NUCLEIC ACID ANALYSIS BY SANDWICH HYBRIDIZATION.

by

Peter John Nicholls.

A Thesis Submitted for the Degree of

Doctor of Philosophy of the

University of London.

Charing Cross and Westminster Medical School.

March, 1989.

Abstract.

The nucleic acid-based tests currently used for the detection of inherited and infectious disease are sensitive and specific, but not always reproducible, because of procedural complexities; this in turn means that they are not amenable to automation. Such tests are not cost-effective, are limited in the number of samples which can be processed at once, and most commonly rely on unstable isotopically labelled probes, which are hazardous to handle and dispose of.

A nucleic acid sandwich hybridization assay has been developed, initially using cloned human papillomavirus (HPV) sequences as a model, and subsequently for the detection and typing of HPV infection in cervical scrapes. The implications of cervical HPV infection are of clinical significance, because of the relatively recent accumulation of evidence linking specific HPV types with the aetiology of cervical cancer. Considering that viral type could have a great influence on the prognosis of infection, and that immunological assays are not capable of differentiating between types, the sandwich hybridization technique, which could handle large numbers of samples, would be an invaluable tool for improving the efficacy of the cervical screening programme.

The assay requires two distinct, non-overlapping DNA fragments, derived from adjacent positions on the viral genome. One fragment (**A**) is covalently attached to a Sephacryl S-1000 bead, and the other fragment (**B**) is suitably labelled. On hybridization of A and B with total DNA extracted from a clinical sample, followed by stringent washing, there are two possible outcomes: (i) HPV DNA in the sample (the **Target** DNA) hybridizes with both A and B, forming the **A-Target-B** complex. Label is effectively immobilized on the bead. (ii) Absence of HPV target in the sample prevents formation of the A-Target-B complex, and the Sephacryl bead is not labelled. The use of probes labelled

with ^{32}P allows signal quantification to be achieved rapidly and simply, using the Cerenkov method. Results are obtained in an easily interpreted numerical form. The application of non-radioactive probes to target detection has been only moderately successful. The advantages of this assay over more conventional hybridization formats are: (i) the technique is reliable and simple; (ii) results can be obtained in under 24 hours; (iii) the technology exists to automate the entire procedure. In this form, the sandwich assay is capable of reliably detecting 10^{-17} moles (6×10^6 molecules) of HPV DNA. Polymerase chain reaction (PCR)-mediated amplification of the number of target sequences prior to hybridization increases the sensitivity of the test considerably. The characteristics of a system involving PCR followed by sandwich assay have been investigated; the procedure, which is complete in under 24 hours, can detect as little as 8.3×10^{-21} moles (5,000 molecules) of target DNA. This represents a significant improvement over conventional nucleic acid hybridization techniques, and will allow large numbers of samples to be screened effectively for the presence of HPV infection.

To Mum and Dad, with all my love.

-Thanks for making this possible-

The known is finite, the unknown infinite; intellectually we stand on an islet in the midst of an illimitable ocean of inexplicability. Our business in every generation is to reclaim a little more land.

T.H. Huxley, 1887.

Acknowledgements.

First and foremost, I would like to thank my supervisor, Professor Alan D.B. Malcolm, for accepting me into the Department of Biochemistry at Charing Cross and Westminster Medical School. His optimism, advice and support were essential for the completion of this project.

I would like to express my appreciation to Cambridge Life Sciences, for providing the financial support required for my studentship.

Over the past three years, a large number of people have passed through the department, and it is impossible to thank each individually for their friendship and help; suffice it to say that I will consider myself lucky if I work with such an amicable bunch of people again.

I would like to express appreciation to a few individuals whose contribution to this project deserves to be recognized: firstly, I would like to thank Dr. Colin Wickenden for supplying me with clones of HPV DNA, and for many useful discussions regarding the molecular biology of papillomaviruses. Thanks are due also to Dr. Neil Bowles (for always being willing to lend a hand, and particularly for his guidance in the early stages of this project), and to Sarah Talbot (for preparing many of the plasmids used in this study).

I would like to acknowledge Dr. Jane Langdale for 'starting the ball rolling' with respect to sandwich hybridization, and Dr. Penny Ward for supplying me with clinical specimens.

I am especially grateful to the Department of Medical Illustration, Charing Cross and Westminster Medical School, for their help in producing illustrations, and also to the library staff for their excellent service (and particularly for allowing me to use their new Medline CD-ROM system). Thanks also to Linda Readings, secretary

to Alan Malcolm, for invaluable advice during the production of this thesis.

My special appreciation goes to a fellow PhD student, Uta Voss, for her helpful advice and criticism, and for always 'lending an ear'. I wish Uta and Ian every success for their new life in the U.S.A.

Thanks to all my colleagues in lab 8L16, for providing a friendly atmosphere to work in. The best of luck to all those submitting for a higher degree in the near future.

Finally, I would like to thank Mum, Dad, Dave and Hilda, and also my friends (especially Jane) for their support over the past three years. Without them, this project would certainly never have been completed.

List Of Abbreviations.

A	Adenine.
AAF	N-acetoxy-N-2-acetylaminofluorene.
Ad	Adenovirus.
AP	Alkaline phosphatase.
APTE	Aminophenylthioether.
ASAC	Amplified signal affinity capture assay.
ASO	Allele-specific oligonucleotide probe.
ATP	Adenosine ribonucleoside triphosphate.
bp	Base pairs.
BPV	Bovine papillomavirus.
BSA	Bovine serum albumin.
C	Cytosine.
^{o}C	Degrees Celsius.
Carbodiimide	1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene sulfonate.
Ci, mCi, uCi	Curie, milli-Curie, micro-Curie.
CIN	Cervical intraepithelial neoplasia.
CIP	Calf intestinal alkaline phosphatase.
CMV	Cytomegalovirus.
CPG	Controlled pore glass.
cpm	Counts per minute.
CRPV	Cottontail rabbit papillomavirus.
dATP	2'-deoxyadenosine 5'-triphosphate.
DBM	Diazobenzyloxymethyl.
dCTP	2'-deoxycytidine 5'-triphosphate.
dGTP	2'-deoxyguanosine 5'-triphosphate.
DIG-dUTP	Digoxigenin-labelled 2'-deoxyuridine 5'-triphosphate.
DMF	Dimethylformamide.

DMSO	Dimethylsulphoxide.
DNA	Deoxyribonucleic acid.
DNAse	Deoxyribonuclease.
DNA-resin	DNA covalently coupled to a resin.
dpm	Disintegrations per minute.
DPTE	Diazophenylthioether.
DTT	Dithiothreitol.
dTTP	2'-deoxythymidine 5'-triphosphate.
dUTP	2'-deoxyuridine 5'-triphosphate.
E.coli	Escherichia coli.
EDTA	Ethylenediaminotetra-acetic acid.
EtBr	Ethidium bromide.
g, mg, ug, ng, pg	Gram, milligram, microgram, nanogram, picogram.
G	Guanine.
g_{av}	Average gravitational force.
GAWTS	Genomic amplification with transcript sequencing.
HEPES	N-2-hydroxyethyl piperazine N'-2-ethane sulphonic acid.
HIV	Human immunodeficiency virus.
HPV	Human papillomavirus.
HRP	Horse radish peroxidase.
IPTG	Isopropyl-beta-D-thio-galactopyranoside.
kb	Kilobase pairs.
l, ml, ul	Litre, millilitre, microlitre.
LMP	Low melting point (agarose).
m, mm, um, nm	Metre, millimetre, micrometre, nanometre.
M, mM, uM, nM	Molar, milli-molar, micro-molar, nano-molar.
MES	2(N-morpholino)ethane sulfonic acid.
mRNA	Messenger RNA
NHS-LC-biotin	Sulfosuccinimidyl 6-(biotinamido) hexanoate.

O.D.	Optical density.
OLB	Oligolabelling buffer.
OPC	Oligonucleotide purification cartridge.
OPD	O-phenylenediamine.
OR	Oligomer restriction.
ORF	Open reading frame.
PBS	Phosphate buffered saline.
PCR	Polymerase chain reaction.
$p[dN]_6$	Hexadeoxyribonucleotide primer.
PIPES	Piperazine-N,N'-bis(2-ethane sulfonic acid).
PV	Papillomavirus.
RFLP	Restriction fragment length polymorphism.
RIA	Radioimmunoassay.
RNA	Ribonucleic acid.
RNAse	Ribonuclease.
rpm	Revolutions per minute.
SDS	Sodium dodecyl sulphate.
SPR	Solid-phase receptacle.
SSC	Saline sodium citrate.
T	Thymine.
TAE buffer	Tris-acetate electrophoresis buffer.
TBE buffer	Tris-borate electrophoresis buffer.
TEMED	N,N,N',N'-tetramethylethylene diamine.
Tris	Tris(hydroxymethyl)amino methane.
u.v.	Ultraviolet.
V	Volts.
v/v	Volume per volume.
w/v	Weight per volume.
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactoside.

CONTENTS. **PAGE.**

List Of Figures. PAGE.

List Of Tables. PAGE.

1. INTRODUCTION.

One of the most significant achievements of the biochemist during the past two decades is the use to which immunologically based assays have been put in clinical diagnosis (Steward, 1985). The problem faced and surmounted by immunologists in effecting the transition from research tool to routine clinical assay bears a remarkable similarity to that confronting the molecular biologist today; that is, how can nucleic acid hybridization, a technique of tremendous potential (Meinkoth & Wahl, 1984; Syvanen, 1986; Matthews & Kricka, 1988), be modified in order to satisfy all of the properties required of a routine diagnostic test? There are several such requirements, and the importance placed on each depends on the objectives of the assay: the technique must be sensitive, specific and reproducible. Other advantages would be cost-effectiveness, ease of manipulation and amenability to automation. Ideally, the signal detection step should be based on a non-radioactive system, because of the instability of probes labelled with isotopes like ^{32}P, and the potential hazards involved in their handling and disposal.

Sandwich hybridization for the analysis of nucleic acid sequences was first used over a decade ago (Dunn & Hassel, 1977), but its potential as a diagnostic assay was not realized until 1983, when it was applied to the detection of adenovirus DNA in nasopharyngeal aspirates from children with acute respiratory infection (Ranki et al., 1983 a,b). It has since been modified and used not only for the detection of microbial infection (Palva & Ranki, 1985), but also for the analysis of nucleotide sequence variations (Langdale & Malcolm, 1985).

The development of the sandwich hybridization assay has resulted in adaptations which make it far more suitable than conventional

techniques for use in routine diagnosis (Nicholls & Malcolm, 1989). Despite these advantages, it still remains relegated to the research laboratory; this reflects the fact that further improvements are necessary before the sandwich assay is widely accepted for clinical use. In order to identify the improvements which need to be made, it is helpful to consider in more detail the advantages and disadvantages of the nucleic acid hybridization techniques currently in use in research laboratories.

1.1 Conventional Methods Of Gene Detection.

The assays used in the laboratory for nucleic acid analysis suit the needs of the research scientist in terms of sensitivity, specificity and reproducibility. It would not, however, be realistic to describe them as ideal for use in routine diagnosis, because they are labour-intensive, not easily automated, and are limited in the number of samples that can be processed at once. The most commonly used methods for detecting specific sequences within a complex, non-homologous nucleic acid mixture are the Southern blot (for DNA) (Southern, 1975) and the Northern blot (for RNA) (Alwine et al., 1977).

1.1.1 Southern Blotting.

The Southern technique involves several stages, some of which require complex manipulations; the first step must always be purification of DNA from the sample of interest. The most common means of achieving this is to incubate the sample with a protease (in order to break down cell membranes, and release the cell contents), followed by extraction with phenol/chloroform (to remove contaminating proteins). Traces of phenol are removed with water saturated ether; this is particularly important if the purified DNA is to be digested

with a restriction enzyme. Clearly, if the number of samples to be analyzed is large, the time-consuming nature of this purification step makes the Southern technique unattractive for use in a routine clinical setting. The second stage in the blotting procedure involves enzymatic cleavage of the nucleic acid sample into specific restriction fragments, which are then size-fractionated by electrophoresis through an agarose gel. The fragments are transferred to a suitable solid support (usually a cellulose nitrate or nylon membrane) and denatured, their positions relative to each other being maintained. There are two problems commonly encountered at this stage in the procedure: firstly, DNA fragments larger than 10 kb in size do not transfer efficiently out of agarose gels. Secondly, small DNA fragments less than around 300 bp do not bind effectively to nitrocellulose (although this becomes less pronounced when a nylon membrane is being used). The first problem can be overcome by depurination of the DNA by treatment with acid; depurination results in a large DNA fragment being broken up into several smaller fragments, and leads to improved transfer out of the gel. The extent of cleavage can be difficult to control, and if depurination is too extensive, the fragments produced may be too small to bind to the membrane (Wahl et al., 1979). The final steps in Southern blotting involve hybridization with a suitably labelled specific probe, washing off of excess label in a low salt buffer, and signal detection by autoradiography. That the Southern technique is invaluable in research laboratories is plain, since it remains today essentially unmodified since arriving on the scene in 1975. There have been attempts to make the process less laborious; most of these modifications are designed to reduce the time taken for transfer of DNA out of the gel. Examples are: (i) electro-blotting, which reduces the transfer time from around 24 hours to 2 hours (Bittner et al., 1980);

(ii) centrifugation at 40 x g_{av}, which allows transfer in 20 minutes (Wilkins & Snell, 1987); (iii) bi-directional blotting, which results in the production of two identical blots from a single agarose gel in 1 hour (Smith & Summers, 1980). Attempts have been made to combine electrophoresis and blotting steps by running DNA fragments off of the end of the gel, directly onto a hybridization membrane, using a procedure known as direct blotting electrophoresis (Beck & Pohl, 1984). The membrane is attached to a rotating drum, so the spatial separation of fragments is maintained as they elute from the gel. The procedure has also been used for DNA sequencing, the band pattern being visualized with high sensitivity using a non-radioactive detection system (Beck, 1987).

In some circumstances, transfer of DNA out of the gel is not required (Purello & Balazs, 1983). After electrophoresis, the DNA is denatured, and the gel is neutralized thoroughly, prior to drying down under vacuum. Gel drying apparatus designed specifically for this purpose are commercially available, because the procedure is used in both of the routinely used DNA sequencing techniques (Sanger et al., 1977; Maxam & Gilbert, 1977). Once dried, the gel can be treated in the same way as a membrane; it can be hybridized sequentially with more than one probe, because hybridized sequences can be washed off under appropriate conditions (usually low salt concentration and/or high temperature). Although there is some loss of small fragments during drying (those below around 300 bp in length), direct hybridization to a gel does have advantages; for instance, when the probe being used is a synthetic oligonucleotide the sensitivity achieved is approximately 5-10 times greater than that obtained by hybridization to DNA which has been transferred to a membrane by Southern blotting (Studencki & Wallace, 1984).

Despite the fact that considerable effort has been put into simplifying the Southern technique, there is no doubt that it is not a practicable way of screening large numbers of samples. This has led to the development of alternatives specifically designed with mass screening in mind; these are considered in more detail below.

1.1.2 Rapid Blotting Techniques.

In many circumstances, it is not necessary to know the size of a nucleic acid sequence, but only whether it is present in, or absent from, a sample. This has led to the development of more rapid hybridization techniques, which do not require the size-fractionation of the sample nucleic acid, thus eliminating the need for running agarose gels, and the subsequent transfer of DNA onto a membrane. Instead, nucleic acid is purified from the sample of interest, and immobilized directly onto the solid support. Normally, deproteination is necessary prior to immobilization, because proteins compete with DNA for binding sites on the membrane. Depending on the nature of the sequence of interest, it is possible to selectively immobilize DNA or messenger RNA (mRNA), using the chaotropic salt, sodium iodide (Bresser et al., 1983). After hybridization with a labelled probe, the membrane is washed, and the presence of radioactivity can be detected by autoradiography. Results are quantitated by densitometric scanning of the resulting exposure. Variations on this theme include dot-blotting (Kafatos et al., 1979), spot-blotting (Cunningham, 1983) and slot-blotting (Bowles et al., 1986); the latter gives the most easily quantitatible result, as the signal can be most readily scanned in a densitometer. This is because samples are applied to the membrane using a commercially available vacuum manifold, and are limited to a well defined area.

Although these 'rapid' techniques have advantages over Southern blotting, they are still not ideally suited for routine clinical analysis: the purification and immobilization of the sample nucleic acid prior to hybridization is time-consuming, and more importantly, autoradiography is used as a means of signal detection. Autoradiography is not an efficient way of measuring a radioactive signal; when there are small amounts of the sequence of interest in a sample (as may be the case in the detection of single-copy genes), autoradiography times can be inconveniently long.

1.1.3 In situ Hybridization.

The blotting procedures described so far involve the purification of DNA or RNA from the sample of interest. By contrast, *in situ* hybridization is a technique which does not involve any purification step, but rather allows the detection and localization of nucleic acid sequences within cell or tissue preparations. In circumstances where it is necessary to detect a specific sequence present in only a very small subset of cells (for example, in the case of an infectious agent which preferentially invades a well-defined cell type), *in situ* hybridization can be more sensitive than blotting. This is because the latter involves extraction of total nucleic acid from all cell types present in the sample, regardless of whether or not they are likely to contain the sequence of interest.

There is no doubt that, as a research tool, *in situ* hybridization is of great importance; this is reflected by the fact that the number of studies incorporating *in situ* results has increased dramatically over the past five years. The technique has been used to identify sites of gene expression (Pardue, 1985), and to analyze the tissue distribution of specific RNA transcripts (Siegel & Scott Young,

1986); it has also allowed specific sequences to be mapped in condensed chromosomes (Harper, 1986). Nevertheless, the procedure is far from suitable for routine diagnostic use, because it is complex and technically demanding; results are commonly obtained several days, or even weeks after the end of an experiment. Although non-radioactive probes have been used successfully, giving results quickly and with excellent resolution, sensitivities are low in comparison with radioactive methods (Szabo & Ward, 1982). Further disadvantages are that the type of sample and the method of tissue fixation and permeabilization have a profound effect on the success of the procedure; despite attempts at standardization, methods vary considerably, and there is, as yet, no consensus as to the best protocol. This effectively means that each time *in situ* is applied to a different tissue type, or for the detection of a different target sequence, the conditions need to be optimized once again. As the number of characterized genetic disorders increases, and more probes become available for clinically or commercially important infectious agents, the diagnostic use of nucleic acid based assays will increase extensively. This is likely to occur much more rapidly if the same assay format can be applied in each case, regardless of the nature of the sample, and the origin of the probes being used.

How can the drawbacks inherent in conventional hybridization techniques be improved upon, in order to produce the most suitable format for accurately, easily, and cheaply handling large numbers of samples? Firstly, there should be no requirement for electrophoretic separation, or the immobilization of the sample material. Results should be obtained rapidly, in numerical form, without the need for extensive sample pre-processing. Finally, the quantitation of signal

should ideally be based on a non-radioactive method, and the same assay format should be applicable, regardless of the nature of the sample and source of the probes being used. The ultimate aim should be the development of a totally automated system (Landegren et al., 1988). Of the nucleic acid hybridization assays described to date, sandwich hybridization appears to be one of those which could be most easily modified to satisfy all of the criteria outlined above. The properties which make the technique a suitable candidate, together with the modifications which will need to be made, are discussed in section 1.3.

1.2 Nucleic Acid Labelling.

An absolute requirement of assays designed to use nucleic acid probes for the detection of specific DNA or RNA sequences is that the probe should be labelled in such a manner that it can be easily detected after hybridization. Several methods for achieving this have been described, and these can be separated into two categories, depending on whether the labelled probe is radioactive or non-radioactive.

1.2.1 Radioactive Nucleic Acid Probes.

The most frequently used methods for labelling nucleic acid probes involve the incorporation of a radioactive tag into the DNA or RNA strand. The isotope most commonly used for this purpose is ^{32}P, although ^{125}I, ^{35}S and ^{3}H (tritium) are suitable in certain circumstances.

Double-stranded DNA can be labelled to a specific activity of around 5×10^{8} dpm/ug by a method known as nick translation (Rigby et al., 1977). Deoxyribonucleoside [^{32}P]-triphosphates are incorporated into the DNA strand by the combined activity of DNAse I and DNA

polymerase I. Random nicks are introduced into the DNA by DNAse I, and nucleotides are removed from the 5'-phosphoryl terminus of the nick by the exonuclease activity of DNA polymerase I. In the presence of all four deoxyribonucleoside triphosphates [dNTPs] (one or more of which is labelled with ^{32}P), the polymerase mediates the addition of nucleotides to the 3'-hydroxyl terminus. Nick translation can utilize any deoxyribonucleotide labelled with ^{32}P in the alpha position, although ^{125}I-, ^{3}H- and biotin-labelled nucleotides can also be incorporated. The method is particularly suitable for the production of large quantities of probe for use in multiple hybridization reactions, and in situations requiring a high probe concentration, because around 1 ug of DNA can be labelled in a single reaction (Cunningham & Mundy, 1987). The main disadvantage of nick translation is that results can be unpredictable with impure DNA substrates, particularly those contaminated with agarose, because there are commonly potent inhibitors of DNAse I in commercial agarose preparations (Feinberg & Vogelstein, 1983).

A second method of labelling double stranded DNA is replacing nick translation, because it is extremely simple, and results in probes with a higher specific activity (typically around 2×10^9 dpm/ug). The technique, known as random hexadeoxyribonucleotide priming (or 'oligolabelling' for short), involves the de novo synthesis of a radiolabelled DNA strand, by priming a denatured DNA template with a random mixture of hexanucleotides (Feinberg & Vogelstein, 1983; 1984). After primer annealing in the presence of the large subunit of E.coli DNA polymerase I (the Klenow fragment), together with a mixture of all four dNTPs (again, one or more of which may be labelled with ^{32}P), a radio-labelled DNA strand is synthesized by the addition of nucleotides to the 3'-end of the primers. The efficiency of the reaction is unaffected by the presence of agarose, so DNA fragments can be labelled

immediately after electrophoresis, without the need for further purification. The method is particularly suitable for labelling small amounts of relatively impure DNA (typically around 50 ng) to a high specific activity. The quantity of probe produced is generally sufficient for only a single hybridization, and the average length of newly synthesized DNA strands is variable. However, since it is known that the size of labelled strands produced is an inverse function of primer concentration, it is possible to adjust the reaction conditions to produce a probe of the desired length (Hodgson & Fisk, 1987).

A disadvantage of both techniques described above is that for every labelled DNA strand produced, there is a complementary strand; on hybridization with a sample, there is a tendency for these strands to reanneal, rather than hybridize to the target sequence, thus resulting in a decrease in the amount of available probe. This problem may be surmounted by using single-stranded probes, which may take the form of DNA, RNA or synthetic deoxyribonucleotides.

Single-stranded probes of high specific activity are produced using the M13 bacteriophage universal probe primer (Hu & Messing, 1982). The primer is complementary to the 5' side of the multiple cloning site of the M13 family of vectors, and is used to initiate the synthesis of a [-] strand from the [+] template strand. This is achieved by the addition of the Klenow fragment of DNA polymerase I, largely as described for oligolabelling. To avoid read-through into the target-specific sequence, the reaction is limited by a low concentration of labelled nucleotide, and terminated by the addition of a chelating agent (EDTA); thus, the inserted probe sequence remains single-stranded. The incorporation of labelled deoxyribonucleotides during elongation of the primer results in a specific activity of around 5×10^8 dpm/ug, and the probe does not need to be denatured

prior to use. Variations on this theme have been reported, including the use of a single-stranded, unlabelled, target-specific probe which is hybridized to samples immobilized on a membrane. After washing, the extent of hybridization is determined by annealing labelled M13 vector DNA to the free M13 region of the target-specific clone (Wolf et al., 1986; Zolg et al., 1987). This technique is considered in more detail in section 1.3.6.

