Occasional article

The polymerase chain reaction

I PEAKE

From the Department of Haematology, University of Wales College of Medicine, Cardiff, Wales

Occasionally a technique is developed in an area of science which so improves the scientist's ability to practice his or her art that it quickly becomes an essential standard technique. An example of such a development would be the ability to produce monoclonal antibodies for an experimental immunologist. The polymerase chain reaction (PCR) represents an equally momentous advance in the field of molecular biology, in particular in the analysis of DNA (or RNA). With this technique, a given sequence of DNA may be amplified to the extent that it may be analysed directly, even from minute amounts of starting material. As a result specific sequences can be rapidly analysed for polymorphisms, point mutations, and other features by various techniques, including direct DNA sequencing, restriction enzyme analysis, and the use of highly specific oligonucleotide probes. The only requirement is that the nucleotide sequence of short regions of DNA flanking the region of interest is known.

PCR technique

DNA polymerase enzymes have been used in molecular biology for many years because they can synthesise the complementary strand of a DNA sequence given a mixture of the necessary deoxynucleotide triphosphates and, more importantly, a priming short double stranded sequence. Synthesis is directed 5' to 3' from the priming region. In the PCR, double stranded DNA sequences are selectively produced by adding a large excess of specific DNA oligonucleotides to the DNA, under appropriate conditions. These oligonucleotide primers can now be readily synthesised in the laboratory using automatic equipment, and are generally made to be 20 to 25 bases in length. In practice, two specific oligonucleotide primers are synthesised and added to the DNA so that they flank the region of interest, primer 1 being a copy of the coding strand, and primer 2 a copy of the non-

coding strand (fig 1). Once the primers have been added, their binding to the DNA (annealing) is achieved by melting the double stranded DNA at 94°C and cooling the mixture to 40-55°C. The addition of the polymerase enzyme at this point will lead to synthesis of complementary DNA strands along both the original coding and non-coding strands from the primer binding sites. If the process is then repeated—that is, melting at 94°C followed by cooling cyclically—then the area of DNA between the two primers will become amplified logarithmically because newly synthesised strands become templates for the excess primers.

In the original report of this technique¹ polymerase enzyme from *Escherichia coli* (Klenow fragment DNA polymerase I) was used, but this enzyme is destroyed at the melting temperature of 94°C, necessitating the addition of fresh enzyme for each cycle. The introduction of a thermostable DNA polymerase enzyme from *Thermus aquaticus* (*Taq*) which can survive extended incubation at 94°C, and which is most active at about 70°C, has recently removed the necessity to add polymerase at each cycle.²⁻⁴ This also means that at the relatively high temperature (70°C) of the synthesis or extension stage the primers will tend to hybridise specifically to their complementary sequence, so reducing the amplification of non-specific areas.

The PCR process is now generally carried out using the *Taq* polymerase (fig 2). Incubation times for the denaturing or melting stage, the reassociation or annealing stage, and the extension stage are in the order of two to three minutes, and more than 30 cycles are used, giving an estimated enrichment of the selected sequence of 10⁵ to 10⁶. The whole process has been automated, using microprocessor controlled heating blocks in which rapid temperature changes can be achieved. The times for the various stages within the process can, of course, be varied, depending on the particular experiment. For example, if a long sequence of DNA is to be amplified (at present the maximum seems to be 2-3 kilobase pairs) then the

Accepted for publication 14 February 1989

Fig 1 An example of the sequence and hybridisation sites of oligonucleotide primers for a hypothetical PCR amplification. Primer 1 is a 19 base (19-mer) copy of the coding strand, binds to the non-coding strand, and is extended by the polymerase reaction to give a second coding strand. Primer 2 is a 19-mer copy of the original non-coding strand, binds to the coding strand, and will yield a copy of the non-coding strand by the polymerase reaction.

POLYMERASE CHAIN REACTION

Fig 2 A diagrammatic representation of the polymerase chain reaction. The original section of double stranded DNA (top right) has the area of interest encircled. The three stages of the PCR process are labelled as (i) heat denature (melt); (ii) reassociate (annealing); and (iii) DNA polymerase (extension with Taq polymerase).

The polymerase chain reaction

extension time is increased. If the amount of starting material is very small then upwards of 50 cycles may be necessary. After PCR the DNA sample is analysed by polyacrylamide gel electrophoresis (PAGE), where a predominant single band equivalent in length to the distance between the 5' ends of the oligonucleotide primers is seen after staining with ethidium bromide and examination of the gel under ultraviolet light.

