

BRITISH JOURNAL OF OPHTHALMOLOGY

Editorial

Molecular biology for the ophthalmologist?

Our knowledge of virological disease has expanded considerably over the past few decades subsequent to some remarkable developments in molecular biology. As each technique has been developed it has moved from a largely research orientated tool to its incorporation within the diagnostic regimen. Ophthalmologically related viral disease has not been immune from such developments. We therefore need to understand the implications and applications of these developments.

Since the discovery of DNA in 1869 and the realisation that DNA is a genetic molecule there has been an exponential growth in biological information. The isolation of DNA polymerase I in 1958 (an enzyme capable of making DNA in vitro), DNA ligase in 1967 (which joins together two DNA chains), and the first restriction enzyme (an enzyme which will cleave the DNA molecule at specific sites) allowed the joining together of DNA fragments created by a restriction endonuclease to form a recombinant DNA molecule. The successful isolation of many different restriction endonucleases that cut DNA at their specific base recognition sites has made it possible to detect changes (mutations) in a DNA sequence by the inability to cut at an expected position in the DNA molecule with a given endonuclease. For example, the restriction enzyme from *Haemophilus aegyptius* (Hae III) will cut the DNA wherever the sequence



occurs, leaving blunt ends to the fragments, whilst that from *Escherichia coli* (Eco R1) will cleave the DNA at and wherever the sequence



occurs, leaving the DNA fragments with overlapping or sticky ends. (*Represents the position where the DNA is

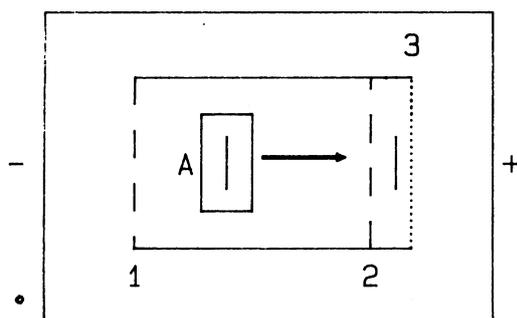


Figure 1 Electroelution. The band of DNA within the excised piece of gel (A), moves into the compartment between membranes 2 and 3.

cut. A=adenine; C=cytosine; G=guanine; T=thymine.)

The fragments generated when viral DNA is cut by an enzyme can be easily separated on gel electrophoresis by comparing the rate at which the fragments move under the influence of an electric current, with marker fragments of known molecular weight. The fragments of DNA can then be extracted from the gel by centrifugation, electroelution (Fig 1), or denatured and transferred to a membrane by capillary action (Figs 2) for further analysis. This can be done by hybridising the denatured DNA fragments to a labelled nucleic acid probe, which is complementary to the fragment under investigation – that is, Southern blotting¹ – or further digesting the extracted DNA fragments with a restriction endonuclease, as described by Kaye *et al* in the *BJO* this month. More definitively, the nucleic acid sequence of the fragments can be determined.

Techniques such as in situ hybridisation, whereby a labelled nucleotide probe of known sequence is hybridised to either RNA or denatured DNA within a preparation of tissue, have allowed the detection and localisation of the respective nucleic acid within the tissue or its components. Restriction endonuclease mapping has allowed the subtyping or straining of various viruses such as herpes simplex type 1 (HSV-1) into some of its component strains. This has clinical applications for the ophthalmologist both from epidemiological aspects and from the knowledge that some strains are clinically more virulent than others.

The concept of HSV latency within the trigeminal ganglion is well known. Here it is beyond the reach of conventional treatment. Support for the idea of corneal HSV latency arose from the isolation of HSV from the corneal discs of patients with previous herpes simplex keratitis by prolonged

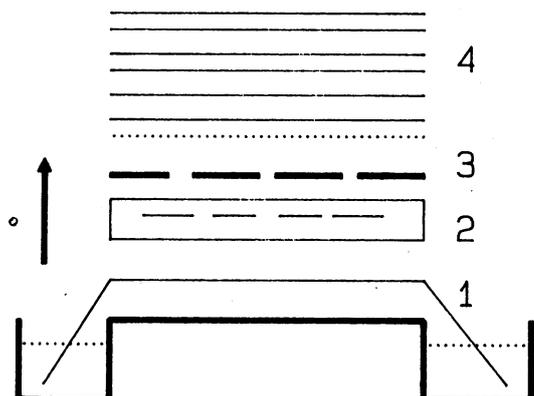


Figure 2 Absorbent towels (4) cause fluid to move up filter paper (1), through the gel (2), carrying denatured DNA bands on to membrane (3).

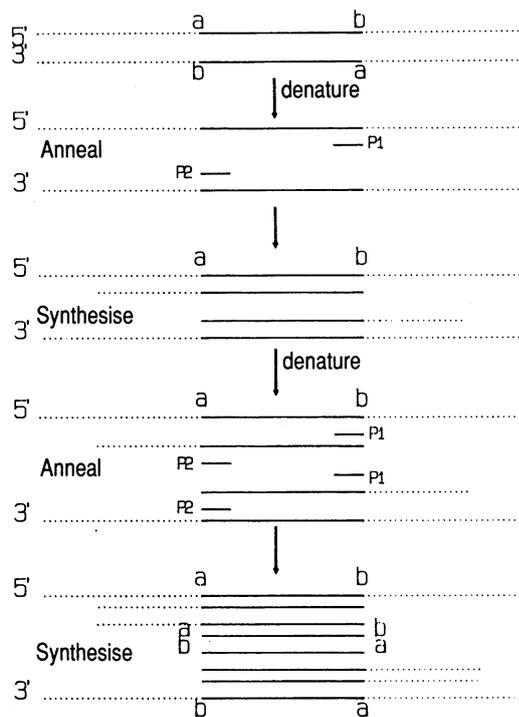


Figure 3 Polymerase chain reaction. *ab* represents segment of DNA which is amplified. Each cycle consists of denaturation of the DNA, annealing of the primers, P1 and P2 to the DNA and the subsequent synthesis of new strands. Segment *ab/ba* accumulates at an exponential rate by acting as its own template.