The RNA polymerases from a number of bacteriophages- those of Salmonella phage SP6, and coliphages T3, T5 and T7 -possess a high degree of specificity for their own promoters in vitro, so transcription can be limited to sequences cloned downstream of an appropriate promoter (Little & Jackson, 1987). RNA transcripts specific for the cloned DNA are prepared using linearized template, the four ribonucleotides (one or more of which may be labelled), and the appropriate RNA polymerase (depending on which promoter is being used). Many transcripts are produced from each template strand. Prior to use in hybridization, the template is removed from the probe by treatment with DNAse I. There are several advantages to using RNA probes: firstly, they are single-stranded, so there is no complementary strand competing with target molecules for hybridization to the probe. RNA-DNA hybrids have been shown to be more stable than those formed between two DNA strands (Casey & Davidson, 1977), so hybridization and washing can be performed under more stringent conditions, thus reducing the non-specific background signal. Finally, excess probe can be removed by treatment with RNAse, because RNA in a duplex with DNA is resistant to RNAse degradation; this also contributes to a decrease in the background signal. The most significant disadvantage of an RNA probe is that it is susceptible to degradation by naturally occuring RNAse. This can result in uncertainty in the interpretation of a

negative result, so it is essential to destroy all contaminating RNAse in a sample prior to addition of the probe.

Non-enzymatic methods for radio-labelling DNA have been described; for example, polynucleotides can be iodinated by heating in an aqueous solution of iodine or phallic trichloride, in the presence of iodide ions, at pH 5 (Commerford, 1971). Iodine is incorporated into cytosine residues via a stable covalent bond. The iodine atom occupies a position sterically equivalent to that of the methyl group in thymidine, so even extensive iodination should not affect the hybridization properties of a labelled DNA strand. Inexplicably, in practice this is not the case, and since the extent of reaction is difficult to control, probes labelled by this method have not been widely used in molecular biology, although their application to sandwich hybridization assays has been reported (Ranki et al., 1983 a,b).

There are several methods available for labelling either the 3' or 5' ends of linear DNA or RNA. For example, DNA (single- or double-stranded) can be labelled at the 3' end using the enzyme terminal deoxynucleotidyl transferase, and single and double-stranded forms of both DNA and RNA can be labelled at the 5' end using T4 polynucleotide kinase. Usually only a single label is introduced, so that the specific activity of the resulting probe is significantly lower than that achieved using the techniques previously described. For this reason, end-labelling is not commonly used for producing probes from cloned sequences, although such methods are used for labelling synthetic oligonucleotides.

There are several disadvantages inherent in the use of radio-actively labelled probes for clinical diagnosis. These include high cost, health hazards, inconvenience in handling and disposal, and the

need for probe manufacture on a regular basis; this is particularly important in the case of ^{32}P-labelled probes, because this isotope has a half-life of only 14.3 days, so the specific activity of probes decays rapidly. Considerable effort has been put into the development of alternative nucleic acid labelling methods, which do not involve the use of radioactive isotopes.

1.2.2 Non-radioactive Nucleic Acid Probes.

The most commonly used methods for detecting non-radioactive nucleic acid probes fall into one of the following categories: i) biotinylated nucleotide analogues incorporated into the probe are detected using enzyme-conjugated avidin or streptavidin (Leary et al., 1983); ii) haptens incorporated into the probe can be detected immunologically (Landegent et al., 1984; Tchen et al., 1984); iii) proteins (for example, enzymes) directly cross-linked to the nucleic acid can be detected by means of their activity on substates (Renz & Kurz, 1984). The most established of these techniques involves the use of analogues of dUTP and UTP, containing a biotin molecule bound to the C-5 position of the pyrimidine ring through an allylamine linker arm (Langer et al., 1981). These analogues have been incorporated into DNA by nick-translation, oligolabelling, or more recently, using the polymerase chain reaction (Lo et al., 1988; see section 1.7); polynucleotides containing low levels of biotin substitution (not more than fifty molecules per kilobase) exhibit hybridization characteristics identical to those of unmodified DNA, and can be detected by means of the extremely high affinity ($Kd = 10^{15}\ M^{-1}$) between biotin and the glycoprotein avidin (Green, 1975). Recently, the bacterial protein streptavidin has become widely used because, unlike avidin, it possesses a near neutral isoelectric point; this leads to a reduction

in non-specific binding to DNA, and a consequent decrease in background signal. In practice, the biotinylated probe is detected by reaction with a pre-formed complex of streptavidin and biotinylated enzyme; since each steptavidin molecule possesses four binding sites for biotin, a single modified nucleotide in the probe can result in the attachment of many enzyme molecules (Leary et al., 1983). The enzymes most commonly used are alkaline phosphatase (AP) and horse radish peroxidase (HRP); these may be detected by their ability to convert a soluble, colourless substrate into a coloured product (Gillam, 1987), or by the enzyme-mediated breakdown of the product they produce, with the emission of visible light (Hauber & Geiger, 1988). An alternative to the enzymatic incorporation of biotin is to use a photo-activatable analogue, photobiotin, which forms stable linkages with single- and double-stranded nucleic acids on irradiation with visible light (Forster et al., 1985). One of the problems associated with this kind of approach is that it is difficult to regulate the frequency with which biotin molecules attach to the nucleic acid; if modification is too extensive, the probe produced may have significantly altered hybridization properties. Alternatively, insufficient modification will result in a probe with unacceptably low sensitivity. These problems may be surmounted by using a technique which does not result in a direct linkage between biotin and nucleic acid. For example, many biotin molecules can be linked chemically to a basic macromolecule like polyethyleneimine; the modified macromolecule may in turn be covalently attached to nucleic acid by means of a bifunctional cross-linking reagent (glutaraldehyde). The result is that for the modification of a single nucleic acid residue, many biotin molecules are incorporated into the probe (Al-Hakim & Hull, 1986). Alternatively, simultaneous biotinylation and iodination of long-chain diamino compounds, (for

example, diaminohexane), produces conjugates that can be linked to DNA without the use of a cross-linking reagent (Al-Hakim & Hull, 1988). The same result can be achieved by using biotinylated nucleic acid binding proteins like histone H1 protein (Renz, 1983), or the single-strand binding protein of E.coli (Syvanen et al., 1985). The nucleic acid-protein complex is stabilized by cross-linking with glutaraldehyde, without significantly affecting the hybridization properties of the modified nucleic acid.

It is possible to label a nucleic acid molecule with a marker group which may be detected immunologically. An example of such a group is a guanine residue modified in vitro by treatment with N-acetoxy-N-2-acetylaminofluorene (AAF) (Tchen et al., 1984). After hybridization, the probe is bound with an antibody (Ab1) directed against acetylaminoflurene-modified guanine; signal detection is achieved by addition of a fluorochrome-labelled second antibody (Ab2), directed against Ab1 (Landegent et al., 1984). Alternatively, Ab2 may be labelled with europium, and the extent of hybridization determined by a technique known as time-resolved fluorometry (see section 1.3.7). The main disadvantage of modifying nucleic acids with AAF is that the reagent is a potent carcinogen, thus complicating probe handling and disposal.

Proteins like AP or HRP may be cross-linked directly to nucleic acids by means of a glutaraldehyde-mediated linkage, to produce probes which may be visualized directly, by addition of the appropriate substrate (Renz & Kurz, 1984). The main problem associated with this approach is that the hybridization conditions must be controlled extremely carefully, in order to prevent denaturation of the enzyme.

With the increased interest in the application of synthetic oligodeoxyribonucleotides to gene detection, several techniques have

been described for labelling oligonucleotides with non-radioactive groups. The most common means by which this is achieved is to attach an aliphatic amino group at the 5' terminus of the oligonucleotide (Smith et al., 1985). Reagents are now available for the incorporation of such a group as the final step in the synthesis procedure on a commercially available DNA synthesizer (Agrawal et al., 1986). The amino group will react specifically with a variety of electrophiles, thereby allowing other chemical species to be attached to the oligo. Labelling at the 5' end has been shown to have little effect on the stability of the hybrid formed between the oligo and its target sequence, and it leaves the 3' terminus free to act as a primer in enzyme-mediated extension reactions; this has been shown to be particularly useful in DNA sequencing techniques which use oligos labelled at the 5' end with fluorescent markers (Smith et al., 1986).

The hybridization properties of oligos 5' end-labelled with fluorescent markers, enzymes (AP or HRP) and chemiluminescent molecules (isoluminol) have been compared (Urdea et al., 1988). Labelling with HRP, in conjunction with an enhanced chemiluminescence detection system (Matthews et al., 1985) was shown to be capable of detecting 3×10^{7} molecules (5×10^{-17} moles) of target DNA; this compares favourably with the sensitivity achieved using probes labelled with ^{32}P, and is far better than that obtained using oligos labelled directly with a fluorescent marker.

An alternative to end-labelling is the incorporation of base-modified nucleosides during synthesis, facilitating the preparation of oligonucleotides carrying one or more internal aliphatic amino group (Haralambidis et al., 1987). It is also possible to incorporate biotinylated nucleotides into an oligo internally, by polymerase-mediated elongation of a specific octanucleotide primer (Langer et al.,

1981).

Recently, non-radioactive probes have been successfully used with great sensitivity, although it is fair to conclude that the improvement is due to the application of techniques like the polymerase chain reaction (Saiki et al., 1985 a; see section 1.7) or oligo-nucleotide-mediated signal amplification (Urdea et al., 1987 a; see section 1.3.7), rather than to better methods of nucleic acid labelling. Nevertheless, once such techniques are fully developed, so that they are totally reliable and routine, there will no longer be a place for radioisotopes in nucleic acid-based clinical diagnosis.

1.3 The Sandwich Hybridization Assay.

1.3.1 Principle And First Use.

The principle of the sandwich hybridization assay is quite simple, and is illustrated in figure 1. It involves the use of two nucleic acid fragments, preferably from adjacent positions on the genome, which do not hybridize to each other to any extent. One of the two fragments is immobilized on to a solid support (Dunn & Hassel, 1977), or labelled in such a way that it can be captured by the support after undergoing hybridization (Syvanen et al., 1986 a); this will be referred to as **fragment A**. The other is suitably labelled for use as a hybridization probe (**fragment B**). When both A and B are mixed with a sample containing the target sequence, they hybridize with it, forming an **A-target-B** complex, and the labelled probe becomes attached to the solid support via the linking bridge formed by the target. In the absence of target, the support is not labelled. The advantages inherent in this are immediately apparent; the sample nucleic acid does not need to be immobilized, and the choice of solid support for the

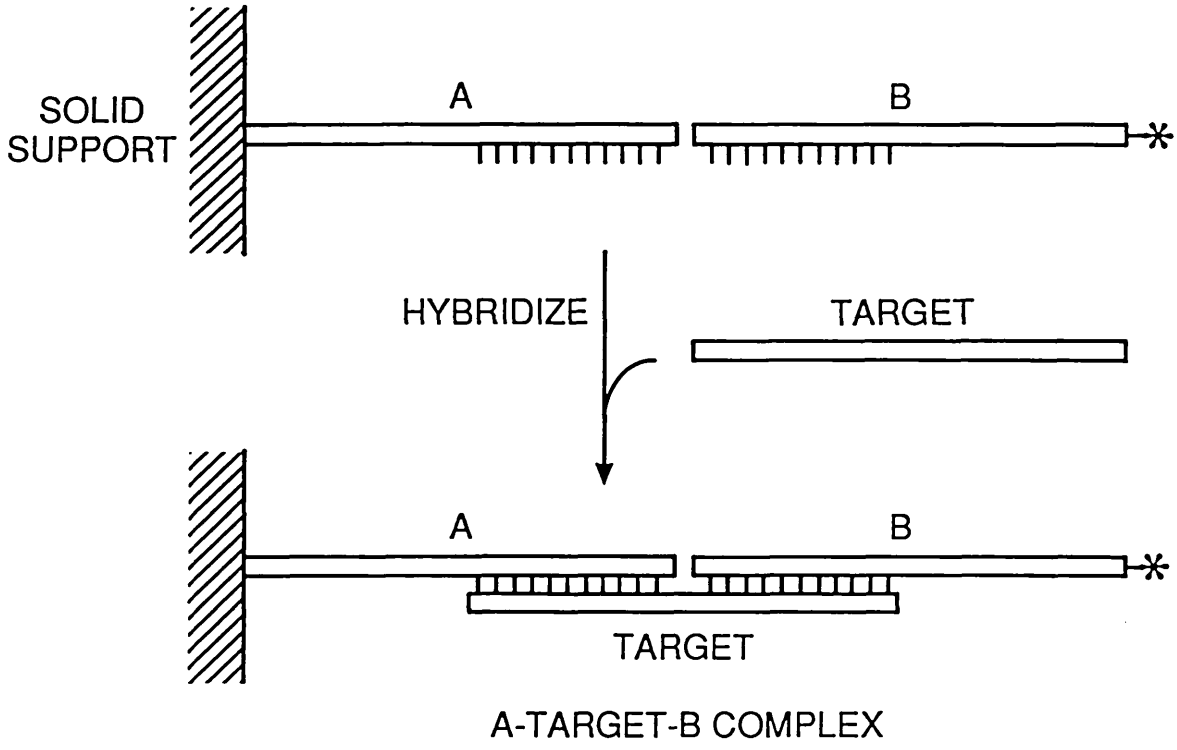


FIGURE 1

THE PRINCIPLE OF NUCLEIC ACID SANDWICH HYBRIDIZATION.

attachment of fragment A is not limited to a membrane, because there is no requirement for transfer out of a gel. The number of manipulations necessary to achieve a result is significantly fewer than for more conventional hybridization formats.

Although sandwich hybridization appears to be an obvious candidate for use in nucleic acid based clinical assay, it was not designed with this in mind. The technique was first described as a two-stage process by which RNA transcripts could be mapped to their origins on the viral genome (Dunn & Hassel, 1977; Dunn & Sambrook, 1980). Adenovirus type 2 (Ad2) DNA was restriction enzyme digested, size-fractionated on an agarose gel, and subsequently Southern blotted onto nitrocellulose; the immobilized Ad2 sequences were to form the 'A' component of the sandwich. The blot was hybridized with poly-(A) RNA isolated from cells 20 hours after infection with an Ad2-Simian virus 40 (SV40) hybrid virus, $Ad2^{+}ND1$ (Lewis et al, 1969). This isolate contains a 0.94 kb insertion of SV40 DNA, which replaces 1.9 kb of the Ad2 genome, located between positions 80.6 and 86.0 on the conventional physical map of Ad2. The SV40 insertion consists of the region located between co-ordinates 11 and 28 on the circular map of SV40 DNA (figure 2A). The molecules acting as 'targets' in the poly-(A) RNA were the chimeric ones derived from the junction between the Ad2 and SV40 DNA sequences in the hybrid. The region of each target molecule complementary to specific Ad2 DNA sequences hybridized to the appropriate band (or bands) on the nitrocellulose membrane. The blot was then washed, and probed with ^{32}P-labelled SV40 DNA (the 'B' component); the free tails of chimeric RNA molecules already hybridized to Ad2 sequences on the nitrocellulose underwent a second round of hybridization. Washing and autoradiography established which Ad2 fragment the chimeric RNA derived from (figure 2B). The results

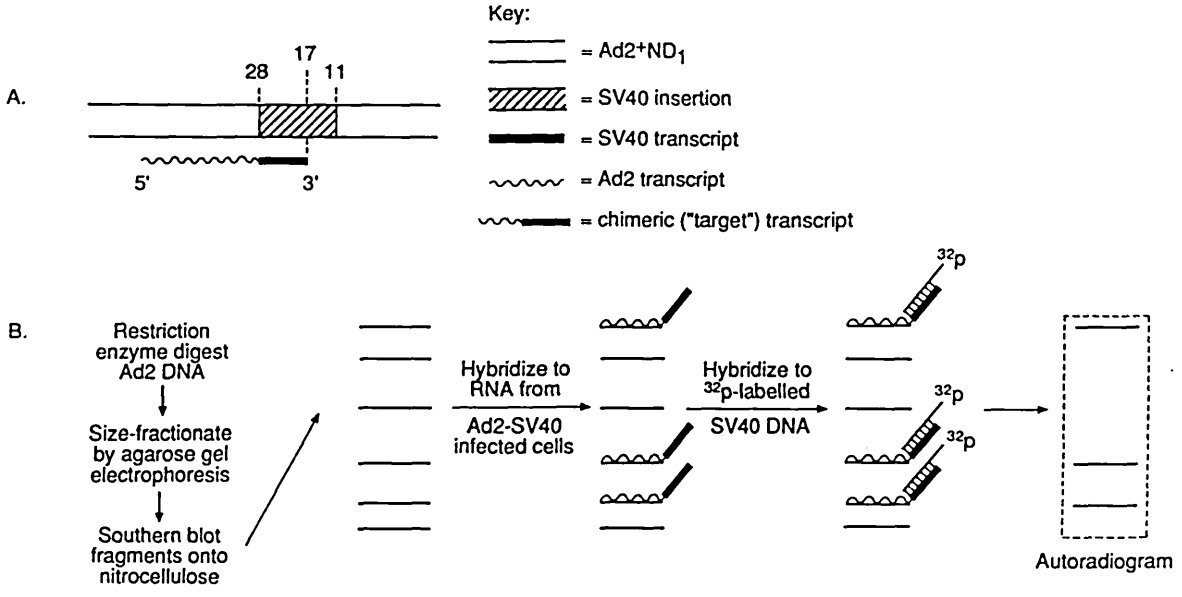


FIGURE 2

MAPPING VIRAL TRANSCRIPTS BY NUCLEIC ACID SANDWICH HYBRIDIZATION.

obtained demonstrated that the Ad2-SV40 RNA molecules hybridized not only to genomic sequences adjacent to the SV40 insertion site in the hybrid virus (as predicted), but also to other multiple discrete regions of the Ad2 genome. This was interpreted in terms of RNA sequences derived from multiple non-contiguous areas of the viral genome becoming covalently linked during, or after transcription. Possible mechanisms were postulated by which such chimeric RNA's could be derived, the simplest involving post-transcriptional splicing of independent RNA chains by RNA ligase. Initiation of transcription occurs within Ad2 sequences, and not within the SV40 sequence integrated in the genome of $Ad2^{+}ND1$. During the course of lytic infection with $Ad2^{+}ND1$, a chimeric viral RNA is produced, whose 5' end incorporates the start of the Ad2 fibre gene and whose 3' end lies in the inserted SV40 sequence. Two forms of this RNA exist in cells at late times of infection with the hybrid virus; these were identified by electron microscope heteroduplex analysis, and also by sandwich hybridization (Dunn et al., 1978). Both forms contain the conventional tripartite leader present on 'late' Ad2 mRNA's, but they differ in the presence or absence of a fourth leader sequence consisting of 180 bp of Ad2 sequences located 2 kb upstream from the gene. In a cell-free translation system derived from rabbit reticulocytes, the fourth leader sequence was shown to have no influence on the ability of the chimeric messenger to direct the synthesis of the protein it encodes. Later, in 1981, sandwich hybridization was utilized in studies to demonstrate that transcriptional events are primarily responsible for the tissue-specific synthesis of liver mRNA, not produced in brain or hepatoma cells (Derman et al., 1981).

Despite this initial success for mapping the chromosomal location of RNA transcripts, the sandwich hybridization technique was

never widely used for this purpose. It remained in the form described above for several years, until its potential for use in clinical assay was recognized.

1.3.2 A Diagnostic Use Of Sandwich Hybridization.

The first report of a diagnostic application of the sandwich assay was in 1983, and described the detection of adenovirus DNA in crude samples (Ranki et al., 1983 a). Two non-overlapping BamHI restriction fragments of the adenovirus type 2 (Ad2) genome were cloned into two different vectors; one fragment was inserted into the double-stranded plasmid pBR322 (Bolivar et al., 1977), and the other fragment was cloned into a DNA phage, M13 (Hu & Messing, 1982). The advantage of using two different vectors, with no extensive homology at the nucleic acid level, is that it eliminates the need for isolation of the fragments from vector sequences prior to immobilization, or labelling. If both fragments A and B were cloned into the same vector, there would be a risk of obtaining a false positive result, due to hybridization of contaminating vector sequences in the immobilized and labelled Ad2 genomic fragments. This is a highly significant problem in sandwich assays, because the probe is used in a large excess over target, in order to improve the kinetics of the hybridization reaction (see section 4.6.1). A fifty-fold excess of probe over target would mean that as little as 2% contamination of the Ad2 probe with vector sequences would result in a false positive result. This can be avoided by cloning fragments A and B into different vectors, or by taking great care to thoroughly purify the insert from vector sequences. For the first diagnostic sandwich assay, pBR322 containing an Ad2 insert was denatured and immobilized onto a nitrocellulose filter by baking at 80^{o}C for 2 hours. Each filter was then used to produce disks, each 10

mm in diameter, for use in the assay. Recombinant phage DNA containing fragment B was chemically iodinated with ^{125}I, to a specific activity of 10^7-10^8 dpm/ug (Commerford, 1971). Such single-stranded probes have a distinct advantage over their double-stranded counterparts- those produced by nick translation (Rigby et al., 1977), or random hexanucleotide priming (Feinberg & Vogelstein, 1983,1984) -because there is no complementary sequence to compete with the target DNA for hybridization to the labelled strand. This results in improved hybridization kinetics, because a large excess of probe can be used to push the reaction to completion, without the danger of probe self-reassociation, and effective withdrawal from the hybridization equilibrium. The assay for the detection of Ad2 sequences was performed by mixing the denatured sample with immobilized and labelled probes, hybridizing, and washing off the excess label. The great advantage of using a separate disk for each sample was that signals could be individually quantitated by gamma-counting, producing results in an easily interpreted numerical form, much more rapidly than by auto-radiography. The number of counts attached to each filter was used to derive the amount of Ad2 DNA in a sample by comparison with a standard curve. Overnight hybridization resulted in a sensitivity capable of detecting 5×10^6 molecules (8×10^{-18} moles) of Ad2 DNA in a crude sample; this is equivalent to the detection of less than 10^2 infected cells. Crude samples cannot be readily analyzed in procedures involving sample immobilization, because contaminating macromolecules can cause high levels of non-specific binding of probe to solid support (Ranki et al., 1983 a).

In summary, the advantages of this sandwich assay format, the first with a diagnostic application, make it attractive because it is rapid and simple to perform, presents results in an easily interpreted

numerical form, and can be used for analyzing crude samples with a minimum of sample pre-processing. It does have important disadvantages however: firstly, the sensitivity achieved is at least ten times less than that attained by Southern blotting. Secondly, because of procedural complexities, it would prove difficult to automate the assay using available technology. Although it is far from ideal for routine diagnostic analysis, this sandwich protocol has been used for the detection of viruses, bacteria and other infectious organisms, in crude biological samples. Nevertheless, although the sandwich technique has distinct advantages over more commonly used nucleic acid detection systems, it has not yet been widely accepted as a successor to them. To overcome this problem, it will be necessary to re-design the protocol, in order to eliminate the inherent disadvantages. The strengths and weaknesses of the sandwich assay are considered in more detail below.

1.3.3 Sandwich Hybridization For Detecting Viral Infection.

Nucleic acid based detection systems are particularly suitable for identifying viral infection, because viruses can persist in the cell as RNA or DNA, in episomal form, or integrated within the cellular genome; there need be no protein expression, so the only means of detecting infection is by recognizing abnormalities in the infected cell (assuming the virus disrupts normal cell function, which is not always the case), or by identifying nucleic acids unique to the infecting organism. For example, potato spindle tuber viroid can only be identified in infected potatoes by nucleic acid hybridization (Owens & Diener, 1981). Viral persistence without protein expression renders immunoassay powerless as a tool for identifying infected tissues (Kulski & Norval, 1985).

Sandwich hybridization assays have been applied with moderate

success to the detection of adenovirus infection in nasopharyngeal aspirates from children (Ranki et al 1983 a,b) and young adults (Lehtomaki et al., 1986) with acute respiratory infection, and also to the detection of cytomegalovirus (CMV) in urine samples (Virtanen et al., 1984). The final reported use of sandwich hybridization for identifying viral infection is for the detection of human papilloma-virus nucleic acid in cervical scrapes and biopsies (Parkkinen et al., 1986; Parkkinen, 1988; Parkkinen et al., 1988). Although the assay systems mentioned above are very similar to that used for the first diagnostic application of sandwich hybridization (section 1.3.2), it is useful to consider each in more detail, in order to highlight the requirements of nucleic acid-based tests for detecting viral infection. It also aids the identification of potential improvements which may bring sandwich hybridization within the realm of routine diagnosis.