Applications of PCR

DETECTION OF DNA POLYMORPHISMS AND POINT MUTATIONS

The ability to amplify selectively a given area of DNA has many potential applications in molecular biology, genetics, and microbiology. In the initial studies PCR was successfully used to amplify regions containing restriction enzyme sites which were either polymorphic (haemophilia A studies using intragenic *BclI* and *XbaI* polymorphic restriction sites³, or were affected by the point mutation implicated in the disease state (sickle cell anaemia for example, where an adenosine to thymine mutation at the second position of the sixth codon of the β globin chain gene results in a glutamic acid to valine change and also loss of a *DdeI* restriction enzyme site.¹² In these studies the effect of restriction enzyme digestion on PCR amplified DNA may be studied directly by PAGE, with the identification of one or two bands, depending on the presence or absence of the restriction enzyme site. In these studies care must be taken to choose primer sites not equidistant from the restriction enzyme site, so that the two bands seen after digestion are well separated.

Single nucleotide changes can also be readily detected in PCR amplified material by probing with specific oligonucleotides for the "new" sequence and comparing its binding with that of the "wild type sequence" or normal sequence. As with PCR oligonucleotide primers, the availability of automatic DNA synthesizers enables such probes to be readily made in the laboratory. In this technique single stranded PCR amplified DNA is blotted on to a membrane (nitrocellulose or nylon based), which is then incubated in a hybridisation solution containing the probe labelled with radioactive [32] P or a non-radioactive marker, such as biotin. By careful adjustment of the hybridisation and washing temperatures differential binding of the two oligonucleotides can be observed. This dot blot procedure has been shown to be of particular use in the screening of large numbers of samples.

DNA SEQUENCING

In many cases the most direct procedure for the detection of a specific gene defect is by DNA sequencing of PCR amplified material either directly using

specific sequence primers,³ or after ligation of the DNA into standard sequencing vectors such as M13.⁶ This latter procedure can be facilitated by the inclusion of a restriction enzyme site at the end of the PCR primers, so allowing for easy ligation into the sequencing vector. Care must be taken with this technique, however, because PCR artefacts (on average one nucleotide is miscopied in the PCR reaction every 20 cycles) may be present in a particular sequencing clone, and it is therefore wise to sequence several independent clones. Direct sequencing of PCR amplified material will result in such artefacts being masked by the "normal" sequence, although the quality of the sequencing gels is often inferior by the direct procedure.

ANALYSIS OF RNA

One of the most interesting uses for PCR has been its application not only in DNA analysis, but also in RNA analysis. As in many cases over 90% of the DNA within a gene is intronic and therefore not directly involved in protein biosynthesis, its analysis is generally of very limited interest. Most gene defects occur in the exonic or coding regions, and it is the analysis of these regions that yields most information concerning structural gene defects. Although PCR amplification of each exon within a gene is possible, especially if some surrounding intronic DNA sequence is known, this can be a considerable task in a large gene such as factor VIII which has 26 exons. Within the cell nucleus, transcription of DNA to RNA is followed by differential splicing out of intronic RNA, so that messenger RNA (mRNA) consists, essentially, of exonic sequence. Analysis of this material instead of genomic DNA therefore has considerable diagnostic potential. PCR of mRNA has recently been reported using residual megakaryocyte mRNA found in platelets to obtain amplified DNA for the coding sequence of the platelet membrane glycoprotein *GP11a* gene.⁷ Clearly, if samples of the tissue or cells producing the particular protein under study are available then mRNA analysis will substantially reduce the effort required to analyse the gene. This approach will also show up defects resulting in abnormally large or small mRNA as a result of DNA changes at intron-exon splice sites, or possibly as a result of DNA gene deletions. It may also help to identify those cells producing low concentrations of protein in which mRNA concentrations are too low to be detected by standard Northern blotting methods.

MICROBIOLOGICAL APPLICATIONS

The use of PCR and specific oligonucleotide probes in routine microbiological screening is an area of great potential. It has been used to detect human immunodeficiency virus 1 (HIV 1) in a variety of samples; and

the results have been compared with the antibody and antigen state of the patient.⁸⁻¹⁰ In general, PCR seems to be a most sensitive technique and has also been used to detect cervical human papillomavirus¹¹ and human rhinovirus.¹² Because the PCR technique is so sensitive and can, theoretically, detect a single DNA strand, great care must be taken to avoid contamination of samples. Suitable controls must be used to assist in the identification of true positive results.