corneal explant culture.² This then became a subject of much debate, the pursuit of which has been hampered by the very low levels of virus with which one may be dealing. Recent developments in molecular biology, however, of which the polymerase chain reaction³ (PCR) is a milestone, has made it possible to overcome this problem. This technique allows a sequence of DNA to be amplified from minuscule amounts of DNA, thus making it amenable to direct analysis.

The PCR is based on the repetitive cycling of three reactions (Fig 3). Each cycle consists of three steps: denaturation of the target DNA by heating to a high temperature, annealing of oligonucleotide primers to the target DNA, and the synthesis of two new strands of DNA by a DNA polymerase. If a sequence of RNA is to be amplified, a copy of it must first be made (cDNA) by a reverse transcriptase before beginning the PCR. The oligonucleotide primers used average 18 to 25 bases in length and can be easily synthesised with automated equipment. In practice two specific primers are used which flank the region of interest. One primer is complementary to the coding strand and the other complementary to the non-coding strand. Synthesis of the DNA occurs along both the coding and non-coding strands from the primer binding sites. As these steps are repeated, the DNA defined by the primers undergoes exponential amplification as the newly synthesised strands themselves become templates.

The discovery and subsequent cloning of the heat stable DNA polymerase of *Thermus aquaticus*⁴ has allowed automation of the procedure, so that it is possible to amplify specific DNA sequences more than a million-fold in the same tube in only a few hours. Because of this simplicity, the PCR has widespread applications from basic science and clinical research to the routine diagnosis of certain diseases, or even the amplification and sequencing of DNA from the brain of an ancient mummy.⁵ Furthermore the ability of the PCR to

detect as little as a single copy of a gene in 10^5 cells makes it particularly suited to the study of corneal latency. The definition of latency is based on an operational definition – that is, the absence of detectable virus from a cell-free suspension but the presence of virus following prolonged organ culture. Kaye *et al* have added to this definition (1) the demonstration of viral DNA; (2) the absence of viral transcripts produced in a productive infection; and (3) the presence of latency-associated transcripts (LATs), which, though not essential for the establishment or maintenance of latency,⁶ have been found in increased amounts in a latent infection. At present they do not appear to code for any known viral protein. (This definition will undoubtedly need further modification as the role of various viral transcripts coding for proteins such as IPCO, 4, and 27 become further defined.)

It is important therefore to consider whether these criteria have been met. Kaye *et al* demonstrated the presence of three pieces of HSV DNA well separated on the viral genome, which argues strongly for the presence of extensive stretches of the viral genome. In nine of the 15 corneas in which HSV DNA was demonstrated evidence for the presence of LAT, in the absence of glycoprotein C transcripts (a late viral protein expressed in a productive infection) was found. Although this is suggestive of a latent infection, the possibility remains that other areas of the viral genome were being transcribed which went undetected. Furthermore, although attempts to reactivate virus from the corneal discs by tissue cocultivation with and without azacytidine (a hypomethylating agent) were made, virus was not detected. This is unfortunate, as the ability to reactivate virus is an important prerequisite for demonstration of a latent infection. Nevertheless this work goes a long way in support of the concept of corneal latency.

The finding of viral DNA in five of 10 non-HSV-diseased corneas from seropositive patients, three of whom had evidence for LAT, is intriguing. Four possibilities exist: (1) They may represent false positives. This would appear unlikely, as equivalent results for the presence of HSV DNA were obtained in two centres with the thymidine kinase primers. (2) The original clinical diagnosis may have been incorrect or have masked a herpetic keratitis. (3) A sub-clinical HSV infection occurred. (4) The non-HSV-related corneal disease triggered the seeding of HSV into the cornea. Although further work is needed to answer these possibilities, the question is raised about possible iatrogenic transfer of HSV following penetrating keratoplasty.

What then is the relevance of corneal latency? From a practical point of view the frequency of recurrent disease may be reduced by identifying and avoiding specific triggers that reactivate virus within the cornea. Furthermore virus in the cornea may be more accessible to suppression and eradication than virus in the trigeminal ganglion. This awaits the development of newer methods of treatment directed at the viral genome.

- 1 Southern EM. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* 1975; 98: 503–17.
- 2 Shimeld C, Tullo AB, Easty DL, Thomsit J. Isolation of herpes simplex virus from the cornea in chronic stromal keratitis. *Br J Ophthalmol* 1982; 66: 643–7.
- 3 Mullis KB, Faloona FA. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol* 1987; 155: 335–50.
- 4 Saiki RK, Gelfand DN, Stoffel S, *et al*. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 1988; 239: 487–91.
- 5 Paabo S, Gifford JA, Wilson AC. Mitochondrial DNA sequences from a 7000-year-old brain. *Nucleic Acids Res* 1988; 16: 9775–87.
- 6 Sedarati F, Izumi KM, Wagner EK, Stevens JG. Herpes simplex virus type 1 latency-associated transcription plays no role in establishment or maintenance of a latent infection in murine sensory neurons. *J Virol* 1989; 63: 4455–9.