1.3.3.1 Adenovirus (Ad).

The adenoviruses are divided into six groups on the basis of nucleic acid homology (Ginsberg et al., 1987); members of a given serotype have an 80%-90% sequence homology with others in the same group, but only 10%-15% homology with those of other groups (Green et al., 1979). Certain serotypes are associated with epidemics of pneumonia, and other respiratory tract infections (Ginsberg et al., 1987). Diagnosis of adenovirus infection was traditionally based on serology and virus isolation, but more recently, immunoassays directed against viral antigens have been used (Halonen et al., 1980; Sarkinnen et al., 1981). Such methods are the most sensitive currently available for the rapid diagnosis of respiratory virus infection.

The sandwich assay designed for adenovirus detection requires two pairs of non-overlapping genomic probes, one pair from the genome

of Ad2, and the other from Ad3. These viral types were chosen because they are members of adenovirus serogroups C and B respectively, and it is these serogroups which cause most respiratory infections among children. One fragment of each probe pair (A) was cloned into pBR322, and the other (B) into phage M13mp7 (Virtanen et al., 1983; Ranki et al., 1983 b). Nasopharyngeal mucus aspirates were collected with a mucus extractor through the nostrils of children admitted to hospital with respiratory infection. Specimens were screened for seven respiratory viruses by radioimmunoassay (RIA) and also by virus isolation, prior to treatment with SDS and proteinase-K. Samples were then heated and rapidly cooled, in order to denature the DNA, prior to mixing with fragment A (immobilized onto individual 10 mm diameter nitrocellulose disks), and fragment B (radiolabelled with ^{125}I to a specific activity of approximately 10^8 dpm/ug [Commerford, 1971]). After hybridization, stringent washing and signal measurement, results were compared with those obtained from an immunoassay designed for the detection of Ad hexon antigen. Two control hybridizations were performed, by which the specificity of the assay was confirmed: firstly, the Ad-specific fragment A was replaced with sonicated calf thymus DNA, and secondly immobilized DNA was omitted entirely. An excellent correlation was observed between results obtained by RIA and sandwich assay, both techniques resulting in a similar sensitivity. Sandwich hybridization allowed the detection of approximately 4.8×10^6 molecules (8×10^{-18} moles) of target DNA; this represents a sensitivity significantly lower than that achieved by virus isolation. It is, however, important to remember that hybridization and RIA provided results in under 24 hours, whereas up to three weeks were required for all positive samples to be identified by virus isolation.

The main strength of the sandwich hybridization assay described

above is that it is unaffected by the crude nature of the sample (Ranki et al., 1983 a). Techniques which require sample immobilization are affected by the presence of biological contaminants, for two reasons: firstly, proteins can compete with nucleic acids for the available binding sites on the solid support, and secondly, macromolecules which become bound to the membrane can result in unacceptably high levels of non-specific background hybridization. This results in a dramatic decrease in the sensitivity of the test. The disadvantages of the sandwich assay for the detection of adenovirus infection are that it relies on the use of a radioactive probe, and certain stages in the procedure involve manipulations complex enough to prevent automation using currently available technology.

1.3.3.2 Human Cytomegalovirus (CMV).

Human cytomegalovirus (CMV) has a complex genome, 230 kb in size, which codes for 250 different proteins. Serological evidence of previous infection with the virus is demonstrable in between 30% and 100% of the individuals in adult populations (Weller, 1971). Most infections are symptomless, but in patients undergoing immunosuppressive therapy, and particularly in those receiving organ transplants, CMV infection can have serious consequences (Glenn, 1981). The presently used methods for diagnozing infections include serological analysis, and virus isolation, but neither is ideal because of the delay in arriving at a result. Monoclonal antibodies to demonstrate the presence of viral antigens are available (Volpi et al., 1983), but a rapid nucleic acid based assay would be an invaluable asset in monitoring the progression of CMV infection.

There are two factors which need to be considered when choosing the nucleic acid probe sequence for the detection of CMV: firstly, genetic variation and antigenic heterogeneity is common amongst

naturally occuring CMV strains (Waner & Weller, 1978), so it is important to select DNA probes from regions not subject to extensive variation. A second complicating factor is that large viral genomes have been shown to contain repeated sequences common to all eukaryotic DNA (Heller et al., 1982; Peden et al., 1982). These must obviously be avoided, or background problems will result from probes hybridizing to human DNA, as well as to CMV sequences. The sandwich hybridization assay designed for the detection of CMV in urine utilizes two adjacent BamHI restriction fragments derived from the long unique region of the CMV genome (Virtanen et al., 1984). One of the fragments (A), 4.2 kb in size, was cloned into pBR322, and the other, 2.5 kb fragment was inserted into phage M13mp7. The assay was performed essentially as described in section 1.3.3.1, resulting in a sensitivity capable of detecting 5×10^6 virus particles in an 18 hour hybridization protocol; this is lower than the sensitivity of direct dot hybridization, in which the sample is immobilized onto a membrane. However, one of the disadvantages of the direct assay is that the method is sensitive to the presence of enterobacteria in urine samples; such bacteria may harbour plasmids or phages related to the commonly used cloning vectors, so it is essential to purify the probe from the vector thoroughly before use (Chou & Merigan, 1983). In sandwich hybridization this problem is circumvented, because the sample is not immobilized (Virtanen et al., 1984).

1.3.3.3 Human Papillomavirus (HPV).

The human papillomaviruses are double-stranded DNA viruses (genome size around 8,000 bp), of the family Papovaviridae. There are currently over 50 recognized types of the virus, which are distinguished on the basis of nucleic acid homology and host specificity. The replication of papillomaviruses is highly dependent on

the differentiation of keratinocytes, and it is not yet routinely possible to propagate virus particles *in vitro*. This makes it particularly difficult to generate antibodies directed specifically against HPV surface antigens (Ostrow & Faras, 1987), so immunoassays for identifying HPV infection are not readily available. The clinical significance of genital infection, together with a detailed analysis of the molecular biology of this group of viruses, is given in section 1.4. The nucleic acid-based assays currently used for the detection of HPV-specific nucleic acids, including Southern blotting (Choo *et al.*, 1987), slot blotting (Wickenden *et al.*, 1985) and sandwich hybridization (Parkkinen *et al.*, 1986; Parkkinen, 1988; Parkkinen *et al.*, 1988), are discussed fully in section 1.5.4, and in chapter 5.

1.3.4 Detection Of Other Infectious Organisms.

Nucleic acid hybridization can be used to demonstrate the presence of bacterial infection, by identification of species or group-specific genes, without the need for cultivation or bacterial typing. There are circumstances where the limitations of conventional typing tests make nucleic acid-based detection systems particularly attractive. The first therapeutic response to bacterial infection is commonly administration of antibiotics, which then interfere with bacterial growth in cell culture. This can render conventional strain-typing useless, thus confusing the choice of optimum treatment. A rapid nucleic acid-based assay would be unaffected by previously administered antibiotics, and could facilitate the identification of virulence-determining genes, thus distinguishing between pathogenic and apathogenic strains of a bacterium. It would also allow the identification of genes encoding proteins responsible for antibiotic resistance (Mosely *et al.*, 1982).

A nucleic acid sandwich assay for the detection of Enterobacteriaceae in crude samples has been developed, using two consecutive fragments derived from the OmpA gene of Escherichia coli K-12 (Palva, 1983). An important consideration in adapting the sandwich technique for bacterial detection is that, in general, the bacterial genome is much larger than that of a virus; the use of an intact bacterial genome as the target was found to have a negative influence on the efficiency of hybrid formation. Fragmentation of the sample DNA by mechanical shearing was found to increase the level of hybridization by around 50%; this may be because the stability of a long DNA fragment hybridized to a short probe is less than that of the hybrid formed by reannealing of the target to its complementary strand. Target reannealing may actually result in the probe being displaced from the probe-target hybrids already formed; such strand-displacement has been used as the basis for nucleic acid detection systems (Ellwood et al., 1986; Vary, 1987). The assay for detecting Enterobacteriaceae was performed largely as described in section 1.3.2. The sensitivity of the test was such that 3×10^6 molecules (5×10^{-18} moles) of E.coli DNA could be detected after overnight hybridization. The presence of 10^9 unrelated bacterial cells had no detrimental effect on the sensitivity, and of six members of the Enterobacteriacea tested, all were identified with the probes derived from E.coli K-12. As predicted, the distantly related members of the family (for example, Proteus mirabilis) give significantly lower signals than more closely related members, such as Salmonella typhimurium. A similar assay for the detection of the obligate intracellular parasite Chlamydia trachomatis has been described (Palva et al., 1984), which is capable of detecting 10^6 molecules (1.7×10^{-18} moles) of target DNA, in a protocol lasting 12 hours. This is ten times less sensitive than a nucleic acid dot-blot

assay using the same probes (Palva, 1985), but both methods are capable of distinguishing between C.trachomatis types A, C, E, G, H, I and L2, which represent the more distinct groups amongst the 15 pathogenic serotypes. Thus, by careful selection of hybridization conditions and probe sequence, it is possible to design assays for the detection of either a single organism, or all of the members of a related group of organisms.

The most common method for identifying infection with Plasmodium falciparum, the parasite which causes human malaria, is the demonstration of circulating organisms in peripheral blood, using light microscopy. This method is sensitive and reliable, but is of limited use if large numbers of samples are involved, because it is time-consuming (Bruce-Chwatt, 1984). Immunological techniques are not an ideal alternative, because they are unable to distinguish between active and previous infections (Avraham et al., 1982). A nucleic acid sandwich hybridization protocol has been developed, using repetitive sequences of P.falciparum as probes (Zolg et al., 1987). Sample DNA is immobilized on to a nitrocellulose membrane, and hybridized with an unlabelled probe consisting of a target-specific insert in phage M13. A second probe, M13 DNA labelled by nick translation with ^{32}P, is subsequently hybridized with the free region of the first probe. The signal obtained is independent of the size of the specific insert, and the assay is at least as sensitive as light microscopy.

1.3.5 Detection Of Restriction Fragment Length Polymorphisms.

An important modification of the sandwich technique, for the detection of restriction fragment length polymorphisms (RFLP's), was first described in 1985 (Langdale & Malcolm, 1985). Prior to this the method had only been used to detect the presence or absence of specific

nucleic acids and not to detect variations within the nucleotide sequence. RFLP's result when a genomic mutation creates or destroys a specific sequence of nucleotides recognized by a restriction enzyme. On binding to this recognition site, the enzyme hydrolyses both DNA strands, so the number and position of such sites within the genome determines the pattern of bands when the enzyme digested DNA is size-fractionated by gel electrophoresis.

Sickle-cell anaemia is a well characterized genetic disorder, caused by a single adenine to thymine mutation in the sixth codon of the beta-globin gene; the mutation results in the substitution of a glutamic acid residue for a valine, on translation of the beta-globin mRNA. The principle of the sandwich assay for the detection of this mutation is shown in figure 3. A 341 bp beta-globin gene restriction fragment from the 5' side of the sickle mutation site was isolated, and immobilized via the bases onto Sephacryl S-500, using a diazotization reaction (Bunemann et al., 1982; Seed, 1982). A second fragment, 201 bp long, immediately adjacent and 3' to the first, was labelled with ^{32}P by random hexanucleotide priming. Sample genomic DNA was digested with DdeI, denatured, and hybridized with the immobilized and labelled probes. The DNA from an individual homozygous for the sickle mutation is not hydrolysed at the mutation site, and the intact globin target sequence forms a linking bridge between the immobilized and labelled DNA, resulting in labelled molecules becoming attached to the Sephacryl S-500. Conversely, the DNA from a normal individual is severed at the mutation site, and is thus not able to form the bridge; the solid support is not labelled. The advantages inherent in using a bead as the hybridization solid support are discussed fully in section 1.6.1. The sensitivity is such that 3×10^{6} molecules (5×10^{-18} moles) of target DNA can be detected in a 12 hour assay, and

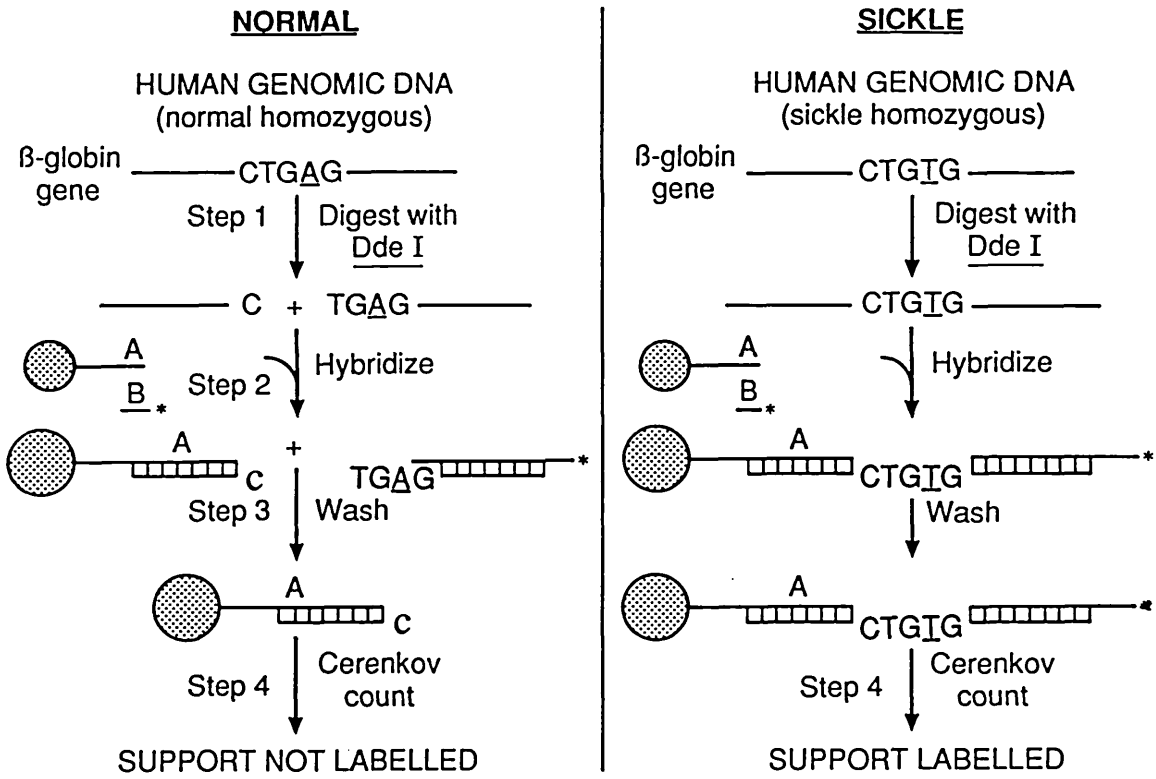


FIGURE 3

SCHEMATIC REPRESENTATION OF SANDWICH HYBRIDIZATION ASSAYS DESIGNED TO DISTINGUISH NORMAL AND SICKLE ß-GLOBIN GENES.

differentiation between samples from normal and sickle cell anaemia patients (homozygotes) as well as those from individuals with sickle cell trait (heterozygotes) can be readily achieved. However, particular attention should be paid to two points: firstly, if the restriction enzyme digestion is incomplete, target sequences in normal samples will remain intact, giving rise to a false-positive signal. Secondly, non-specific degradation of a sample could result in target sequences being severed regardless of the nature of their restriction sites, giving rise to a false negative signal. Errors such as these are not normally encountered in techniques involving size-fractionation, because the physical state of the sample DNA can be ascertained from the positions of the bands, relative to markers, on a gel. Another important consideration is that only a minority of point mutations actually result in the disruption or formation of a restriction site. As the number of recognized restriction enzymes increases, so it becomes more likely that an appropriate one will be available for a given mutation, but there will still undoubtedly be cases where this is not so. This problem can be surmounted by utilizing the differential melting properties of matched and mis-matched synthetic oligonucleotides (see section 1.7.2 for the principle of this method). The advantages of using synthetic probes are obvious; they can be made cheaply and in high yield on commercially available equipment. Oligonucleotides can be labelled with radio-isotopes (Studencki & Wallace, 1984; Collins & Hunsaker, 1985), or with non-radioactive marker groups (Cook et al., 1988; Urdea et al., 1988), and are single-stranded; this means that the kinetics of a hybridization reaction can be improved by increasing the concentration of probe, without the danger of probe re-association, and removal from the hybridization equilibrium. Studies are currently in progress to optimize the

immobilization of oligonucleotides to beaded solid supports (Ghosh & Musso, 1987; Voss & Malcolm, 1988 a,b), and to use labelled oligos as probes in differential melting sandwich hybridization assays for the detection of the point mutations resulting in sickle cell anaemia and alpha$_1$-antitrypsin deficiency (Uta Voss, personal communication).

1.3.6 Variations On The Sandwich Theme.

As the spectrum of nucleic acid based assays expands, the number of different probes required will increase, resulting in logistical problems of manufacture and storage. A technique has been developed for surmounting this difficulty, using sandwich hybridization in conjunction with a universally applicable probe (Wolf et al., 1986). An unlabelled M13 construct (termed 'X') containing a 3 kb insert specific for cytomegalovirus (CMV) AD169 DNA was hybridized to a sample immobilized on a nitrocellulose membrane. The presence of CMV target in a sample results in hybridization with the CMV-specific insert of X, leaving the remaining M13 sequence single-stranded. Subsequently, a labelled M13 probe is applied, which hybridizes with the free M13 part of X; only if the first, CMV-specific, hybridization is successful will the nitrocellulose become labelled. The signal obtained from such an assay will clearly be related to the number of target molecules present, but not to the size of the sequence-specific insert, so it should allow for standardization between tests for different organisms. The main disadvantage of this system is that it requires the immobilization of the sample nucleic acid; when a large number of assays are necessary, this step can be very time-consuming. It is possible that this problem could be overcome by using a 'double' sandwich hybridization, in which a suitable fragment A is immobilized, to act as a capture reagent. On hybridizing A, X and target, the first

sandwich would form, resulting in the attachment of X to the solid support via the target molecule. Labelled M13 could then be directed against the single-stranded M13 part of X, thus producing the signal. The main problem likely to be encountered with this approach is that a large excess of labelled probe would be required in order to push the hybridization equilibrium in favour of the 'double' sandwich; this may result in an unacceptably high background signal, and unfavourable hybridization kinetics.

Sandwich hybridization assays for the determination of plasmid copy number in Bacillus subtilis (Nyberg et al., 1985), and also for the quantitation of alpha-amylase mRNA in this organism (Palva et al., 1988), have been developed. Both techniques (which are identical in principle to that reported by Ranki et al. in 1985 [section 1.3.2]) may eventually play an important role in the fermentation industry, where the optimization of the parameters affecting gene expression is of considerable financial significance.

The rate at which two complementary DNA strands hybridize to each other is dependent, amongst other things, on their concentrations (Wetmur & Davidson, 1968; see section 1.6.3). In conventional assays involving the immobilization of the sample, the hybridization rate of a single-stranded probe can be enhanced by using it in a vast excess over the target. The alternative is to use rate-enhancers like dextran sulphate (Wetmur, 1975; Wahl et al., 1979) or, more effectively, polyethylene glycol (Amasino, 1986). These function by effectively increasing the concentration of nucleic acid in solution, due to the exclusion of probe molecules from the volume occupied by the hydrated polymer. This effect can be variable when double-stranded probes are being used, because the polymer can increase probe self-reassociation (potentially decreasing the signal), but can also enhance the formation

of networks of labelled molecules at the point of hybridization to the target nucleic acid strand (potentially increasing the signal) (Wahl et al., 1979). Neither dextran sulphate nor polyethylene glycol are effective at increasing the rate of hybrid formation in sandwich assays where the target is double-stranded, because they have the greatest effect on the self-reassociation of denatured target (Ranki et al., 1983 a; Langdale & Malcolm, 1985); this problem will be encountered in any assay where the target is double-stranded and free in solution, rather than immobilized in single-stranded form on the solid support.

The rate at which mixed-phase hybridizations occur (that is, those in which one strand is immobilized and the other is free in solution), is significantly lower than the rate of an identical reaction performed entirely in solution (Wetmur & Davidson, 1968). The advantages of both solution hybridization and a sandwich reaction have been combined in a recently developed technique known as affinity-based hybrid collection (Syvanen et al., 1986 a). It has been applied to the detection of resistance genes in uropathogenic strains of E.coli (Syvanen & Korpela, 1986). The identification of resistance genes, and those encoding pathogenicity factors and toxins, can be vital in influencing the choice of an optimal course of medical treatment. The principle of affinity-based hybrid collection is shown in figure 4; the technique involves hybridizing the denatured sample nucleic acid with two sequence-specific probes, one of which is chemically iodinated with ^{125}I (Commerford, 1971), and the other labelled with biotin. The hybridization reaction is allowed to procede to completion in solution, and the hybrid complex is collected by mixing with a streptavidin-agarose support. The affinity between biotin and streptavidin is extremely high; the association constant for the interaction is

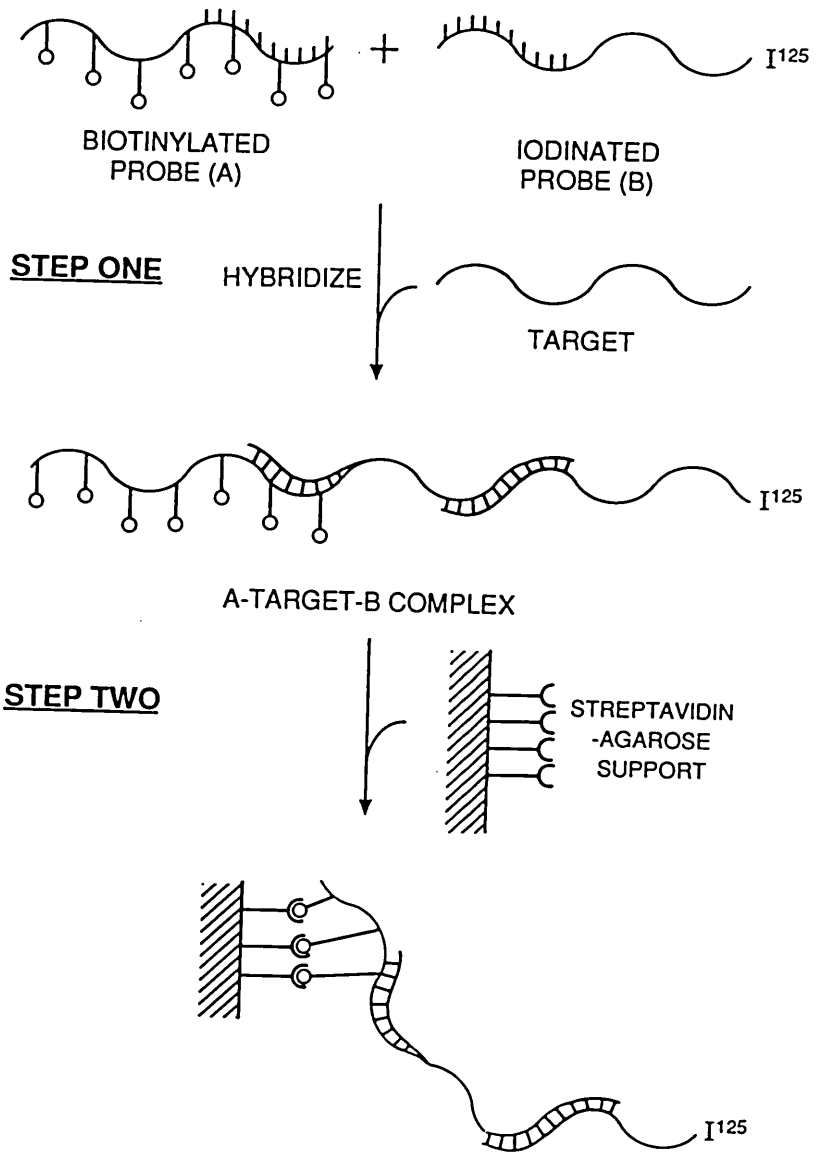


FIGURE 4

THE PRINCIPLE OF AFFINITY BASED HYBRID COLLECTION.

10^{15} M^{-1}, and is approximately 10^6 times greater than the typical affinity of an antibody for an antigen (Green, 1975). This ensures that the collection of the complex is quantitative. After washing stringently, the results are determined by gamma-counting. In order to test the accuracy of the assay, DNA from single colonies of twenty different E.coli strains was analyzed without purification; in all cases, the relevent resistance genes were correctly identified in around three hours.