POTENTIAL PROBLEMS WITH PCR

Before PCR is possible, DNA sequence for at least the primer regions must be known, and for this the standard recombinant DNA techniques of preparing DNA libraries, screening, and sequencing of clones will probably be necessary. Given the enormous amount of sequence data now available, however, the application of PCR to examine particular sequences in a large number of genes is now possible, using published sequences.

The greatest potential problem with the PCR reaction is related to the specificity of the oligonucleotides and the possibility of amplification of non-specific sequences. In polymorphism studies this is not a common problem because the size of the expected amplified bands will be known, but it is important in DNA sequence studies, particularly by the direct method, that the amplified fragment is clearly the major sequence present. Non-specific binding of oligonucleotide probes can also be a problem, although if the PCR reaction results in efficient amplification of the expected band, this can be minimal. It is, of course, advisable to check any primer or probe sequence for the presence of any repetitive sequences which might cause the problems outlined above.

Conclusion

PCR represents a major technical advance in the general field of molecular biology. The ability to analyse minute amounts of DNA or RNA—for example, that present in a single hair follicle or in the few cells obtained after a buccal mouth wash—has considerable importance in genetic or forensic studies.¹³ PCR is also replacing the Southern blot procedure for RFLP analysis, and is allowing non-radioactive analyses to be introduced which will give a result in 24 hours or less. It has also revolutionised the approach to DNA sequencing, allowing for the

detailed examination of the specific genes much more easily than was possible just a few years ago. The identification of bacteria and viruses will be revolutionised—with dot blot procedures utilising specific oligonucleotide primers and probes, potentially contaminated samples will be able to be screened rapidly. Within the next few years further exciting developments based on the PCR technique can be anticipated, specifically, perhaps, in the field of automated DNA analysis.

References

- 1 Saiki RK, Scharf S, Faloona F, *et al.* Enzymatic amplification of β globin genomic sequences and restriction site analysis for diagnosis of sickle cell anaemia. *Science* 1985;230:1350-4.
- 2 Chehab FF, Doherty M, Cai S, Kan YW, Cooper S, Rubin EM. Detection of sickle cell anaemia and thalassaemia. *Nature* 1987;329:293-4.
- 3 Kogan SC, Doherty M, Gitschier J. An improved method for prenatal diagnosis of genetic diseases by analysis of amplified DNA sequences: Application to hemophilia A. *N Engl J Med* 1987;317:985-90.
- 4 Saiki RK, Gelfand DH, Stoffel S, *et al.* Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 1988;239:487-91.
- 5 Wong C, Dowling CE, Saiki RK, Higuchi RG, Erlich HA, Kazanian HH. Characterization of β -thalassaemia mutations using direct genomic sequencing of amplified single copy DNA. *Nature* 1987;330:384-6.
- 6 Levinson B, Janco R, Phillips J, Gitschier J. A novel missense mutation in the factor VIII gene identified by analysis of amplified hemophilia DNA sequences. *Nucleic Acids Res* 1987;15:9797-805.
- 7 Newman PJ, Gorski J, White GC, Gidwitz S, Cretney CJ, Aster RH. Enzymatic amplification of platelet specific messenger RNA using the polymerase chain reaction. *J Clin Invest* 1988;82:739-43.
- 8 Ou C-Y, Kwok S, Mitchell SW, *et al.* DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells. *Science* 1988;239:295-7.
- 9 Loche M, Mach B. Identification of HIV-infected seronegative individuals by a direct diagnostic test based on hybridisation to amplified viral DNA. *Lancet* 1988;ii:418-21.
- 10 Hart C, Schochetman G, Spira T, *et al.* Direct detection of HIV RNA expression in seropositive subjects. *Lancet* 1988;ii:596-9.
- 11 Young LS, Bevan IS, Johnson MA, *et al.* The polymerase chain reaction: a new epidemiological tool for investigating cervical human papillomavirus infection. *Br Med J* 1989;298:14-18.
- 12 Gama RE, Hughes PJ, Bruce CB, Stanway G. Polymerase chain reaction amplification of rhinovirus nucleic acids from clinical material. *Nucl Acids Res* 1988;16:9346.
- 13 Lench N, Stanier P, Williamson R. Simple non-invasive method to obtain DNA for gene analysis. *Lancet* 1988;i:1356-8.

Requests for reprints to: Dr I Peake, Department of Haematology, University Hospital of Wales, Heath Park, Cardiff CF4 4XN, Wales.