of the most prevalent hereditary diseases in adults. The age of onset of symptoms is 20–25 years, but a potentially-fatal maternally-transmitted form of the disease can affect neonates. Manifestations of the disorder include muscle wasting, myotonia, cataracts, intellectual impairment and cardiac conduction defects. Myotonic dystrophy demonstrates an increase in severity accompanied by earlier age of onset in successive generations, a phenomenon termed 'anticipation' and for this reason prenatal diagnosis may be required when an affected woman becomes pregnant (Harper, 1989). The gene for myotonic dystrophy was identified in 1992 as a possible serine–threonine protein kinase sited on chromosome 19 at region 19q13.3. The mutation associated with the disease is known as a triplet repeat. At one end of the protein-kinase gene a sequence occurs which has the form $(CTG)_n$ where n in normal chromosomes lies between 5 and 35. As few as fifty repeats are present in mildly-affected individuals and those who are severely affected may have over 2000 repeats (Aslanidis *et al.* 1992; Harley *et al.* 1992). Although it is not yet clear exactly how an expansion in the triplet number affects the gene function, possession of an expansion is diagnostic for the disease. The question asked of a molecular genetics laboratory by the clinician might then be 'Here is a blood sample from a woman affected by myotonic dystrophy and a chorion villus biopsy from her unborn child. Will the baby be affected by the same disease?'

DIAGNOSTIC PROCEDURE

DNA is extracted from both samples and each sample is digested with the restriction enzyme EcoRI. When the gene was discovered, the triplet region was examined carefully and found to lie in a region of DNA flanked by EcoRI restriction sites. The triplet region lay in a fragment 10 000 bp long. If the triplet is expanded then the size of this fragment

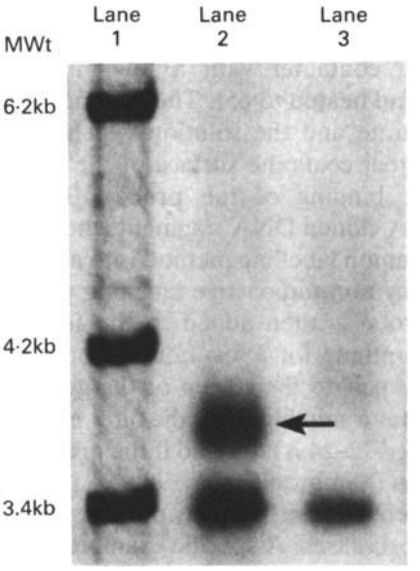

Fig. 2. This photograph shows an autoradiograph of a Southern blot used to diagnose myotonic dystrophy. Lane 1 contains DNA fragments of known molecular weight. Lane 2 shows DNA from a patient suspected of having myotonic dystrophy. The DNA is digested with restriction enzyme BgII, resolved on a 10 g agarose/l gel, blotted and probed with a myotonic gene probe. Two bands are visible, one at 3·4 kb and another (←) at 3·6 kb. This additional band is caused by an expansion of approximately 200 base pairs or seventy triplet repeats in the myotonic dystrophy gene. This patient has mild symptoms of the disease. Lane 3 shows DNA from a normal individual similarly analysed. Only one band is visible since both myotonic dystrophy gene regions are identical and have no expansions.

increases so that, for example, in a severe case where there are 1000 repeats the fragment would be increased by 3000 bp (3×1000) to 13 000 bp. This size variation can be determined easily by Southern blotting. The digested DNA is separated on an agarose gel and transferred to nylon membrane for hybridization. The probe used in the test is a small piece of DNA from the region adjacent to the triplet repeat. This probe should detect two fragments of EcoRI digestion in the DNA from the mother and unborn child, the normal fragment from the normal chromosome and the expanded fragment from the mutated chromosome. If a fragment of greater than 10 000 bp is detected in the material from the unborn child then this indicates that the child will be affected. Another restriction enzyme, BgII, has sites which are very close to the triplet expansion region. This enzyme is very useful for detecting small mutations in the gene since even very small expansions may be clinically significant (Shelbourne *et al.* 1992). Fig. 2 shows how a small myotonic dystrophy mutation can be detected using the Southern technique.

CONCLUSION

This account of the application of Southern blotting has been illustrated by reference to one particular disorder, but similar types of analyses can be carried out for other diagnostic purposes and for research in all aspects of molecular biology. The technique has proved to be so valuable that one commentator noted 'For the past ten years,

Southern blotting has been the foundation stone of gene analysis in inherited disease' (Brock, 1993). The introduction of DNA amplification and automated DNA fragment analysers will certainly reduce the use of Southern blotting in years to come, but for certain applications there are no substitutes in view.

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