An automated sandwich hybridization procedure for the detection of Salmonella DNA in biological samples was first described by Polsky-Cynkin et al, in 1985. Two consecutive restriction fragments derived from the genome of S.typhimurium were isolated, and one of these fragments (A) was immobilized on to the inner surface of a polypropylene solid-phase receptacle (SPR), by well characterized protein-DNA complexing procedures (Welsh & Cantor, 1984). The second DNA fragment (B) was labelled with ^{32}P; denatured sample was mixed with A and B simultaneously, and hybridization and washing were performed in a commercially available automated immunoassay system (Parsons et al., 1983). Cycles of pressure variation force reagents in and out of each SPR, resulting in a 30% increase in hybridization rate. Results were quantitated by scintillation or gamma-counting, and in a 4 hour protocol, 8.4×10^6 molecules (1.4×10^{-17} moles) of target DNA could readily be detected in a non-homologous mixture of cellular nucleic acid. Investigation has shown that the immobilized DNA is stable during the pressure cycling, and does not leach off of the SPR surface; it is particularly important in sandwich assays that this is the case, because vast excesses of immobilized probe over target are used, and loss of even a small percentage of the bound DNA would lead to a solution-phase competition, resulting in a sensitivity decrease.

1.3.7 Sandwich Hybridization With Non-radioactive Detection Systems.

All of the hybridization systems described so far rely on nucleic acid probes labelled with radio-isotopes in order to achieve the necessary levels of sensitivity. Isotopes like ^{35}S, ^{125}I and particularly ^{32}P are far from ideal, because they result in probe instability; there are at least three mechanisms by which the decay of an isotope can destroy a labelled nucleic acid strand. Firstly, strand scission results from decay of an atom integral to the nucleic acid backbone (this is particularly important in the case of ^{32}P). Secondly, radiolysis of neighbouring strands can result due to bombardment of solutes with beta-particles. Finally, the kinetic energy released on decay of ^{32}P or ^{125}I can cause a recoil sufficient to break up the nucleic acid molecule ('Nucleic Acid Labelling' booklet, Amersham International plc). One of the most significant barriers preventing hybridization-based assays from being accepted for routine diagnostic use is that special laboratory facilities are required for the safe handling and disposal of radio-isotopes. Not surprisingly, this has generated considerable interest in the development of alternatives to isotopic labelling (Gillam, 1987). A non-radioactive probe should have high stability, a long shelf-life, be perfectly safe to use, and preferably be easy and economical to manufacture.

A modification of affinity-based hybrid collection, for the non-radioactive detection of hybrids bound to microtitre plate wells, has been recently described (Syvanen et al.,1986 a). The model method was developed for the detection of pBR322; one plasmid restriction fragment was modified by a sulfonation reaction (Verdlov et al., 1974), and a different fragment labelled by nick-translation with biotin-11-dUTP. Denatured target material was hybridized with A and B, and the

hybrid complex was captured on to the surface of a microtitre plate well precoated with a commercially available monoclonal antibody directed against sulfone-modified DNA. After washing, the hybrid complex was detected by addition of a horseradish peroxidase (HRP)-streptavidin conjugate, washing again, and finally adding a substrate for HRP which produces a coloured product (in this case, o-phenylene-diamine with hydrogen peroxide). The absorbance of the coloured product is measured with a spectrophotometer at 492 nm. The values obtained can be compared with a standard curve, in order to determine the amount of target DNA present in the sample. The assay can detect 10^7 molecules (1.7×10^{-17} moles) of target, in a 1.5 hour hybridization reaction; although this represents a sensitivity considerably lower than that achieved by conventional blotting, there are a number of characteristics incorporated into the assay which warrant further attention. Firstly, results are determined using a commercially available spectrophotometric microtitration plate reader, which presents them directly in numerical form. A 96-well plate can be scanned in a matter of seconds, and the results can be fed directly into a computer for immediate analysis. Hardware and software ideal for this purpose is already routinely used in diagnostic immunoassay; novel nucleic acid based tests using instruments already familiar in the clinical laboratory will be far more easily integrated for everyday use than those relying on new, unfamiliar technology (Hansen, 1985).

It is becoming obvious that in the development of nucleic acid based assays for the detection of genetic or infectious disorders, we can learn a great deal from immunoassay formats which have proven successful in a clinical setting. An excellent example is that of a technique known as time-resolved fluorometry, which has been successfully applied to immunochemical assays (Soini & Kojola, 1983),

and has now been adapted for use with DNA probes (Syvanen et al., 1986 b). The signal detection principle revolves around the properties of the element europium which, when excited with ultraviolet radiation, releases energy at a longer wavelength, in the form of visible light. This is a characteristic of all members of the lanthanide group of elements. There is a delay of as long as a millisecond between excitation and emission; this is extremely important, because it allows a signal to be measured after the initial burst of short-lived background fluorescence has decayed. This background has previously imposed serious limitations on the sensitivity of assays using fluorometry for measurements on biological samples. The principle of time-resolved fluorometry for the detection of nucleic acids is shown in figure 5; the technique requires two consecutive, non-overlapping sequences derived from the genome of the organism of interest. One of the fragments (A) is immobilized on to a nitrocellulose filter, and the other (B) is modified with immunogenic fluorene, or sulfone. On hybridization of A and B with denatured sample nucleic acid, the modified probe becomes attached to the filter only in the presence of the correct target sequence. After washing, an antibody (Ab1) directed against hapten-modified DNA is allowed to bind to the captured probe; further washing, and incubation with a europium labelled antibody (Ab2) directed against Ab1 results in attachment of europium to the filter. After a final stringent washing step, bound europium is released into solution, and chelated to diketones prior to quantitation of fluorescence in an automatic time-resolved fluorometer. The sensitivity of the technique is such that 10^6 molecules (1.7×10^{-18} moles) of adenovirus type 2 DNA could be detected in an assay lasting 4 hours, by comparison with a standard curve. The assay may be simplified by the use of a DNA probe labelled directly with the metal ion chelator

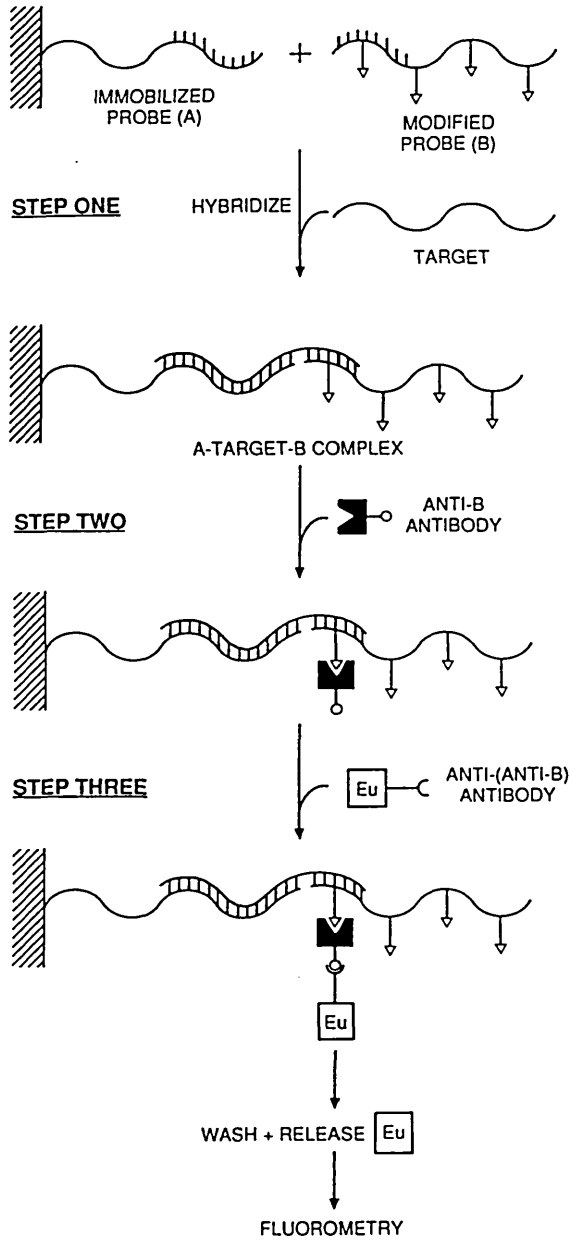


FIGURE 5

THE PRINCIPLE OF TIME-RESOLVED FLUOROMETRY, APPLIED TO NUCLEIC ACID SANDWICH HYBRIDIZATION.

diethylentriamine penta-acetate (DTPA) (Oser et al., 1988). Such probes have been used for the detection of target DNA immobilized in polystyrene microtitre plate wells (Oser & Valet, 1988). After affinity-based hybrid collection using a probe (B) labelled with DTPA, europium ions could be chelated directly by the hybrid-bound DTPA ligands, thus totally avoiding the need for using antibodies as part of the signal detection system. An alternative means of achieving this, labelling probe B with biotin, followed by detection with europium labelled streptavidin, proved to be less sensitive than the immunologically based detection system (Dahlen, 1987).

A novel non-radioactive application of a sandwich-type assay, for the rapid detection of specific nucleic acid sequences in crude biological samples, has recently been reported (Urdea et al., 1987 a,b). It does not rely on electrophoresis or blotting, but uses oligonucleotide hybridization, together with signal amplification steps and a horseradish peroxidase labelling scheme, to achieve a sensitivity comparable to that of Southern blotting. The technique has been applied to the detection of hepatitis B virus (HBV) in human serum, and the principle is shown in figure 6. The system utilizes five sets of oligonucleotides: members of set A (12 different oligos) and set B (36 different oligos) both have regions complementary to different parts of the HBV genome. Set A oligos also have a region complementary to members of set C, and set B oligos have a region complementary to members of set D. Set C oligos are biotinylated and bound quantitatively to avidin-containing polystyrene beads (Urdea et al., 1988), whereas members of set D are chemically cross-linked together to form a complex. If there is HBV DNA present in a sample, A and B oligos will hybridize to it; addition of C, coupled to the polystyrene bead, allows the capture of the A-B-HBV complex on to the solid support, via

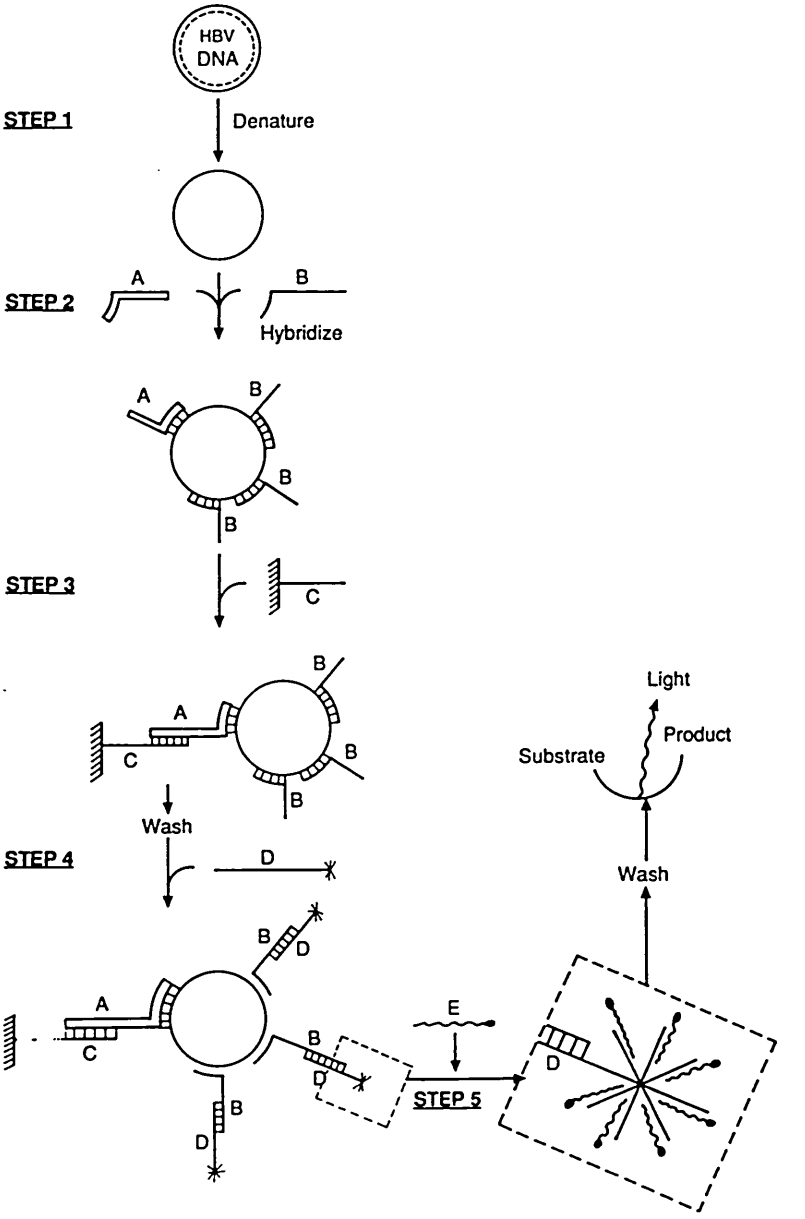


FIGURE 6

NON-ENZYMATIC AMPLIFICATION OF SIGNAL, USING MULTIPLE OLIGONUCLEOTIDE SANDWICH HYBRIDIZATION.

the hybridization between C and the free region of A. Subsequent addition of the D complex results in its hybridization to the free regions of B. In effect, the D complex becomes attached to the bead. Stringent washing steps, and a final hybridization with E, a horseradish peroxidase (HRP)-labelled oligo complementary to D, allows the result to be quantitated by a modification of the enhanced chemiluminescence method (Matthews et al., 1985). There are several important features which contribute to the success of this format; the most significant is that there are three built in signal amplification steps, involving oligo sets B, D and E. For each HBV target molecule, there are 36 binding sites for oligos of set B. Each of these B oligos can hybridize to a D complex, and each D complex can capture several HRP-labelled oligos. The effect of these signal-enhancing steps is that as little as 6×10^4 molecules (10^{-19} moles) of HBV DNA can be detected in 4 hours; this represents a 50- to 100-fold signal enhancement compared to using one labelled probe, and is comparable to the most sensitive dot-blot techniques. Results are best expressed as a signal/noise ratio (S/N); of the samples initially tested, no negative had an S/N value greater than 1.7, whereas the lowest positive (0.2 pg, or about 0.1 amole of HBV DNA) had an S/N of 4.1. Application of the methodology to the detection of other target sequences would only require the synthesis of new oligonucleotide sets A and B, because sets C, D and E are universally applicable and are not target-specific.

1.4 The Papillomaviruses (PVs).

The papillomaviruses were originally classified as a genus of the Papovaviridae family, because of the rough similarities between their viral genome and capsid and those of the polyomaviruses; the latter form the only other genus of the Papovaviridae. Both genera are

characterized by a covalently closed, circular, double-stranded genome, enclosed within an icosahedral capsid; the capsid consists of seventy two capsomeres, in a skew arrangement. The main differences between the genera lie in the size of the genome (relative molecular mass 5 x 10^6 in the case of papillomaviruses [approximately 8 kb in length], and 3 x 10^6 in polyomaviruses [approximately 4.8 kb in length]), and also in capsid size (50-55 nm diameter in papillomaviruses, but only 40-45 nm in polyomaviruses). Although both genera possess a double-stranded genome, only the polyomaviruses have open reading frames (ORFs) on both strands (Broker, 1987), suggesting that the genomic organization of the papillomaviruses differs significantly from that of the polyomaviruses. This has been verified by nucleotide sequence and transcript analysis (see section 1.4.1).

The papillomaviruses share genus-specific antigens, which can be detected by antisera directed against detergent disrupted viral particles (Jenson et al., 1980). On the nucleic acid level, DNA isolated from different papillomavirus types cross-hybridizes under conditions of low stringency (Law et al., 1979), indicating regions with more than 70% homology; under the same conditions, no cross-hybridization is observed with DNA isolated from simian virus 40 (SV40).

The first papillomavirus to be described was that infecting the cottontail rabbit (Shope, 1933), and subsequent studies have characterized papillomaviruses from a wide range of vertebrate hosts. There are currently over fifty different recognized types of human papillomavirus, and at least six recognized types that infect cattle. The criteria used to differentiate between different types of the virus are discussed in section 1.4.1. Each new virus isolated is named after its natural host; this is usually unequivocal, because most

papillomaviruses have a very restricted host range (Pfister et al., 1986).

The replication of papillomaviruses is highly dependent on the differentiation of keratinocytes (Broker, 1987), and it is still not routinely possible to propagate viral particles in vitro. Consequently, biological samples are the only source of viral material. The efficiency of virion production in vivo is dependent on virus type, and in circumstances where the amount of clinical material available is limited, it may not be possible to isolate sufficient viral particles for protein analysis or serological study. For this reason, it is not practicable to distinguish between virus types on the basis of immunological difference, and at present differentiation is achieved by studies of nucleic acid homology and host specificity.

The papillomaviruses are oncogenic viruses in their natural hosts, which induce hyperproliferation in epithelial cells of the skin or mucosa; certain types can also infect fibroblasts. The proliferation of infected cells leads to the formation of benign tumours, papillomas or warts. In some cases, environmental and/or genetic factors can stimulate a benign proliferation to progress towards malignancy. One of the most well characterized examples is that of cattle in the Western Highlands of Scotland, which are affected by papillomatosis of the upper alimentary canal; it has been demonstrated that such animals are at much higher risk than their lowland counterparts for the development of squamous cell carcinomas. Ingestion of bracken fern, a plant commonly found in highland, but not in lowland areas, has been identified as a critical factor in the malignant conversion of the papillomas (Jarrett et al., 1978), and all of the intermediate stages between papilloma and carcinoma have been identified (Campo & Jarrett, 1987). Bracken-grazing cattle have been shown to be highly immuno-

suppressed, and bovine papillomavirus type 4 (BPV 4) has been isolated from papillomas of the upper alimentary canal. A comprehensive study has resulted in a hypothesis to explain the sequence of events during carcinogenesis (Campo et al, 1985), and experimental result has recently confirmed that BPV 4 infection leads to the initiation of the cell transformation process, although continued presence of the virus is not required for malignant progression (Smith & Campo, 1988). This is corroborated by the fact that BPV DNA is absent from the majority of cancer-derived tissue. Quercetin, a chemical present in bracken fern, causes immunosuppression and allows the spread and persistence of papillomas, thus increasing the target size for subsequent (undefined) neoplastic events.

It is clear that the papillomaviruses provide us with an excellent opportunity to study the molecular mechanism of viral carcinogenesis, particularly in the case of those viruses which infect animals, because there are no ethical limitations in studying the progression of deliberately induced infection.

1.4.1 Molecular Biology Of Papillomaviruses.

Most of the papillomavirus types currently under investigation were isolated by cloning directly from biopsy material, rather than from purified virus particles. In order to be classified as a member of the papillomavirus genus, an isolate must satisfy the following criteria: firstly, the genome must be in the order of 7-8 kb in length, and must persist in the source tumour as extrachromosomal covalently closed elements. The isolated nucleic acid must have a degree of sequence homology with DNA extracted from previously characterized papillomavirus types, and share a similar genomic organization (Gissmann et al., 1984). If viral nucleic acid isolated from two

different sources exhibits less than 50% homology, the two isolates are considered to be different papillomavirus 'types'; if the homology is greater than 50%, but is incomplete the isolates are considered to be 'subtypes', and if the two differ only in the presence or absence of specific restriction enzyme recognition sites, they are referred to as 'variants' of the same virus type (Coggin & zur Hausen, 1979). It is important to point out that homologies are determined by allowing the uncharacterized nucleic acid strand to reassociate in solution with the single-stranded genome of a known viral type; this is followed by removal of single-stranded (non-hybridized) regions either by digestion with S1 nuclease, or by hydroxylapatite chromatography. This allows the proportion of hybridized (and therefore homologous) DNA to be expressed as a percentage of the total originally present. However, an accurate representation of homology on the nucleic acid level is not obtained, because certain areas of the genome may possess a degree of similarity, albeit insufficient for them to form a duplex under the conditions of the solution hybridization. Virus types that show no cross-hybridization at all under stringent conditions may still possess conserved regions with more than 50% nucleotide homology, as determined by sequence analysis. HPV 6b and HPV 11, for example, are classified as different virus 'types' even though homology at the nucleic acid level determined by direct comparison of their nucleotide sequences is 82% (Dartmann et al., 1986). Thirty one of the recognized HPV types have been separated into groups on the basis of DNA sequence homology, as shown in table 1 (Pfister et al., 1986). Virus types which show no appreciable homology with any other isolate are assigned their own group (for example, HPV 18 is the only member of group VII). HPV 16 and HPV 31 share some homology with each other, but not with any other type, so they are assigned a separate group (VI). The remaining twenty

four types fall into four groups, which are in turn separated into sub-groups; the members of a given sub-group share more sequence homology with each other, than they do with members of adjacent sub-groups. It is interesting to note that all members of group IV were isolated from patients with the rare skin disease epidermodysplasia verruciformis (see section 1.4.2). The rapidly expanding number of recognized HPV types has led to the proposal that the criteria used for type assignment are unsuitable. However, the suggestion that HPV

TABLE 1: Human papillomavirus types grouped according to DNA sequence homology.

I	IIa	IIb	III	IVa	IVb	IVc	V	VI	VII	VIII
1	2	6	4	5	9	24	7	16	18	30
	3	11		8	15	15		31		
	10	13		12	17					
		26		14						
		27		19						
	28			20						
	29			21						
				22						
				23						
				25						

types with greater than 80% homology (as determined by nucleotide sequence analysis) should be merged as a single type is unrealistic, because homologies are not evenly distributed over the genome, and significant differences in certain areas may reflect important variations in biological activity (Pfister et al., 1986). Despite the extraordinary heterogeneity of papillomaviruses, individual types do not appear to be subject to a rapid genetic drift. For example, the sequences of two BPV 1 isolates cloned separately in the USA and in Sweden revealed just five nucleotide variations (Ahola et al., 1983).

Thus, although a given virus type exhibits a considerable genetic stability, a large number of different types have emerged, possibly by divergent evolution and interviral DNA recombination (Pfister et al., 1986)

Although it is not yet routinely possible to culture the viruses in vitro, the development of molecular cloning techniques has enabled the molecular biology of the papillomaviruses to be studied in great detail. This has no doubt been facilitated by the fact that the genome is small, and therefore easily manipulated. The complete nucleotide sequence of the genome of at least 13 papillomavirus types has been determined, and with the exception of BPV type 2, all have been published. The available sequences are those of: HPV types 1a (Danos et al., 1982), 5 (Zachow et al., 1987), 6b (Schwarz et al., 1983), 8 (Fuchs et al., 1986), 11 (Dartmann et al., 1986), 16 (Seedorf et al., 1985 a), 18 (Cole & Danos, 1987) and 33 (Cole & Streeck, 1986); BPV types 1 (Chen et al., 1982) and 4 (Patel et al., 1987); cottontail rabbit (or Shope) papillomavirus (Giri et al., 1985) and deer fibroma virus (Groff & Lanacster, 1985). Since the sequence of BPV 1 was obtained first, much of the information currently available regarding the molecular biology of papillomaviruses was determined by studying this virus type. If the sequences of the papillomavirus genomes listed above are aligned with that of BPV 1 (linearized at the unique HpaI site), similar genomic organization is observed in each case. Approximately 90% of the papillomavirus genome can be assigned to one of the several open reading frames (ORFs); the major ORFs are located on one strand, with all three possible translational frames being represented. The remaining 10% of the genome consists of a non-coding region (alternatively known as the upstream regulatory region [URR], or long control region [LCR]) of about 800 bp, which harbours the origin of

replication, and transcriptional control signals; sequences suggestive of enhancers, promoters and polyadenylation sites are also present in this region. For most DNA viruses, it is possible to sub-divide the ORFs into two functional groups: the 'early' genes, which are expressed before the onset of viral DNA replication, and the 'late' genes, which are expressed after DNA replication has commenced (Pettersson et al., 1986). Since it is not yet possible to propagate papillomaviruses in tissue culture, it is not realistic to make the distinction between early and late genes on the basis of experimental result; nevertheless, by analogy with other papovaviruses, it has been assumed that the early genes are those which are expressed in transformed cells. In the case of BPV 1, deletion studies have allowed the transforming region to be localized to a fragment representing 69% of the total genome size (Lowy et al., 1980). Eight ORFs of significant size have been identified within this sub-genomic fragment, and these have been designated E1 to E8 (Broker, 1987). Counterparts of five of these, namely E1, E2, E4, E6 and E7, have been identified in all of the papillomavirus genomes so far sequenced, so it is likely that they code for functional polypeptides. The late region consists of two ORFs, termed L1 and L2, which respectively code for the major and minor viral capsid proteins. In an attempt to assign a function to each of the putative viral proteins, the predicted amino acid sequences were compared with a protein sequence database. The carboxy-terminal moiety of the E1 protein was found to possess significant homology with the large T antigens of polyomaviruses, and a number of other enzymes with ATPase and nucleic acid binding properties (Clertant & Seif, 1984). Since the large T protein is required for viral DNA replication, this data fits the hypothesis that the E1 gene product is involved in

maintaining the BPV 1 genome as an episome in transformed cells (Nakabayashi et al., 1983). A distant relationship between the carboxy-terminus of the E2 protein and a sequence in the proteins encoded by viral and cellular mos oncogenes has been observed (Giri et al., 1985). Similar studies on other papillomavirus types have been undertaken; for example, the putative E4 protein of HPV 8 has some homology with the EBNA-2 protein of Epstein-Barr virus (Pfister et al., 1986), and the E6 protein of CRPV resembles the beta-chain of ATP synthase (Giri et al., 1985).

The main body of the experimental studies to elucidate possible transforming functions of the early ORFs have involved disrupting the ORF coding sequence in a predetermined position, by one of three methods (Howley et al., 1986): i) by deletion of a specific viral sequence; ii) by insertion of a linker at a restriction site (in order to throw translation of mRNA 'out of frame'); iii) by oligonucleotide-directed mutagenesis. The expression of the modified sequence is then placed under the regulation of a control region like the Harvey viral long terminal repeat (LTR). This is preferred to using BPV derived control elements, because transforming activity is less likely to be affected by mutation in viral genes whose products are not actively involved in transformation, but which positively regulate the expression of the transforming gene. This is followed by determination of the ability of the modified sequence to induce non-productive focal transformation in, for example, NIH 3T3 mouse fibroblast cells. The infected cells are considered fully transformed if they are capable of anchorage independent growth, and form tumours in nude mice (Nakabayashi et al., 1983). Studies on the effect of ORF disruption have revealed the function of several BPV type 1 ORFs: E1 is involved in maintenance of the viral episome (Sarver et al., 1984); E2 is

associated with trans-activation of transcription (Spalholz et al., 1985), and also encodes a protein which is involved either directly or indirectly in transformation, and maintenance of viral DNA as an episome in transformed cells (DiMaio, 1986); E5 (Schiller et al., 1986; Yang et al., 1985 a) and E6 (Yang et al., 1985 b) constitute the transforming region of the genome (Schiller et al., 1984); E7 is involved in maintenance of high extrachromosomal copy number (Lusky & Botchan, 1985). Further evidence implicating the product of the E5 ORF in cell transformation has come from a fascinating study in which microinjection of a synthetic BPV 1 E5 protein induces cellular DNA synthesis in growth arrested cells (Green & Loewenstein, 1987). It has been postulated that this induction is the underlying basis for cellular transformation. By producing a series of deletion mutants, it has been demonstrated that the protein can be broken down into two functionally distinguishable domains: the first, comprising 13 amino acids from the C-terminal, is alone sufficient to induce cellular DNA synthesis. The second domain comprises the hydrophobic middle region of the protein, which by itself fails to induce DNA synthesis, but increases the activity of the first domain 1,000-fold. Investigations such as this rely directly on primary sequence data, so there is no reason why similar studies should not be performed on other papillomavirus types.

Although the majority of data concerning ORF function has been obtained for BPV type 1, studies are currently in progress to assign functions to the proteins potentially encoded by PV types infecting humans, particularly those which are thought to play a role in the aetiology of genital cancer. For example, transfection of the cloned intact HPV 16 genome into mouse NIH 3T3 cells leads to their morphological transformation at low frequency (Yasumoto et al., 1986).

It has also been reported that a genomic DNA sample from cervical cancer tissue containing HPV 16 was able to induce malignant transformation in NIH 3T3 cells (Tsunokawa et al., 1986). Similarly, a cloned intact HPV 18 genome has been shown to cause transformation of NIH 3T3 and rat-1 cell lines; the transforming region has subsequently been localized to the E6/E7 ORFs (Bedell et al., 1987)

Over the past five years, a considerable amount of effort has been put into studies designed to elucidate the mechanism by which papillomaviruses induce cellular transformation; the clinical aspects of this work, together with the evidence implicating specific HPV types in the aetiology of cervical cancer, are worth considering in more detail.

1.4.2 Clinical Aspects Of PV Infection.

1.4.2.1 Viruses And Cancer.

Cancer is a multistage process, and it is believed that several discrete events are required to transform a normal cell into a malignant one; the clonal proliferation and expansion of the transformed cell results in carcinoma. Different forms of human cancer show marked geographic variations in incidence; this predominantly reflects social rather than genetic differences in the populations at risk. This has lead to the conclusion that variable environmental factors play an important role in the aetiology of cancer. These environmental risk factors comprise three categories: i) physical agents (for example, X-rays, or u.v. light); ii) chemical agents (either directly carcinogenic, or metabolized to produce a carcinogen); iii) infectious agents. Of the infectious agents, viruses have received most attention as risk factors associated with neoplasia in vertebrates. Several human cancers have been linked to viral infection:

for example, Burkitt's lymphoma and nasopharyngeal cancer are frequently concurrent with the presence of Epstein-Barr virus DNA, and seroepidemiological studies support the hypothesis that the virus has a causal role (Griffin et al., 1985); hepatitis B virus infection is inextricably linked with primary liver cell carcinomas (Zuckerman, 1985); adult T-cell leukaemias, endemic in southern Japan and in the Caribbean Islands are clearly related to a retrovirus, human T-cell leukaemia virus type 1 (HTLV-1) (Ratner et al., 1985). Existing data have revealed three important characteristics which all cancers associated with viral infection exhibit: firstly, only a small proportion of individuals carrying the virus eventually develop a malignant tumour. Secondly, the latency period between initial infection and development of cancer indicates that the process requires years of continuous host cell/virus interaction, and finally, the resultant tumours originate from a single cell (that is, they are monoclonal) (zur Hausen, 1986).

1.4.2.2 A Viral Aetiology For Cervical Cancer?

Invasive squamous cell carcinoma of the uterine cervix is one of the most commonly found cancers of the female genital tract. It is most frequent in women between the ages of 50 and 60, although it may appear at any age after puberty. A recent observation that the incidence of cervical cancer is increasing among younger women has lent a new impetus to research aimed at increasing our understanding of this disease (Crawford, 1984). Cervical cancer is, potentially, one of the most easily prevented cancers because, unlike many other forms of the disease, there is an easily detectable and normally prolonged pre-malignant stage. Nevertheless, approximately 2,000 women die of cervical cancer each year in England and Wales, attesting to the poor record of Britain's cervical screening programme (British Medical

Association Report, 1986). There are two principal forms of cervical cancer: adenocarcinoma and squamous cell carcinoma (SCC). The latter constitutes 95% of all cervical cancers, and is characterized by a 'precancerous' phase known as cervical intraepithelial neoplasia (CIN). During this stage, morphological changes are restricted to the epithelium, and the condition is graded from CIN1 (mild dysplasia), through CIN2 (moderate dysplasia) to CIN3 (severe dysplasia, sometimes referred to as carcinoma-in-situ). Beyond CIN3, abnormal cells invade the underlying tissue, and cancer develops.

The involvement of an infectious agent in the development of human cancer of the uterine cervix has been implicated for many years. Studies undertaken in 1842 showed that the incidence of cervical cancer in prostitutes was vastly higher than that observed in nuns, immediately implicating a sexually transmitted agent in the aetiology of the disease (Rigoni-Stern, 1842). Since, there have been many studies on the behavioural characteristics of women with cervical cancer, and both sexual and non-sexual factors have been shown to be significant (Singer & French, 1984). For example, early age of first intercourse, multiple sexual partners, unstable marital status, early age of marriage and previous genital herpetic infection have been implicated as sexually-related factors which increase the risk of a woman contracting cervical neoplasia; smoking, use of oral contraceptive steroids, race and ethnicity are other contributing factors (Singer & French, 1984). Agents transmitted by intercourse which have been implicated in the neoplastic process include smegma, spermatozoa, Herpes simplex virus type 2 (HSV 2) and specific HPV types. However, there is no doubt that the papillomaviruses are currently considered to be the most likely agents to play a role in the development of cervical carcinoma. Although there are still reports of

an association between HSV 2 infection and cervical neoplasia (Kitchener, 1988), many workers favour the hypothesis implicating the HPVs (zur Hausen, 1987).

In order to be unequivocally implicated as the cause of a given disease, an infectious agent must satisfy four criteria: firstly, it should be possible to isolate the organism from every case of the disease, and once isolated, the organism should grow in an artificial medium. Inoculation into a suitable host should generate a disease state with the predicted pathology, and it should be possible to re-isolate the infectious agent from the lesion produced on inoculation. Since it is not possible to grow papillomaviruses in artificial media, and it is not ethical to induce deliberate cervical infection in humans, the association between specific HPV types and cervical cancer cannot be proven on the basis of these postulates (Crawford, 1986). However, types of evidence which would indicate that a virus is tumorigenic in humans include an association between the virus and the tumour in the form of virus particles, viral DNA or RNA, or the presence of antiviral antibodies. This, together with the ability of the virus to induce tumours in animals, and to transform cells in vitro is good circumstantial evidence that the virus is tumorigenic. The data which implicates specific HPV types in the aetiology of cervical cancer is discussed in detail below.

1.4.2.3 HPVs And Cervical Cancer.

There are several reasons why HPVs have been postulated as candidates playing an oncogenic role in the cervix (Gissmann & Schwarz, 1986). In 1976, it was recognized that the morphological features previously interpreted on Papanicolau smears and biopsies as constituting dysplasia were manifestations of a papillomavirus infection of the cervix; it had already been noticed that such lesions

may eventually progress to invasive cancer (Meisels & Fortin, 1976). Malignant conversion of virally induced human papillomas, including genital warts, laryngeal papillomas and lesions associated with epidermodysplasia verruciformis, have been frequently reported (zur Hausen, 1987). These observations spurred the molecular analysis of genital wart lesions and cervical carcinomas for associated HPV types. HPV 6 DNA was cloned from a condyloma acuminatum (de Villiers et al., 1981), and using an HPV 6 derived probe in conjunction with one derived from the genome of the closely related HPV type 11, an association between these virus types and genital warts (or mild dysplasia) was demonstrated. Using non-stringent hybridization techniques, zur Hausen and his colleagues were able to clone HPV 16 and HPV 18 DNA directly from cervical carcinomas, and other HPV types associated with genital lesions have since been cloned using the same technique (Gissmann & Schwarz, 1986).

Recent epidemiological and molecular studies (reviewed in Wickenden et al., 1987 c) have demonstrated that a distinct sub-set of HPV types can be identified in more than 90% of cervical tumours. HPV 16 is found most frequently, having been detected in approximately 50% of cervical tumours harbouring HPV DNA. HPV types 6, 11 (Gissmann et al., 1983; Rando et al., 1986), 18 (Boshart et al., 1984), 31 (Lorincz et al., 1986), 33 (Beaudenon et al., 1986), 35 (McCance, 1986), and 39 (Beaudenon et al., 1987), together with as yet uncharacterized types, are present in the remainder. It is important to point out that HPV types 6 and 11 are rarely associated with genital cancer, but are frequent in condylomata acuminata (Gissmann et al., 1983). In addition, HPV 16 has been found associated with verrucous carcinoma of the larynx (Brandsma et al., 1986), squamous cell carcinoma of the tongue (Lookingbill et al., 1987), and carcinoma of

the lung (Stremlau et al., 1985). In general, HPV DNA is found integrated into the cellular genome in cervical carcinoma tissue, whereas it is extrachromosomal in benign or pre-malignant lesions (Gissmann & Schwarz, 1986). Most investigators have found that viral DNA is actively transcribed in tumour cells, and that the expressed region corresponds to the ORFs which have been associated with transformation; thus, it is possible that viral gene products may be involved in the establishment of the malignant character of tumour cells (Gissmann & Schwarz, 1986).

Another observation which indicates a connection between HPV infection and genital cancer is that virally-derived sequences have been found to persist in cell lines established from cervical carcinomas. For example, HPV 18 DNA is present in an integrated state within the chromosomal DNA of HeLa, 756 and C4-1 cell lines (Schwarz et al., 1985), and HPV 16 DNA is present in SiHa and CaSki cell lines, as well as in four others more recently characterized (Spence et al., 1988). In all cases, the integrated viral DNA has been shown to be transcriptionally active, and detailed studies to clarify the structure and expression of the integrated sequences are presently in progress (Lazo, 1987). Investigation has shown that a common opening point of the viral genome prior to integration lies within the early region comprising the E1/E2 ORFs; as a consequence, only the E6 and E7 ORFs, together with part of E1 and adjacent cellular sequences, can be transcribed from the early viral promoter. Viral integration may play an important role in carcinogenesis, because viral sequences could become regulated by the host, or the normal regulation of host genes may be altered by viral regulatory elements.

The proposed carcinogenic potential of specific HPV types is supported by evidence of the role of the papillomavirus in the

development of cancers in mammals. In the presence of a co-factor (methylcolanthrene, for example), the Shope papillomavirus induces the malignant progression of papillomas to skin carcinomas in about 25% of infected cottontail rabbits (the natural host), and in around 70% of infected domestic rabbits (Rous & Beard, 1935). Likewise, in the presence of BPV type 4 and a co-factor contained in bracken fern, bovine papillomas of the oesophagus and intestine progress to carcinoma (Jarrett et al., 1978; see section 1.4). Recent studies of the rare skin lesion epidermodysplasia verruciformis (EV), a condition in which papillomas undergo malignant conversion almost exclusively in sun-exposed sites, have clarified the role of specific HPV types in the progression of the disease. EV is a multifactorial condition involving genetic, immunological and extrinsic factors, in addition to HPV infection. At least 18 recognized HPV types have been characterized in benign EV lesions, although only HPV types 5, 8 and 17 are associated with progression to malignancy (Orth, 1986). It is interesting to note that, even in malignant lesions, the HPV DNA remains extrachromosomal, and does not become integrated into cellular DNA. The average delay between onset of EV and development of cancer is approximately 25 years; this points to a multistage process, involving additional co-carcinogenic factors. The preferential location of cancers in light-exposed sites suggests that ultraviolet radiation plays a vital role in the malignant conversion of EV lesions, and the disease provides an excellent opportunity for studying the interaction between potentially oncogenic HPV types and genetic, immunological and extrinsic factors in the production of human cancers (Orth, 1986).

It is important to remember that in the three examples given above, a co-factor is required in addition to the virus before full malignant conversion is achieved.

1.4.2.4 Mechanisms Of Virus Induced Neoplasia.

Although there appears to be no precise chromosomal site for virus integration, there is evidence that a specific domain is involved. Results from a study of the chromosomal location of cellular sequences flanking integrated papillomavirus DNA in cell lines, and in primary cervical carcinomas suggest that, in at least some genital tumours, cis-activation of cellular oncogenes by the virus may be involved in malignant transformation of cervical cells (Durst et al., 1987). Another study has shown that in approximately 35% of cervical tumours, the c-myc proto-oncogene is over-expressed, and that patients whose tumours show c-myc over-expression have an eight-fold greater incidence of early relapse than other patients (Riou et al., 1987). It has been suggested that the c-myc proto-oncogene is associated with cellular proliferation and that its inappropriate expression may be involved in carcinogenesis and tumour progression.

Recently, it has been proposed that the development of cervical cancer is associated with failing host cell control of persisting viral genes (zur Hausen, 1987); this breakdown of cellular control could be due to a co-factor which modifies a cellular gene (for example, a carcinogen associated with smoking), rendering it non-functional. This could explain long latency periods between primary infections of cancer-linked viruses and tumour appearance. It also provides an explanation for the fact that only a relatively small number of infected individuals develop a carcinoma.

The role of the papillomaviruses in the progression of cervical cancer is still uncertain; it is possible that they act with co-factors in promoting invasive cancer, or they may be passive 'passengers' in the cervix, although the latter seems increasingly unlikely in view of the consistency with which HPV DNA can be demonstrated in invasive

lesions. Investigation of the transforming properties of the virus are in progress, as are extensive prospective studies designed to clarify the part played by papillomaviruses in the progression of a mild cervical lesion to malignancy. Other areas requiring more study include mode of virus spread, involvement of the immune system, and the molecular mechanism of cellular transformation, including the role of co-factors. The implications of integration of viral DNA into the chromosome must be determined; it could be that integrated viral genes which are consistently transcribed in cervical carcinoma-derived cell lines, and in some genital tumours, code for trans-acting factors which activate cellular genes or pathways that lead directly or indirectly to the malignant phenotype. Alternatively (or additionally), viral sequences could act as insertional cis-acting promoter/enhancer mutagens that activate nearby cellular proto-oncogenes.

Clearly, the increasing body of evidence implicating papillomaviruses in the aetiology of cervical cancer suggests that an efficient means of detecting cervical HPV infection could be an invaluable tool for identifying the pre-cancerous stage of the disease. The techniques used for detecting cervical HPV infection are critically discussed in the following section.

1.5 Methods Of HPV Detection.

Until relatively recently, genital HPV infection was a neglected sexually transmitted disease in terms of clinical concern, largely because of the widespread belief that infection resulted in only mild epithelial changes. The recent evidence to suggest that this is an incorrect assumption has led medical investigators to develop a variety of methods to allow the detection of macroscopically invisible papillomavirus infections; since it is not yet possible to reliably

propagate the virus in tissue culture, alternative methods for accomplishing this have been devised.

1.5.1 Immunological Detection.

The papillomaviruses share genus-specific antigens, which can be detected by antisera directed against detergent disrupted viral particles (Jenson et al., 1980). Immunoperoxidase detection systems have been applied successfully to the identification of HPV infection in cervical and other genital tissues (Kurman et al., 1981). One of the problems associated with this technique is that antibodies produced from a disrupted virus are not capable of distinguishing between different papillomavirus types; for instance, an antibody prepared from BPV type 1 particles is broadly reactive against the common antigens of all HPV types (Lancaster & Jenson, 1987). Since there is strong evidence to suggest that viral type should be an important consideration in the prognosis of genital HPV infection, it is no longer sufficient merely to demonstrate the presence or absence of the virus. A second problem associated with immunoassays designed to detect virus structural proteins is that the late ORFs are only transcriptionally active in well differentiated cells of the epithelium, and structural proteins are commonly not produced at all in higher grades of neoplasia. Despite successful attempts to produce early ORF fusion proteins in bacterial expression systems, and the subsequent production of virus type-specific monoclonal antibodies directed against these fusion proteins (Seedorf et al., 1985 b; Tomita et al., 1987; Thompson & Roman. 1987), nucleic acid hybridization remains a far more sensitive and reliable tool for rapidly and simply distinguishing between HPV types (section 1.5.4). Nevertheless, it is important to remember that antibodies directed against a single protein of a specific HPV type

will be invaluable tools in efforts to determine the role of the virus in the mechanism of cell transformation (Androphy et al., 1987; Banks et al., 1987). A complete analysis of the papillomavirus life cycle will require antibodies for the identification of the various viral proteins in keratinocytes at different stages of differentiation (Thompson & Roman, 1987).

1.5.2 Light And Electron Microscopy.

1.5.2.1 Histological And Cytological Diagnosis.

Infection may result in changes in cellular morphology which are characteristic of a particular virus type; such changes are commonly detected by light microscopy, in Papanicolau-stained smears. Histology and cytology have been used as the gold standards for diagnosing HPV infection in many studies; the methods depend on the detection of koilocytes (squamous cells with a typical halo appearance around an enlarged, irregular, hyperchromatic nucleus), dyskeratosis (intracellular keratinisation), basal-cell hyperplasia and mild nuclear atypia (Meisels & Fortin, 1976). Exophytic HPV-induced warts are characterised by marked proliferation in the cells of the epithelium, which consequently becomes extensively folded, producing upward papillomatous lesions and downward extensions of the dermal papillae. In circumstances where all of these abnormalities occur in a clinical specimen, diagnosis is generally unequivocal. However, there are many lesions in which these changes are less pronounced, or are associated with other significant nuclear atypia, so diagnosis is complicated considerably. Unusual mitoses, abnormalities in the basal cell layer, grading of nuclear atypia and determination of ploidy are a few examples of criteria helpful in establishing whether a lesion is HPV-induced cervical intraepithelial neoplasia, or just a benign HPV

infection. The role of histological examination in the diagnosis of HPV infection is becoming clearer with the increased use of new immunocytological staining techniques; nevertheless, the identification of HPV nucleic acid in dysplastic lesions which do not exhibit the typical morphology associated with HPV infection suggests that histology is of limited use as a diagnostic method.

1.5.2.2 Diagnosis By Electron Microscopy.

A prerequisite to the examination of clinical specimens by electron microscopy is that the sample must be fixed, and embedded in an epoxy resin matrix. Sections are then cut with an ultramicrotome, and mounted in an evacuated chamber, prior to examination with the microscope. This technique has been used to identify characteristic icosahedral HPV particles in biopsy specimens, and occasionally in smears with changes suggestive of HPV infection (Reid et al., 1980). However, the success of such studies has proved to be limited (Morse et al., 1988), and the inconvenience and expense of electron microscopy suggest that the technique will play a very minor role in the diagnosis of HPV infection, particularly as viral particles are produced only in mature, differentiated epithelial cells.

1.5.3 Colposcopic Diagnosis.

Colposcopic identification of cervical HPV infection involves examination of the magnified surface epithelium of the cervix and surrounding tissues, after the application of dilute acetic acid. Areas of the cervix which have undergone epithelial changes characteristic of CIN appear white; other factors indicative of this condition are variations in the surface contour and mild vascular alterations. Vaginal manifestations of HPV infection are less well described, but include vascular abnormality and white epithelium after acid staining

(Coppleson, 1987). The main advantage of colposcopy is that, unlike the techniques described so far, it allows a large area of epithelium to be examined, potentially increasing the chance of detecting abnormality. However, in comparison with alternative techniques, the sensitivity and specificity of colposcopy for detecting HPV infection is uncertain, and confusing terminolgy has resulted in significant variation in results from group to group. Since the point in its natural history at which HPV infection can first be identified colposcopically is unknown, it is unlikely that the technique will exclusively be used for the identification of infection (McDougall et al., 1986).

1.5.4 Nucleic Acid Hybridization.

There are several advantages inherent in the use of nucleic acid hybridization for the detection of clinical HPV infection: firstly, and most importantly, it is possible to identify the virus in circumstances where there is no transcriptional or translational activity. Secondly, nucleic acid hybridization is the only reliable method for distinguishing between different types of the virus (Wickenden et al., 1987 a). The sensitivity and specificity of the technique, together with the speed with which results may be obtained makes it particularly attractive. A variety of sample types may be examined, including biopsies (Gissmann et al., 1983), cervical scrapes (Wickenden et al., 1985) and even formalin-fixed, paraffin-embedded specimens (Mark et al., 1987). Depending on the information which is required, the appropriate hybridization technique may be applied; for example, Southern blotting can determine whether or not the infecting virus is present as extrachromosomal elements, or integrated within the host genome (Choo et al., 1987; Durst et al., 1987). The high sensitivity associated with polymerase chain reaction-mediated target

amplification allows detection of the virus in specimens where there are too few invading genomes to be identified by any other method (Shibata et al., 1988; Young et al., 1989). In situ hybridization preserves cellular detail and tissue architecture, and thus provides information regarding the intracellular localization of viral DNA (Crum et al., 1986; Wells et al., 1987). Sandwich hybridization allows relatively crude samples to be analyzed without the time-consuming immobilization of the extracted nucleic acid, and presents results in an easily interpreted numerical form (Parkkinen et al., 1986; Malcolm et al., 1987; Parkkinen, 1988; Parkkinen et al., 1988). A further advantage of nucleic acid hybridization is that, under conditions of reduced stringency, as yet uncharacterized virus types can be identified using a probe derived from a known HPV type (Ostrow et al., 1987).

Although these techniques are all of use in the research laboratory, they have not yet been applied to large scale screening for the identification of cervical HPV infection, probably because none of them fully satisfy all of the requirements of a routine diagnostic assay (see section 1.1). A report published by the British Medical Association in 1986 concluded that 'at present it is neither practicable nor economic to test all smears and cervical biopsies for infection with these [human papilloma] viruses', even though it is accepted that 'there is growing evidence linking infection by a papillomavirus with cervical cancer' (British Medical Association Report, 1986). The development of a nucleic acid-based test amenable to routine diagnostic use would undoubtedly improve the efficacy of the cervical screening service, and this has been a major aim throughout the course of this study.

1.6 Immobilization Of Nucleic Acids.

As discussed in section 1.1, the majority of the blotting techniques which have been applied to the identification of a specific nucleic acid sequence involve the use of a nitrocellulose or nylon membrane as the solid support. The use of such supports results in procedural complexities, which in turn prevent blotting assays from satisfying all of the requirements of a routine diagnostic assay. How could this problem be circumvented, in order to produce a procedure which would be more suited for routine diagnostic use? If a bead, rather than a membrane, were to be used as a support in a sandwich hybridization assay, many of the complex manipulations associated with membranes (for instance, sample immobilization and post-hybridization washing) could be eliminated, or considerably simplified. In order to develop such an assay, the following questions must be answered: (i) What is the most suitable type of bead to use as the support; (ii) What is the most suitable mechanism by which nucleic acid can be immobilized on to the support; (iii) What are the hybridization characteristics of the immobilized DNA. These questions are considered in detail in the following sections.

1.6.1 Choice Of Solid Support For Immobilization.

A considerable amount of research has been directed at identifying the most suitable beaded solid supports (or resins) for use in affinity chromatography and gel filtration. In fact, nucleic acids were first covalently immobilized onto supports like powdered cellulose in order to be used for the purification of nucleic acid binding proteins, rather than to participate in nucleic acid hybridization assays. Nevertheless, a great deal of useful information regarding the ideal support and coupling chemistry can be derived from

these attempts at affinity chromatography. In order to be suitable, a resin particle should consist of an open, porous network, and be uniform in size and porosity. Chemical and biological inertness are essential, although stable derivatives possessing reactive groups suitable for ligand coupling should be easy to produce. Other useful properties would be physical stability in extremes of pressure, temperature and pH, and inertness in organic solvents.

The majority of the resins which have been found to be suitable for affinity chromatography and gel filtration are porous polysaccharides. Amongst the most commonly used are Sephadex, Sepharose, Sepharose CL and Sephacryl, all of which are manufactured by Pharmacia. In order to ascertain whether any of these supports would be suitable for nucleic acid-based hybridization assays, it is useful to consider the physical and chemical properties of each resin in more detail.

Sephadex is a bead-formed gel, prepared by cross-linking dextran with epichlorohydrin. The mechanical strength of the particles (diameter 40-120 um) depends on the degree of cross-linking; the highly cross-linked Sephadexes G-10 and G-15 are particularly rigid. Sephadex is stable in alkaline and weakly acidic conditions, although hydrolysis of the glycosidic linkages may occur in strong acids.

Sepharose is a bead-formed gel, prepared from agarose. The gel structure is stabilized by hydrogen bonding rather than covalent cross-linking, so it is only stable in buffers in the pH range 4-9, in the absence of oxidizing agents. The mechanical strength of the particles depends on the agarose concentration; Sepaharose 6B (approximately 6% agarose) is considerably stronger than Sepharose 2B (2% agarose).

Sepharose CL is prepared by the reaction of 2,3-dibromopropanol with Sepharose, under alkaline conditions; this cross-linking results

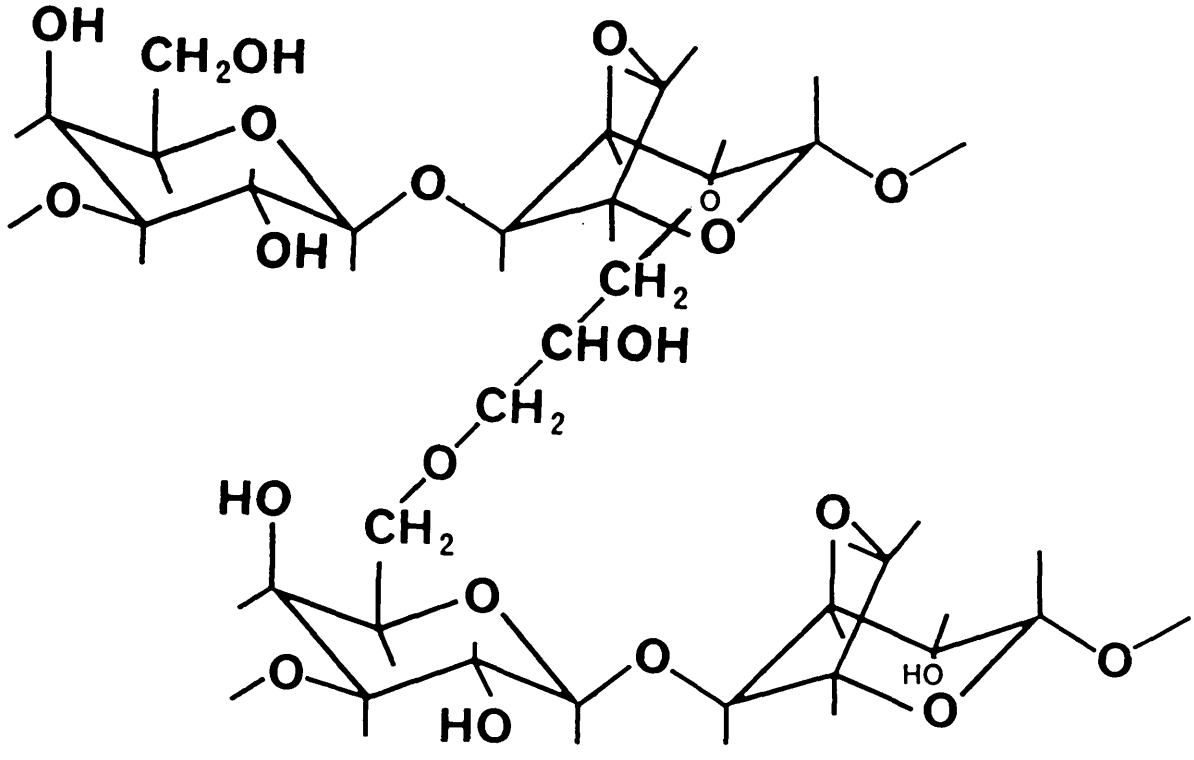


FIGURE 7

THE CHEMICAL STRUCTURE OF THE CROSS-LINKS IN SEPHAROSE CL4B. (Reproduced with permission from "Gel Filtration- Theory And Practice", Pharmacia Laboratory Separation Division).

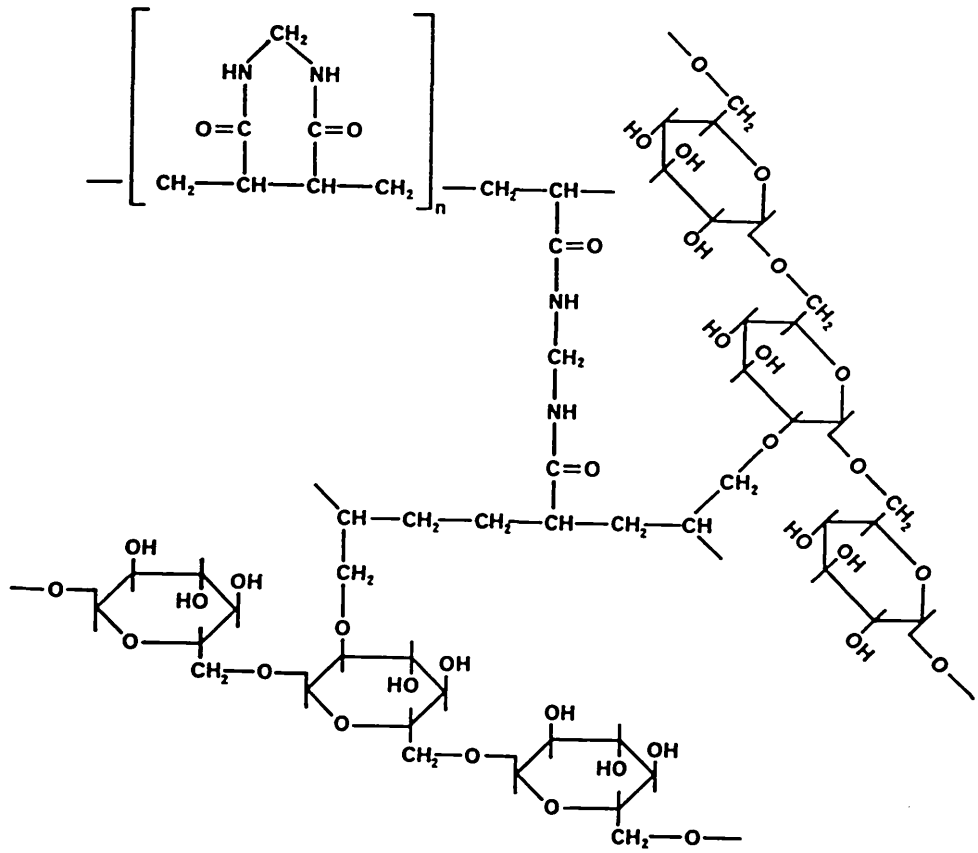


FIGURE 8

THE HYPOTHETICAL PARTIAL STRUCTURE OF SEPHACRYL.
(Reproduced with permission from "Gel Filtration- Theory And Practice", Pharmacia Laboratory Separation Division).

in enhanced stability, so unlike Sepharose, Sepharose CL is stable in buffers in the pH range 3-14. The chemical structure of Sepharose CL is shown in figure 7. Under oxidizing conditions, limited hydrolysis of the polysaccharide chains may occur. Sepharose CL contains an extremely low content of charged groups.

Sephacryl is prepared by covalently cross-linking allyl dextran with N,N'-methylene bisacrylamide, thus producing an extremely rigid gel with a carefully controlled range of pore sizes. The partial structure of Sephacryl is shown in figure 8. The wet bead diameter is 40-105 um, and the average bead diameter is 70 um. The Sephacryls are stable in buffers in the pH range 2-11, although at lower pH, limited hydrolysis of the dextran chains may occur. Sephacryl S-1000 has an extremely large pore size; particles greater than 300-400 nm in diameter are excluded.

Sephadex, Sepharose CL and Sephacryl are all stable in organic solvents (for example, acetone, DMSO and formamide), and may be autoclaved at 120^{o}C. The physical and chemical stability of these supports suggests that they may be ideal for the development of a novel nucleic acid hybridization assay. However, Sepharose melts on heating above 40^{o}C, and is clearly unsuitable for this purpose.

Examples of other supports which have been used in biological purification processes include controlled pore glass (CPG), particulate alumina, powdered cellulose (non-porous), nylon, paper, sodium alginate, magnetic particles (Dynal Dynospheres, for example) and insolubilized proteins; these may also be worth considering as supports for nucleic acid hybridization.

1.6.2 Methods Of Nucleic Acid Immobilization.

In order to be suitable for the covalent immobilization of a

nucleic acid fragment, a chemical procedure should result in irreversible binding, and allow the coupled molecule to retain biological activity; in the case of DNA, this means that the immobilized strand should be available for hybridization with a complementary sequence. This is clearly an absolute requirement if the DNA-resin is to be used in hybridization experiments.

There are three methods by which nucleic acids can be physically (rather than chemically) immobilized. The first of these involves adsorption of DNA (either single- or double-stranded) onto powdered cellulose. After adding the nucleic acid to a paste of cellulose, the mixture is air dried, prior to lyophilization (Alberts & Herrick, 1971). However, DNA-cellulose prepared by this method is only moderately stable to desorbing conditions such as extremes in temperature, or low ionic strength, so it is not suitable for use in hybridization-based assays. A second method of immobilization, the precise mechanism of which is unknown, involves u.v. irradiation of cellulose in the presence of single-stranded DNA (Litman, 1968). The DNA-cellulose produced has been reported to be unaffected by strong desorbing conditions and elevated temperature, although the hybridization properties of the immobilized DNA have not been investigated. The final non-chemical method of nucleic acid immobilization involves entrapment of high molecular weight, single-stranded DNA within cellulose acetate or agarose gels (Bolton & McCarthy, 1962; Purrello & Balazs, 1983), or within acrylamide beads (Cavalieri & Carroll, 1970). The pore size of the beads is such that the large DNA molecule cannot escape, although small nucleic acid binding proteins may readily move in and out of the pores. Studies designed to determine the hybridization efficiency of entrapped DNA give variable results, presumably because of the inability to reliably

regulate the size of the pores.

There are several chemical methods which have been reported for the covalent immobilization of polynucleotides; these can be separated into two groups, depending on whether the nucleic acid molecule is immobilized at multiple points, or just at one point (the 5' or 3' end).

The first of the multiple-attachment methods involves condensation of single-stranded DNA, RNA or polyribonucleotides with a cyanogen bromide (CNBr) activated polysaccharide support (like Sepharose 4B, for example). Activation of the resin results in the formation of a reactive imidocarbonate, which in turn couples with the nucleotide base moiety at mildly alkaline pH. Consequently, each nucleic acid strand is cross-linked with the support in many positions, rather that specifically at the 5' or 3' end (Poonian et al., 1971). A more recent investigation was designed to determine the hybridization properties of DNA immobilized by the CNBr method (Bunemann & Westhoff, 1983); the results suggest that coupling efficiencies of around 50%, 75% and 80% are achieved with activated Sephadex, Sepharose CL and Sephacryl respectively. The availability of immobilized DNA for hybridization was estimated at 60% and around 30% for material immobilized on Sephacryl S-500 and Sephacryl S-1000 respectively. The main disadvantages of using this method are that CNBr is extremely toxic, and CNBr activated material has been reported to be sensitive to impurities in the DNA to be immobilized (Arndt-Jovin et al., 1975).

In order to avoid the use of highly toxic reagents, a number of alternative immobilization chemistries have been developed. For example, cyanuric chloride has been used to covalently couple DNA to cellulose beads, via the amino groups present on the bases (Biagioni et al., 1978). Activated dichlorotriazinyl cellulose is prepared by

soaking cellulose powder in 3 M sodium hydroxide for 15 minutes; the powder is then added to a 5% solution of cyanuric chloride, in a mixture of dioxane and xylene (1:1 w/w). After washing in suitable solvents, DNA coupling is achieved simply by mixing the activated cellulose with an unbuffered DNA solution; the most likely reaction mechanism involves attack of dichlorotriazinyl cellulose by the nucleophilic amino groups of adenine, guanine and cytosine. An immobilization efficiency of 80% has been reported, and 85% of this DNA is double-stranded. DNA-cellulose prepared by this method is extremely stable, particularly in extremes of pH (Biagioni et al., 1978).

Nucleic acids can also be bound to polysaccharide supports activated by reaction with a bifunctional oxirane, like 1,4-butanediol diglycidyl ether (Potuzak & Dean, 1978). Such bisoxiranes have been used previously for the introduction of reactive oxirane groups into agarose, for the subsequent coupling of proteins, peptides, and aliphatic or aromatic amines (Sundberg & Porath, 1974). Studies using nucleotide homopolymers indicate that the order of binding efficiency is poly(dT) > poly(dC) = poly(dA) > poly(dG). Binding efficiencies in excess of 90% have been observed with single-stranded DNA (although just 16% of double-stranded DNA is coupled), and approximately 90% of the immobilized molecules are available for hybridization with complementary RNA (Moss et al., 1981). The size of the nucleic acid fragments, within the range 600 bp up to 48 kb, has no significant effect on coupling efficiency, which is optimum at around 50^{o}C, and pH 11-11.5 (Nagasawa et al., 1985 a). A comparison of coupling efficiencies attained with the most commonly encountered procedures, including bisoxirane-, CNBr-, carbodiimide-, and diazo-activation, indicate that oxirane activation is the most efficient method (Nagasawa et al., 1985 b). Double-stranded DNA immobilized by this procedure onto

epoxy-cellulose is accessible to restriction endonucleases (Lazo, 1984). The loss of some recognition sites is consistent with a 3% modification of nucleotides. The hypothesis that A and T residues are more likely to be involved in covalent linkage than G or C residues is supported by the fact that enzyme recognition sites with a high GC content are less affected by immobilization than those with a high AT content (Lazo, 1984).

The most commonly used method for covalently immobilizing a nucleic acid strand at multiple positions involves the use of diazotizable aromatic amines. DNA has been coupled to diazobenzyloxy-methyl (DBM) cellulose (Noyes & Stark, 1975), and also to diazophenyl-thioether (DPTE) cellulose (Seed, 1982). The first step in both of these procedures involves the covalent attachment of an aromatic amine to the solid support; the amine group is subsequently diazotized by reaction with nitrous acid, and the resulting diazonium ion reacts with single stranded nucleic acids, via the bases. For example, the manufacture of DPTE-cellulose first involves the production of the aminophenylthioether (APTE) derivative, by modification of cellulose with 1,4-butanediol diglycidyl ether (Sundberg & Porath, 1974); after reaction with the strongly nucleophilic thiophenoxide anion of the aromatic amine 2-aminothiophenol (Seed, 1982), the resulting APTE-cellulose is converted to DPTE-cellulose by treatment with nitrous acid. The chemistry of this reaction scheme is shown in figure 9. The principle use of DBM- and DPTE-celluloses is to serve as the solid support for the Northern blotting technique (Bittner *et al.*, 1980); nitrocellulose is not appropriate for this procedure, because binding between RNA and cellulose is poor (Alwine *et al.*, 1977). DBM-cellulose has also been used for the production of single-stranded hybridization probes (Ashley & MacDonald, 1984; Hansen *et al.*, 1987). Single-stranded

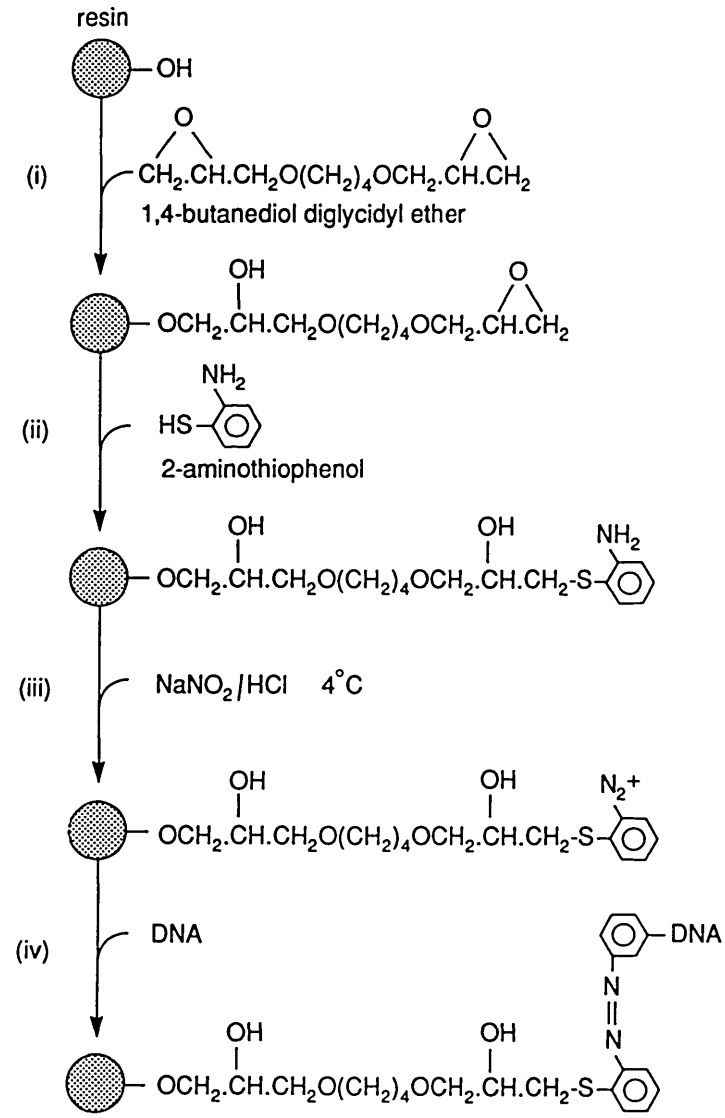


FIGURE 9

CHEMISTRY OF DNA IMMOBILIZATION TO DIAZOTIZED RESINS.

recombinant M13 phage DNA is bound to the DBM-cellulose, such that synthesis of the complementary strand is possible. This is achieved by adding reverse transcriptase (Ashley & MacDonald, 1984), or DNA polymerase (Hansen et al., 1987), in the presence of either oligo(dT), or M13 primer. After synthesis, unincorporated labelled nucleotides are washed away, and the labelled single-strand is eluted with formamide.

Several recent investigations have demonstrated that nucleic acids can be efficiently immobilized by the diazotization procedure to alternative solid supports, like Sephacryl, Sephadex and Sepharose CL (Bunemann et al., 1982; Nicholls et al., 1987). For example, Bunemann reported coupling efficiencies of approximately 70% with DBM-Sepharose CL, DBM-Sephacryl S-500, DPTE-Sepharose CL and DPTE-Sephacryl, and 25% with DBM-Sephadex and DBM-Sephacryl S-1000 (Bunemann et al., 1982). Coupling efficiencies of between 60% and 74% have been reported for DPTE-Sephacryls S-200, S-400 and S-500, although 82% of the DNA attached to DPTE-Sephadex G-50 was bound non-covalently (Langdale & Malcolm, 1985). A modification of this method, designed for the attachment of DNA to uniform, magnetic beads (DPTE-Dynospheres M450), was unsuccessful (Lund et al., 1988).

An alternative method for covalently attaching double-stranded DNA to a diazotized support involves alkylation of the DNA with 4-bis-(2-chloroethyl)amino-L-phenylalanine; the primary amino group of the phenylalanine unit subsequently reacts with the diazotized solid support, with a coupling yield of around 75% (Macdougall et al., 1980). The advantage of attaching a ligand to the DNA prior to coupling to the solid support is that the number of attachment points can be carefully controlled. Although the stability of the DNA-resin is high, hybridization data are not available, because of the double-stranded nature of the immobilized molecules.

Of the methods designed for the end-attachment of nucleic acids to solid supports, 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate (carbodiimide)-mediated attachment via the 5' phosphate group is probably the most frequently encountered. This method initially involved the condensation of synthetic polynucleotides with cellulose, under anhydrous conditions (Gilham, 1964), but more recent modifications allow synthetic or natural polynucleotides to be immobilized in aqueous solution (Gilham, 1968; Rickwood, 1972). The chemistry of the reaction (illustrated in figure 30, section 4.4.1) involves the formation of a phosphodiester bond between the 5' phosphate group on the nucleic acid, and the hydroxyl group on the support. Although it has been reported that additional linkages between the support and the bases T and G sometimes occur at a pH greater than 8.0, these adducts are easily destroyed by exposure to mild alkali (Astell & Smith, 1972). The temperature at which the reaction occurs has a profound effect on the extent of immobilization, and double-stranded DNA is immobilized with a greater efficiency than single-stranded DNA (Mykoniatis, 1985); nevertheless, synthetic oligo-nucleotides have been successfully coupled to AH-Sepharose 4B using this procedure (Voss & Malcolm, 1988 a). The advantage of immobilizing an oligonucleotide via the terminal residue, rather than at an internal position is obvious; a covalent linkage between an internal residue and the support would severely affect the ability of the oligonucleotide to hybridize with a complementary nucleic acid strand. The efficiency with which nucleic acid fragments are immobilized by the carbodiimide method decreases with increasing fragment size. For example, mononucleotides can be coupled to cellulose with an efficiency of 60%-70% (Gilham, 1968), whereas only 30%-45% of SV40 DNA (5,243 bp) is covalently attached to the same support (Shih & Martin, 1974). DNA fragments of

sizes 2,700, 879, 341 and 201 bp have been coupled using carbodiimide to Sephacryl S-500 with respective efficiencies of 25%, 43%, 41% and 39% (Langdale & Malcolm, 1985). Lambda DNA digested with the restriction enzyme HinfI (fragment sizes in the range 400-500 bp) and oligonucleotides (36 residues) can be attached to magnetic Dynospheres (carrying an amino group at the termini of polyethylene glycol linkers) with efficiencies of 65% and 60% respectively (Lund et al., 1988), although the amount of non-specifically bound material appears to be high in the case of the oligonucleotide (40%). Approximately 65% of the immobilized DNA is available for hybridization. Oligonucleotides with a 5' phosphate group have been coupled to amine coated controlled pore glass (CPG) supports, in the presence of water soluble carbodiimide, with an efficiency of around 75%; 94% of the immobilized material is attached via the 5' end (Ghosh & Musso, 1987), and around 75% is available for hybridization (Gingeras et al., 1987).

A novel method for the attachment of oligonucleotides to cellulose via the 3' hydroxyl group has been reported (Panet & Khorana, 1974). The first step in the procedure involves condensation of poly(dT) (approximately 80 residues in length, and possessing 3' phosphate and 5' hydroxyl groups) with cellulose, using water soluble carbodiimide; the 5' end of the immobilized polynucleotide is then phosphorylated with polynucleotide kinase. This ensures that immobilization occurs only via the 3' end. The oligonucleotide of interest is then tailed at the 3' end with dT, using the enzyme deoxypolynucleotidyl-transferase; the terminal residue of this tail is covalently coupled to the 5' end of the immobilized poly(dT) chain, using polynucleotide ligase, and a poly(dA) template. The efficiency of coupling is approximately 50%, but the time-consuming nature of this method has hindered its widespread use.

A number of other methods have been developed for immobilizing nucleic acids using enzymes. For example, a technique for attaching single-stranded DNA via the 5' end to oligo(dT) cellulose, using T4 DNA ligase was recently reported (Goldkorn & Prockop, 1986). A 3' poly(dA) tail is added to a double-stranded DNA fragment, using terminal transferase, and this tail is subsequently hybridized with the oligo(dT) chains on the cellulose. A covalent link between the non-tailed strand of the DNA fragment and the oligo(dT) is constructed, by extending the recessed end of the fragment with Klenow polymerase, and subsequent ligation with T4 DNA ligase. The non-coupled strand is removed by heat denaturation, centrifugation and stringent washing. An overall immobilization efficiency of 85% was achieved, and the method has since been adapted for the immobilization of synthetic oligonucleotides (Voss & Malcolm, 1988 b).

A third enzymatic method for the immobilization of single-stranded DNA involves the coupling of a synthetic oligonucleotide (a 16-mer), modified at the 5' end with an electrophilic aldehyde or carboxyl group, to nucleophilic hydrazine residues on the surface of latex microspheres (Kremsky et al., 1987). The single stranded DNA fragment of interest (either a cloned sequence recovered from M13, or a synthetic oligonucleotide) is attached to the 3' end of the immobilized 16-mer by T4 polynucleotide ligase, using an oligonucleotide 'splint' (a 20-mer, with regions complementary to the 3' end of the 16-mer, and the 5' end of the fragment of interest). Immobilization efficiencies of around 60% have been achieved using this technique.

Other methods which have been used for the immobilization of nucleic acids include labelling a cloned sequence (Syvanen et al., 1986 a) or oligonucleotide (Urdea et al., 1987 a; 1988) with biotin, followed by capture onto a streptavidin-containing solid-support (see

section 1.3.6), covalent attachment of alkylamino oligonucleotides to passively adsorbed proteins on microtiter dish wells (Urdea et al., 1988), and attachment of heat-denatured DNA to microtiter wells by u.v cross-linking (Nagata et al., 1985).

1.6.3 Hybridization Properties Of Immobilized DNA.

The majority of the data available concerning the hybridization properties of DNA covalently immobilized on beads was obtained for the carbodiimide, CNBr and diazotization coupling procedures (Bunemann, 1982; Langdale & Malcolm, 1985; Lund et al., 1988). A limited amount of information is also available concerning nucleic acids immobilized by enzymatic methods (Goldkorn & Prockop, 1986; Wolf et al., 1987). The results can be summarized as follows:

(a) Three parameters should be considered in the choice of the most suitable support: efficiency of coupling, stability and accessibility of coupled DNA and the degree of mismatch on hybridization resulting from the immobilization procedure.

(b) DNA or RNA coupled to macroporous supports (for example, Sepharose CL or Sephacryl) hybridizes more efficiently than that attached to non-porous supports (for example, powdered cellulose), regardless of the immobilization chemistry (Bunemann, 1982). No substantial difference in hybridization rate of DNA immobilized on the two types of support is apparent; in general, such heterogeneous hybridizations proceed an order of magnitude slower than an identical reaction performed in homogeneous solution (Bunemann & Westhoff, 1983).

(c) An extremely high coupling efficiency is not always advantageous; reduced coupling yields may be outweighed by excellent availability of immobilized sequences for hybridization (Bunemann & Westhoff,

1983).

(d) The CNBr coupling method is not ideal, because of the poisonous nature of CNBr, and the fact that the chemistry results in a residual positive charge on the support, potentially generating background signals due to ionic interactions with a negatively charged nucleic acid probe. In addition, variable coupling efficiencies with this method may be obtained, due to partial reassociation of DNA strands prior to immobilization (Arndt-Jovin et al., 1975).

(e) The accessibility of immobilized DNA depends on the method of immobilization, rather than on the type of support. Providing over-activation of the solid support is strictly avoided, 90%-100% of the nucleic acid immobilized by CNBr and diazotization methods should be available for hybridization (Bunemann & Westhoff, 1983).

(f) DPTE-Sephacryl S-500, DBM-Sephacryl S-500 and CNBr-activated Sephacryl S-500 have all been recommended as supports for the immobilization, and subsequent hybridization, of DNA or RNA (Bunemann, 1982; Langdale & Malcolm, 1985); background caused by non-specific adsorption of probe is especially low for this support. On average, 50% of the DNA immobilized on DBM-activated supports, and 60% of that on DPTE-activated supports, is available for hybridization; the respective figures using Sephacryl S-500 are 95% and 100%.

(g) A $1^{o}C$ decrease in hybrid melting temperature results for each 1% of residues in the immobilized strand attached to the support; coupled DNA may be totally inaccessible when just 3% of its bases are involved in coupling. Hybrids incorporating DNA immobilized via long spacers (like 1,4-butanediol diglycidyl ether) have been reported to show no appreciable decrease in stability (Bunemann,

1982).

In summary, the diazotization and carbodiimide coupling procedures appear to be the most suitable for the covalent immobilization of DNA, because they result in reasonable coupling yield, and subsequent availability of immobilized DNA for hybridization. The chemical reagents involved are not as toxic as CNBr, and no expensive (and potentially fragile) enzymes are involved. The most suitable solid supports appear to be of macroporous structure; the Sephacryls are most frequently found to be effective.

As discussed in section 1.3, most of the sandwich hybridization assays described to date satisfy all of the requirements of a routine clinical assay, except that they are not particularly suited to automation, and are not as sensitive as certain conventional techniques (Southern blotting, for example). In some circumstances, sensitivity is not the main priority when choosing a suitable assay format; it may be more important to use a method which is automated and fast, and therefore capable of handling large numbers of samples. An example of such a system is the sandwich hybridization performed on Sephacryl beads, described in section 1.3.5 (Langdale & Malcolm, 1985). The relative insensitivity of the test -the lower limit of detection is 3 x 10^6 target molecules, compared to around 3 x 10^5 in the case of Southern blotting- is outweighed by the advantages of speed and amenability to automation. In such circumstances, where the objective is to detect the presence or absence of a particular genomic defect, the overall sensitivity can be improved by increasing the amount of sample added to each assay, assuming that this does not result in a

significant increase in the background signal. Since it is possible to isolate approximately 40 ug of genomic DNA from just 1 ml of human blood (Kunkel et al., 1977), availability of sample does not tend to be the limiting factor in the case of assays where whole blood is a suitable sample source. However, in circumstances which require the detection of an infectious agent in a clinical sample of limited size (for example, a cervical scrape or biopsy), the amount of nucleic acid available for assay may be small, so it is not practicable to increase sensitivity by simply increasing the amount of sample used. In the case of human papillomavirus infection of the cervix, it has been shown that a determination of the number of viral genomes per infected cell is not always of prognostic value (Wickenden et al., 1987 b); HPV infections which lead to carcinoma have been associated with viral integration, rather than with episomal persistence (see section 1.4.2), even though viral copy numbers may be much higher in the latter case. In essence, this means that it would be more useful to identify positive samples reliably, regardless of how little HPV nucleic acid is present, than it would be to quantitate levels of viral DNA accurately. If necessary, the integrational status of the virus in infected samples could be subsequently determined by Southern blotting.

Any procedure which results in an increase in the signal to noise (S/N) ratio will result in higher sensitivity. There are two basic mechanisms by which this could be achieved: firstly, if the total amount of label attached to each target molecule after hybridization were to be increased, the signal generated would increase proportionally. This idea was used to great effect in a recently described technique in which multiple sandwich hybridization of oligonucleotides, together with a non-radioactive detection system, was used for the diagnosis of hepatitis B virus infection in human serum

(section 1.3.7) (Urdea et al., 1987 a). The second method of increasing sensitivity would be to increase the number of target sequences in a sample. The most obvious means of achieving this is to utilize the polymerase chain reaction (PCR), which was first described in 1985 (Saiki et al., 1985 a); the technique results in a specific amplification of a pre-determined double-stranded DNA sequence (Saiki et al., 1986), and is worth considering in more detail.

1.7 The Polymerase Chain Reaction (PCR).

1.7.1 Principle Of PCR.

The principle of the PCR technique is shown in figure 10. Each cycle of the 'chain' involves denaturation of the target sequence (usually by heating to around 95^{o}C for 2-4 minutes) followed by annealing of a pair of oligonucleotide primers; one of the primers is complementary to the 3' end of the target region on the sense DNA strand, and the other complementary to the 3' end of the target region on the anti-sense strand. The third, and final, stage involves DNA polymerase-mediated elongation of the hybridized oligonucleotide primers. The addition of nucleotides occurs at the 3' end of each primer, so it is vital to design the reaction scheme such that elongation occurs in the correct direction. The cycle is then repeated, starting at the denaturation stage, and the result of amplification is an exponential increase in the amount of target sequence present in a sample. Although the theoretical increase in the number of target sequences is 2^{n}, where n is the number of PCR cycles, in practice, maximum efficiency is never achieved, possibly because of reannealing of amplified sequences to each other, rather than to the primers; after 20 cycles of amplification of a 110 bp beta-globin sequence, a 220,000-fold increase in target was observed, as opposed to a theoretical maximum of around 1,050,000-fold (Saiki et al., 1985 a). This

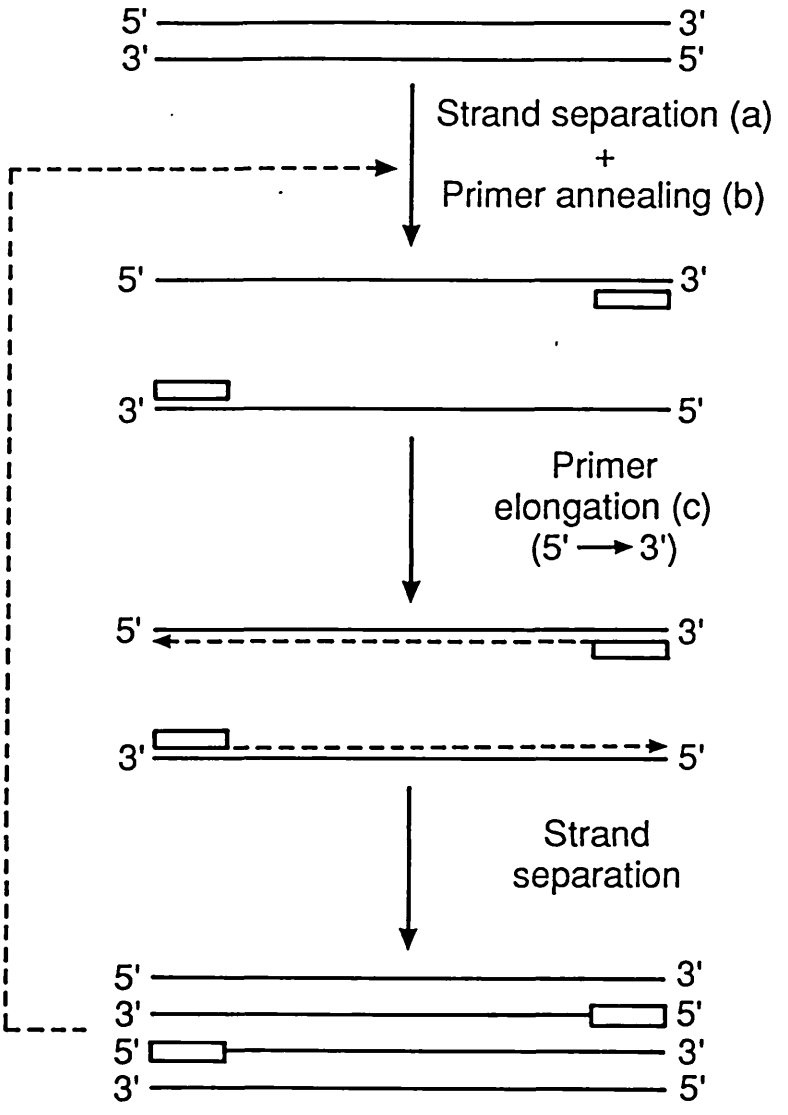


FIGURE 10

THE PRINCIPLE OF THE POLYMERASE CHAIN REACTION (PCR).

represents an efficiency of around 85% at each cycle. The most simple means of detecting the amplified sequence is by agarose or polyacrylamide gel electrophoresis, or by hybridization with a suitably labelled probe, using any of the available hybridization formats.

The PCR technique was initially developed using the Klenow fragment of DNA polymerase I for primer elongation (Mullis et al., 1986); the main disadvantage of using this enzyme is that polymerase activity is destroyed after each cycle, due to the high temperature essential for the denaturation step. This in turn means that fresh enzyme needs to be added after every cycle, increasing the expense and complexity of the procedure. An obvious means of avoiding this problem was to use a thermostable DNA polymerase, such as that isolated from the thermophilic bacterium Thermus aquaticus (Chien et al., 1976). The stable polymerase improves the PCR technique in two ways: firstly, it allows the procedure to be automated. This was initially achieved using a series of water baths (Rollo et al., 1988) and subsequently using a microchip controlled heating block, which automatically controls the temperature of the contents of the PCR reaction vessel (Saiki et al., 1988 b). The second advantageous property characteristic of the 'Taq' polymerase is that it results in a substantially increased specificity of the reaction (Kogan et al., 1987). Klenow-catalysed PCR was found to yield a variety of reaction products, only some of which were the required fragment. The non-specific products probably result because annealing and elongation of oligonucleotide primers is performed at around 30^{o}C, well below the optimum hybridization temperature; this could result in primers hybridizing to an incorrect sequence, so the strand produced on elongation need not have the same nucleotide sequence as the predicted product. This problem may be minimised by size-fractionation of restriction enzyme-digested template genomic DNA,

prior to amplification (Beck & Ho, 1988), although this is time-consuming, and impossible if the size of the restriction fragment carrying the gene of interest is unknown. The optimal working temperature range for Taq polymerase is 70-75^{o}C, so primers can be allowed to anneal at a more suitable temperature (usually around 55^{o}C for a 20-mer), thus reducing the probability of incorrect annealing. Elongation at around 70^{o}C ensures that the reaction is specific (Erlich et al., 1988). Studies on the fidelity of DNA synthesis by Taq polymerase suggest that single-base substitution errors are produced at a rate of 1 for each 9,000 nucleotides polymerized (Tindall & Kunkel, 1988). Since the target sequence is amplified exponentially during PCR, individual sequences containing errors may represent a significant proportion of the reaction product, depending on how early in the amplification process the error occurs. In spite of this potential limitation, experimental conditions can be designed to limit the generation of mutations occuring during PCR, resulting in a final mutation rate that is only twice that observed in a single round of DNA synthesis (Saiki et al., 1988 b). Mutations resulting during PCR are only significant if it is necessary to clone a single amplified sequence, and may result in unacceptable alterations within the isolated clone.

The efficiency of amplification is not significantly affected by the presence of nucleotides or label molecules at the 5' end of the primers, even if this results in an area of mismatch with the original template DNA. This allows the incorporation of additional sequence information into the PCR product through the oligonucleotide primers (Mullis et al, 1986); examples of additions which may be useful include restriction site linkers to allow direct cloning and sequencing of amplified genomic sequeneces (Scharf et al., 1986), nucleotide

substitutions, insertions, deletions or regulatory elements.

1.7.2 Applications Of PCR.

The first reported diagnostic use of the PCR technique was for the rapid prenatal diagnosis of sickle-cell anaemia (Saiki et al., 1985 a). As discussed in section 1.3.5, the sixth codon of the gene coding for beta-globin possesses a recognition site for the restriction enzyme DdeI; the mutation which results in sickle-cell anaemia destroys this site. After PCR amplification, one of two techniques was used for discriminating between normal and sickle alleles. The first of these, termed oligomer restriction (OR) (Saiki et al., 1985 b), involves stringently hybridizing a ^{32}P end-labelled normal allele-specific oligonucleotide in solution to the PCR product; the labelled oligonucleotide (a 40-mer) spans the informative restriction site, and an exact match will result only if the product derives from an individual possessing at least one normal allele, thus generating a double-stranded region containing a recognition site for the restriction enzyme DdeI. If the match is not exact, cleavage with DdeI is inhibited. After digestion with this enzyme, polyacrylamide gel electrophoresis and autoradiography can be used to establish the size of the digestion products, and therefore the nature of the alleles present in the original sample. DNA from normal individuals, sickle-cell anaemia patients and sickle-cell carriers (heterozygotes) can be readily distinguished by the PCR-OR method in under 10 hours, and the incorporation of suitable controls confirms complete endonuclease digestion and methodologic accuracy (Embury et al., 1987). Following the initial report, the PCR-OR technique was used for the identification of human immunodeficiency virus (HIV) in human samples (Kwok et al., 1987).

The second method used for distinguishing normal and abnormal alleles, using allele-specific oligonucleotide probes (ASO), is appropriate regardless of whether or not the mutation creates or destroys a restriction enzyme recognition site. Oligonucleotides can be used for the identification of single base mutations in nucleic acids, because the hybrid formed between an oligonucleotide and its exactly complementary target sequence is more stable than that formed between the same oligo and a target sequence which contains a single mutation (Studencki & Wallace, 1984). A single base mismatch out of a total of less than twenty (the usual length of an oligonucleotide probe) results in a hybrid melting temperature between 5°C and 10°C lower than that of the perfectly matched pair of sequences. By careful arrangement of the hybridization conditions, it is possible to distinguish between normal and mutant target alleles. After PCR amplification of the region of the beta-globin gene surrounding the sickle-cell anaemia mutation site, an aliquot of each sample was spotted on to a nylon hybridization membrane. After stringent hybridization with ^{32}P-labelled ASO probes as described above, genotypes could be readily distinguished following autoradiography (Saiki et al., 1986). Since this initial report of PCR in conjunction with an ASO probe detection system, the technique has been applied to the identification of the mutations associated with haemophilia A (Kogan et al., 1987), phenylketonuria (DiLella et al., 1988), and alpha$_1$-antitrypsin deficiency (Bruun Petersen et al., 1988). It has also been applied to the detection of somatic ras mutations in peripheral blood cells from patients with acute myelogenous leukaemia (Farr et al., 1988), and in the study of colorectal tumours (Bos et al., 1987). Since PCR results in a large increase in the amount of target sequence present in a sample, it has been possible to use ASO probes labelled with horseradish peroxidase, rather than with a

radio-isotope, for the identification of the mutations associated with sickle-cell anaemia and beta-thalassaemia (Saiki et al., 1988 a). The most simple means of detecting mutations which create or disrupt a restriction enzyme recognition site does not involve hybridization of a labelled probe; instead, the sample is first subjected to PCR amplification, and the PCR product is digested with the appropriate enzyme. After agarose or polyacrylamide gel electrophoresis, the correct genotype may be ascertained by analysis of the size of the DNA fragments produced. This approach has been used for the identification of the mutations associated with $alpha_1$-antitrypsin deficiency (Abbott et al., 1988), sickle-cell anaemia and alpha-thalassaemia (Chehab et al., 1987).

Several groups have used PCR for the identification of infectious agents; this is much more simple than the cases described above, because it is not necessary to use the differential melting properties of matched and mismatched oligonucleotide probes to distinguish between alleles. A single probe (either a synthetic oligonucleotide, or cloned sequence, specific for the organism of interest) is used to determine whether or not the sample is infected. This procedure has been used for the identification of HIV-infected individuals (Loche et al., 1988; Ou et al., 1988), and also for the detection of toxigenic E.coli using biotin-labelled probes (Olive et al., 1988). A recently described technique for detecting closely related HPV types in DNA recovered from cervical smears does not rely on hybridization of a probe; instead, HPV type-specific primer pairs are chosen so that the size of the PCR product is different depending on which virus type is present. Thus, the nature of the infecting virus can be determined directly by electrophoresis of the PCR product (Young et al., 1989).

It is clear that the PCR technique is a powerful means of increasing the sensitivity of nucleic acid-based diagnostic assays, not least because it is flexible, and is effective on many types of sample. For example, it has been used for the analysis of DNA extracted from formalin-fixed, paraffin-embedded tissues (Impraim et al., 1987; Lai-Goldman et al., 1988), and for the detection of human papilloma-virus in paraffin-embedded specimens, without DNA extraction (Shibata et al., 1988). There are several advantages inherent in the ability to use such samples for nucleic acid analysis: for example, formalin fixation and paraffin-embedding render the tissue non-infectious, so routinely obtained biopsy and post-mortem material may be studied. Of particular interest in the case of infectious agents (HIV or the HPVs, for example) is the fact that PCR performed on archival tissue samples obtained prior to the identification and isolation of the virus will allow a more detailed analysis of viral epidemiology (Lai-Goldman et al., 1988). PCR amplification of DNA released from a crude lysate of a small number of cells occurs with an efficiency only two- to three-fold less than that obtained with an equivalent amount of purified DNA. However, as the number of cells increases beyond around 5×10^3, there is a marked reduction in efficiency, probably due to inhibition of the amplification process by cellular debris (Saiki et al., 1986). This does not represent a significant problem in the case of analysis of genomic sequences, because each cell is certain to contain the region of interest; for example, the use of unfractionated lysates for the detection of the mutation associated with sickle-cell anaemia reduces the total time required for the assay to less than 8 hours. However, it would not be realistic to use a crude cell lysate for the detection of infectious agents, because the number of infected cells may be small, amongst a large background of uninfected ones. The selection of a small

number of cells for amplification may not be representative of the cell population as a whole. Hence, it is not possible at present to avoid purification of DNA from a sample in the case of PCR-based diagnosis of infectious disease.

It is possible to PCR amplify a single molecule of DNA (Saiki et al., 1988 b), so a single hair contains more than enough nucleic acid for amplification of genomic sequences (the root end of a freshly plucked hair may contain as much as 0.5 ug of DNA). Thus, it has been possible to detect genetically variable mitochondrial and nuclear DNA sequences from the root region of shed, as well as freshly-plucked single hairs (Higuchi et al., 1988); this may be of great use in forensic science, because it will allow victims and suspects to be positively identified by hairs shed at the scene of a crime.

In the research laboratory, the PCR technique is becoming a useful tool for improving some of the methods used by the molecular biologist. For example, it has been used for the rapid production of plasmid insert, without the need for large-scale plasmid preparation, restriction enzyme digestion and insert isolation (Liang & Johnson, 1988); a similar procedure can be used to produce vector-free biotinylated probes by replacing dTTP with biotinylated dUTP in the reaction buffer (Lo et al., 1988).

The development of rapid and reliable techniques for the direct sequencing of enzymatically amplified DNA (Engelke et al., 1988) will bypass the need for gene cloning, library construction and screening, thus dramatically improving procedures designed for the detection of mutations and polymorphisms in individual subjects. For example, direct genomic sequencing of PCR amplified DNA has been used for the characterization of new mutations resulting in beta-thalassaemia (Wong et al., 1987). An alternative sequencing method based on PCR

amplification has been recently described; the technique, known as genomic amplification with transcript sequencing (GAWTS) involves the attachment of a T7 phage promoter onto at least one of the PCR primers (Stoflet et al., 1988). The segments amplified by PCR are transcribed with T7 RNA polymerase to further increase the signal, and to provide an abundance of single-stranded template for reverse transcriptase-mediated dideoxy sequencing. An end-labelled reverse transcriptase primer complementary to the desired sequence generates the additional specificity required to generate unambiguous sequence data. The method is capable of producing results at least five times as fast as conventional sequencing, and since GAWTS is amenable to automation, further increases in the rate of sequence aquisition are likely. The technique has recently been modified for directly obtaining sequence data from RNA (Sarkar & Sommer, 1988).

Although in its original form PCR allows the amplification of segments of DNA between two regions of known sequence, it is possible to amplify segments that lie outside the boundaries of known sequences (Triglia et al., 1988). The approach simply requires inversion of the restriction fragment of interest by circularization with DNA ligase and re-opening at a different restriction enzyme recognition site. This may be particularly useful for sequencing the genomic DNA flanking a viral integration site. An ingenious PCR-based assay for detecting the targetted modification of chromosomal genes by homologous recombination between exogenous DNA and a target locus has been reported (Kim & Smithies, 1988); the procedure is not dependent on the phenotype of the target gene, or on its expression in target cells, so it has overcome one of the major constraints preventing the widespread use of gene targetting.

In summary, the polymerase chain reaction results in a dramatic

increase in the amount of target sequence present in a sample; this means that the main priority in chosing the assay for subsequent sample analysis need not be high sensitivity. In fact, amenability to automation and the capability of handling large numbers of samples are more important; there would be little advantage in using PCR if subsequent procedures for analysis of amplified sequences were time-consuming and technically demanding. There is no doubt that some kind of signal amplification step will be used if nucleic acid-based assays are to take their place as invaluable tests in the clinical laboratory. It will not be acceptable to compromise on sensitivity, because techniques like PCR or oligonucleotide-complex based amplification systems (Urdea et al., 1987 a; see section 1.3.7) not only dramatically improve the detection limits within which an assay can operate, but also result in a more rapid result. In the case of detection of genetic defects, sufficient human DNA for PCR amplification can be isolated from buccal epithelial cells obtained by mouthwash (Lench et al., 1988). This greatly simplifies the ease with which a large number of samples could be obtained, because the need for medical supervision of sample collection, and the concomitant risk of infection are eliminated. For these reasons, PCR amplification followed by sandwich hybridization appears to be an obvious candidate for development of a technique which would satisfy all of the requirements of a routine diagnostic test; the development of such an assay has been a major objective of this study.

2. AIMS.

The methods of gene detection most commonly used in research laboratories can be precise and sensitive, but are not suitable for routine diagnostic use, because they are labour intensive and time consuming. They are also limited in the number of samples which can be processed at once, are impossible to totally automate, and rely on lengthy autoradiographic procedures as a means of signal detection. Although there are circumstances in which *in situ* hybridization can provide useful information regarding the intracellular location of specific nucleic acid sequences, the technique is not suitable for large-scale diagnostic use for the reasons outlined above.

Nucleic acid sandwich hybridization appears to be an ideal candidate for the development of a suitable alternative to conventional techniques, because it eliminates the need for electrophoresis and sample immobilization, and is therefore amenable to automation (possibly using equipment originally designed for sandwich immunoassay). There is no requirement for signal detection by autoradiography, so results can be obtained in an easily interpreted numerical form. Although sandwich hybridization techniques have been reported for the detection of infectious agents, and also for the identification of nucleotide sequence variations (reviewed in Nicholls & Malcolm, 1989), none of these methods have yet made an impact on the realm of routine diagnostics. Probably the main reason for this is that, even using probes labelled with radioactive isotopes, the sensitivity of the sandwich assay is around ten times less than that of Southern blotting. However, with the recent advent of signal amplification procedures like the polymerase chain reaction (PCR) (Saiki *et al.*, 1985 a), lack of sensitivity becomes relatively insignificant when compared with the advantages inherent in sandwich

hybridization.

An excellent example of a condition which is most effectively diagnosed by nucleic acid analysis is human papillomavirus (HPV) infection of the uterine cervix. The HPVs have been firmly implicated in the aetiology of cervical cancer, although the precise mechanism for this interaction is as yet poorly understood (Pfister, 1987). Since viral nucleic acid can persist within an infected cell without transcriptional or translational activity, immunoassay is not an effective means of screening for infection (Pfister, 1987). A recent report by the British Medical Association conceded that 'there is growing evidence linking infection by a papillomavirus with cervical cancer', although it was concluded that 'at present it is neither practicable nor economic to test all smears and cervical biopsies for these [human papilloma] viruses' (British Medical Association Report, 1986).

With these facts in mind, the major aim of this study was to develop a sandwich hybridization assay suitable for detecting human papillomavirus infection of the cervix. The following points regarding the development of the assay were considered important:

(1) Although there is evidence to suggest that integration of viral nucleic acid into the host cell chromosome may initiate the neoplastic process, the evidence is not conclusive. For this reason, the assay should be capable of detecting viral DNA, regardless of its integrational status and abundance.

(2) In order to be suitable for widespread use, the method of obtaining a sample should be simple and non-invasive. The cervical scrape would be ideal, because it is an accepted non-invasive procedure, and there are no ethical limitations in obtaining control material

from normal individuals. Scrapes have been used previously for the successful identification of HPV infection (Wickenden et al., 1985).

(3) Specific HPV types have been associated with cervical tumours, whereas other distinct types are more frequently associated with lower grade cervical abnormalities. It is essential that the assay should be capable of discriminating between 'high risk' and 'low risk' types of the virus. In order to achieve this, it is necessary to isolate a pair of non-homologous nucleic acid probes for each important virus type, and at least one probe of the pair must hybridize just to its source virus, and not to any other virus type.

(4) To produce a format which would lend itself most readily to automation using currently available technology, the assay should be developed using DNA probes covalently immobilized to particulate resins, rather than to nitrocellulose or nylon filters. A principal aim was therefore to identify the most suitable resin, in terms of the efficiency with which DNA could be irreversibly bound, and the availabilty of bound nucleic acid for subsequent hybridization (with a low non-specific background signal). Chemical methods which have been reported as being the most consistently successful for covalently immobilizing DNA by the 5' end, or via the base moieties, must be considered.

(5) In order to achieve high sensitivity, the possibility of including a PCR-mediated signal amplification step prior to sandwich assay should be considered. An alternative procedure involving the affinity capture of biotinylated PCR products on streptavidin-agarose, followed by detection of the amplified region with a probe in a 'one-step' assay, may prove successful.

(6) The signal amplification associated with PCR should allow the application of non-radioactive detection systems to be studied.

The overall aim of this investigation was to produce a rapid, reliable, safe, sensitive and simple technique for economically identifying HPV infection in large numbers of cervical scrape samples. The procedure should be capable of distinguishing between the virus types associated with cervical abnormalities, and also be amenable to automation. Once developed, it should be simple to adapt the procedure for the detection of other infectious organisms.

3. MATERIALS AND METHODS.

3.1 Materials.

Chemicals.

With the exception of the reagents mentioned below, all chemicals were Analar grade, supplied by British Drug Houses Ltd. (BDH), Poole, Dorset, England.

The following were obtained from the Sigma Chemical Co., St. Louis, MI, U.S.A: bovine serum albumin (BSA) fraction V; dithiothreitol (DTT); ethidium bromide (EtBr); N-2-hydroxyethyl piperazine N'-2-ethane sulphonic acid (HEPES); 2-mercaptoethanol; 2(N-morpholino)ethane sulfonic acid (MES); piperazine-N,N'-bis(2-ethane-sulfonic acid) (PIPES); Sigmacote; sodium chloride (NaCl); Tris(hydroxymethyl) amino methane hydrochloride (Tris-HCl); Tris(hydroxymethyl) amino methane (Tris-base).

Caesium chloride (CsCl) and Ficoll were obtained from Pharmacia Biotechnology International AB, Uppsala, Sweden.

Isotopes.

The isotope phosphorous-32 (^{32}P) was supplied by New England Nuclear (NEN), DuPont (U.K.) Ltd., Southampton, Hampshire, England, at an activity of >3,000 Ci/mmole (10 mCi/ml, in 10 mM tricine).

Enzymes.

a) Restriction Enzymes.

BamHI, DdeI, EcoRI, HaeIII, HindIII, PstI and TaqI were supplied (with appropriate x10 reaction buffers) by Bethesda Research Laboratories (Gibco-BRL), Rockville, MD, U.S.A.

b) Other Enzymes.

DNA polymerase I 'Klenow fragment', streptavidin-alkaline phosphatase (AP) and streptavidin-horse radish peroxidase (HRP)

conjugates, T4 polynucleotide kinase, and T4 DNA ligase were obtained from Gibco-BRL. Calf intestinal alkaline phosphatase (CIP) was from Pharmacia. Lysozyme and RNAse A were supplied by Sigma, and both micrococcal nuclease (from Staphylococcus aureus) and proteinase K were from the Boehringer Corp., Mannheim, W. Germany. Finally, DNA polymerase from Thermus aquaticus YT1 was purchased from Perkin Elmer Cetus, Norwalk, CT, U.S.A.

Nucleic Acids.

Salmon sperm DNA and calf thymus DNA were obtained from Sigma. 2'-deoxyadenosine 5'-triphosphate (dATP), 2'-deoxycytidine 5'-triphosphate (dCTP), 2'-deoxyguanosine 5'-triphosphate (dGTP), 2'-deoxythymidine 5'-triphosphate (dTTP) and hexadeoxyribonucleotide primer ($p[dN]_6$) were from Pharmacia. Bacteriophage lambda DNA and phiX174 DNA were supplied by Gibco-BRL. Plasmid pUC8 was from Amersham International Plc., Amersham, Buckinghamshire, England. Reagents for oligonucleotide synthesis (including beta-cyanoethyl phosphoramidites, and Aminolink-2 reagent) were obtained from Applied Biosystems, Warrington, Cheshire, England. Total human DNA was the kind gift of a colleague, Uta Voss.

Chromatography And Electrophoresis.

Sephadex G-50 (medium grade) was obtained from Pharmacia. Normal and low melting point (LMP) agaroses were from Gibco-BRL. NuSieve GTG agarose was supplied by FMC BioProducts, Rockland, ME, U.S.A. Nensorb 20 affinity chromatography columns were from NEN.

Blotting And Hybridization.

Whatman chromatography 3MM paper was from Whatman Ltd., Maidstone, Kent, England. Nitrocellulose blotting membranes were supplied by Schleicher and Schuell GmbH, Dassel, W. Germany. Hybond-N was from Amersham International.

Autoradiography And Photography.

FP4 film, Phenisol and Autophen developer, and Hypam fixer were supplied by Ilford, Basildon, Essex, England. X-OMAT XAR-5 X-ray film was from Eastman Kodak, Rochester, NY, U.S.A. Okamoto film cassettes and screens were from X-Ograph Ltd., Malmesbury, Wiltshire, England.

Reagents For Bacterial Culture.

Agar, yeast extract and tryptone were supplied by Difco Laboratories, Detroit, MI, U.S.A. Ampicillin was from Sigma, and 5-bromo-4-chloro-3-indolyl-beta-galactoside (X-gal) and isopropyl-beta-D-thio-galactopyranoside (IPTG) were obtained from Northumbria Biologicals Ltd. (NBL), Cramlington, Northumberland, England. Sterile 90 mm Petri dishes were purchased from Sterilin Ltd., Teddington, Gloucestershire, England.

Materials For Nucleic Acid Immobilization.

Sephacryls S-500 and S-1000, Sepharose CL4B and epoxy-Sepharose 6B were from Pharmacia. Dynospheres M450 were the kind gift of Dyno Industrier A.S., Oslo, Norway. Streptavidin-agarose was from Gibco-BRL. 1,4-butanediol diglycidyl ether (95%), 2-aminothiophenol and 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene-sulfonate (95%) were purchased from the Aldrich Chemical Co. Ltd., Gillingham, Dorset, England.

Labelling And Detection Of Non-radioactive DNA Probes.

Sulfosuccinimidyl 6-(biotinamido) hexanoate (NHS-LC-biotin) was obtained from the Pierce Chemical Co., Oud-Beijerland, The Netherlands. Biotin-11-dUTP, and a Blu-GENE non-radioactive detection kit were purchased from Gibco-BRL. A non-radioactive DNA labelling and detection kit was a gift to the laboratory from Boehringer. O-phenylenediamine dihydrochloride was obtained from Sigma.

3.2 Bacterial Stocks: Strains And Storage.

All plasmids were propagated in Esherichia coli (E.coli) K-12 strains HB101 (Boyer & Roulland-Dussoix, 1969), JM101 or JM103 (Messing et al., 1981).

The genotypes for these strains are as follows:

HB101 F^-, hsdS20, rB^-, mB^-, $recA^-$, supE44, ara-14, gal K-2, lacY1, proA2, rps L20, str^r, xyl-5, mtl-1.

JM101 (lacpro), thi, F'traD36, proAB, supE, lacIqZ M15.

JM103 (lacpro), thi, strA, endA, sbcB15, hsdR4, F'traD36, proAB, supE, $lacI^qZ$ M15.

All bacterial stocks were stored as exponential cultures, both in 50% v/v glycerol at -20°C, and in 15% v/v glycerol, at -70°C. The latter were not used routinely as a source of viable cells, but were retained as replacements, should stocks stored at -20°C die, or become contaminated.

3.3 Plasmids.

Cloned DNA from HPV types 6b, 11, 16 and 18 were the kind gift of a colleague, Colin Wickenden; the clones were originally obtained from Professor H. zur Hausen, Institut Fur Virologie, Heidelberg, W. Germany. With the exception of type 6b, HPV DNA was supplied as the complete genome, cloned into the BamHI site (HPVs 11 [7,931 bp] and 16 [7,904 bp]), or EcoRI site (HPV 18 [7,857 bp]) of pBR322 (Bolivar et al., 1977). HPV 6b (7,902 bp) was supplied as a 5,368 bp BamHI/EcoRI sub-genomic fragment, cloned into pBR322. All constructs were propagated in E.coli K-12 strain HB101.

Plasmid beta f5.5 is a pUC8 (Viera & Messing, 1982) derived construct, which contains a 1.9 Kb BamHI restriction fragment from the 5' end of the human beta-globin gene (Maingon, 1982). Plasmids KJ7

(Johnson, 1985) and JL1 (Langdale & Malcolm, 1985) are pUC derived recombinants, containing a 341 bp HinfI beta-globin gene fragment, and a 201 bp MstII beta-globin gene fragment respectively. Both pKJ7 and pJL1 were the kind gift of a colleague, Hermia Figueiredo.

Plasmids containing inserts derived from the genomes of human papillomavirus types 6b, 11, 16 and 18 (all constructed using pUC8) were as follows; figures in brackets refer to the genomic nucleotide positions of each HPV fragment:

pJN1 contains a 1,776 bp HPV 16 PstI fragment (7,003-875).

pJN2 contains a 483 bp HPV 16 PstI fragment (4,755-5,238).

pJN3 contains a 216 bp HPV 16 PstI fragment (6,787-7,003).

pJN4 contains a 555 bp HPV 18 PstI fragment (5,770-6,325).

pJN5 contains a 441 bp HPV 18 PstI fragment (6,325-6,766).

pJN7 contains a 1,042 bp HPV 11 PstI fragment (5,432-6,474).

pJN8 contains a 435 bp HPV 11 PstI fragment (6,474-6,909).

pJN9 contains a 3,601 bp HPV 6b PstI/EcoRI fragment (6,489-2,188).

pJN10 contains a 1,081 bp HPV 6b PstI fragment (5,408-6,489).

pJN11 contains the full-length HPV 11 genome (7,931 bp), cloned into the BamHI site.

pJN12 contains a 686 bp HPV 6b PstI/BamHI fragment (4,722-5,408).

pJN16 contains the full-length HPV 16 genome (7,904 bp), cloned into the BamHI site.

All pJN plasmids were constructed during the course of this investigation.

3.4 General Methods.

Many of the methods used in this study were adapted from those described in 'Molecular Cloning: A Laboratory Manual' (Maniatis et al.,

1982).

3.4.1 Extraction And Precipitation Of DNA.

The most common extraction procedure performed on DNA samples was for the removal of contaminating protein; this protein can derive from the cells which were the source of the nucleic acid (in the case of plasmids propagated in E.coli, or DNA extracted from clinical samples). Alternatively, it may be necessary to remove enzymes which have been used to modify DNA, prior to subsequent procedures (for example, removal of a restriction enzyme from a DNA digest prior to ligation). Many of the enzymes which use nucleic acid as substrate are inhibited by contaminating proteins, particularly those which bind to the DNA molecule. Deproteination was performed by extraction with phenol/chloroform. Prior to use, phenol was saturated with TE buffer, pH 8.0 (10 mM Tris(hydroxymethyl) amino methane hydrochloride [Tris-HCl], 1 mM ethylene diamine tetra-acetic acid [EDTA]), and was added in equal volume to the DNA sample. After thorough mixing (without vortexing), the sample was centrifuged for 5 minutes, either in a micro-centrifuge (MSE Microcentaur, 13,000 rpm [13,400 x g_{av}]), or in a Sorvall RC5B superspeed centrifuge (HB-4 rotor, 10,000 rpm [10,815 x g_{av}]), depending on the volume of the sample. The upper (aqueous) layer was transferred to a clean tube, and extracted twice in an identical manner with chloroform/isoamyl alcohol (24:1). A final purification step, extraction twice with water saturated diethyl ether, was performed in the same fashion, except that the upper layer (ether) was discarded instead of the lower (aqueous) layer. Traces of ether were removed from the sample by loosening the cap of the tube, and incubating at 65^{o}C for 5 minutes.

Precipitation of DNA was achieved by addition of 0.1 volumes of

3 M sodium acetate (NaOAc), pH 4.5, 0.1 volumes of 0.1 M $MgCl_2$ and 2.5 volumes of ice-cold absolute ethanol. The mixture was incubated either at -20°C overnight, or at -70°C for 1 hour, prior to pelleting the DNA by centrifugation for 10 minutes. This was performed at 4°C, either in a micro-centrifuge (MSE Microcentaur, 13,000 rpm [13,400 x g_{av}]), or in the Sorvall RC5B (HB-4 rotor, 10,000 rpm [10,815 x g_{av}]). The supernatant was discarded, and the pellet washed by vigorous resuspension in cold 70% ethanol. After re-centrifugation, the supernatant was once again discarded, and the pellet dried either in a vacuum desiccator, or in a Speed Vac concentrator (Savant Instruments Inc., Hicksville, NY, U.S.A.). Dry pellets were resuspended in an appropriate volume of TE buffer, pH 8.0, only immediately prior to use. For long term storage, pellets were kept dry, or under 70% ethanol, at -20°C until required.

3.4.2 Random Shearing Of High Molecular Weight DNA.

Salmon sperm or calf thymus DNA was dissolved in deionized distilled water to a concentration of 5 mg/ml, by gentle stirring overnight at 4°C. Random shearing of the DNA was achieved by sonication in an MSE Soniprep 150; this was carried out on ice, using 10 x 30 second pulses, with at least 60 seconds between each pulse. An aliquot of the sonicated DNA was electrophoresed on a 1% agarose gel, alongside known molecular weight standards, in order to determine the extent of sonication. This entire procedure was repeated until the average size of the fragments was around 500 bp. Once sonication was complete, 1 ml aliquots of the DNA solution were dispensed into 1.5 ml screw-cap Eppendorf tubes, and denatured by heating in a boiling water bath for 10 minutes. The aliquots were rapidly frozen in a dry ice/ethanol bath, and stored at -20°C until required.

3.4.3 Restriction Endonuclease Digestion.

Digests were performed in pre-sterilized 1.5 ml polypropylene Eppendorf tubes. The components of the reaction were added in the following order: DNA (dissolved in TE buffer, pH 8.0); 0.1 volumes of x10 restriction enzyme buffer; nuclease-free BSA (to a final concentration of 100 ug/ml); distilled, deionised water (to make up the reaction volume); restriction enzyme (1-5 units/ug of DNA). Care was taken to ensure that the final DNA concentration was between 0.1 and 0.2 mg/ml, and that the volume of enzyme added did not exceed one tenth of the total reaction volume. The latter is particularly important, because enzyme storage buffers contain glycerol, which can inhibit enzyme activity. Maximum activities are only obtained if the glycerol is sufficiently diluted in the reaction mix. The temperature and buffer composition for each digest was as recommended by the enzyme manufacturer. After incubation (1-16 hours, depending on the nature of the DNA sample), an aliquot was removed from each digest, and the remainder was left on ice. The aliquot was subjected to agarose gel electrophoresis, in order to confirm that the digest was complete. Samples were then either electrophoresed immediately, or stored at $-20^{o}C$ until required.

3.4.4 Gel Electrophoresis.

3.4.4.1 Agarose gels.

All agarose gel electrophoresis was performed in horizontal submerged gel apparatus (either model H4, or model H6) supplied by Bethesda Research Laboratories, MD, U.S.A. 0.5%-2.0% agarose gels were made by dissolving the appropriate mass of solid agarose powder in Tris-acetate electrophoresis (TAE) buffer (40 mM Tris-HCl, pH 8.0, 20

mM NaOac, 2 mM EDTA). Higher percentage gels (2.5%-4.0%) were made by dissolving NuSieve GTG agarose in Tris-borate electrophoresis (TBE) buffer (89 mM Tris-HCl, pH 8.0, 89 mM sodium borate, 2 mM EDTA). Care was taken to ensure that solid agarose was fully dissolved by thoroughly boiling the solution in a microwave oven. Prior to pouring, the solution was cooled to approximately 45^{o}C, in order to prevent damage to the apparatus. The gel was poured, a slot forming comb was placed in position, and the agarose was allowed to solidify at room temperature (or at 4^{o}C for low melting point agarose). The comb was carefully removed, and the electrophoresis tank was filled with buffer to a level 0.5 cm above the surface of the gel. TAE was used for running normal agarose gels, and TBE was used for NuSieve GTG agarose gels. Ethidium bromide solution (5 mg/ml) was added to the buffer, to a final concentration of 0.5 ug/ml. Prior to loading, 0.1 volumes of 'stop' buffer (0.5% w/v sodium dodecyl sulphate [SDS], 0.025% w/v bromophenol blue, 0.025% w/v xylene cyanol, 5 mM EDTA, 10% w/v Ficoll) was added to each sample. After loading, samples were electrophoresed in a field of 2-10 V/cm (40-170 mA), until the required separation of fragments was observed, on exposure of the gel to short wave u.v. light (generated by a Fotodyne model 3-3002 transilluminator, Fotodyne, WI, U.S.A.). An indication of the extent of separation could be obtained from the position of the tracking dyes included in the stop buffer. Gels were photographed on Ilford FP4 negative film, using a Plan Polaroid MP-4 Land camera.

3.4.4.2 Polyacrylamide Gels.

Polyacrylamide gel electrophoresis for the analysis of ^{32}P labelled oligonucleotides was performed in a vertical slab gel apparatus (model V16-2), supplied by BRL. The glass gel plates were cleaned with ethanol, and thoroughly dried before the apparatus was

assembled, as described by the manufacturer. The following stock solutions for making gels were prepared: 40% w/v acrylamide; 2% w/v N,N'-methylene-bisacrylamide; 0.9 M Tris-borate, pH 8.3, 25 mM EDTA; N,N,N',N'-tetramethylethylene-diamine (TEMED). The protocol described below is for a 20% w/v polyacrylamide gel, containing 7 M urea; for gels of a different percentage, the volumes of acrylamide and bisacrylamide added were varied accordingly. 50 ml of stock acrylamide solution and 33 ml of bisacrylamide were added to 10 ml of stock Tris-borate/EDTA buffer, and the mixture was degassed using a vacuum pump; this was done in order to remove oxygen, which inhibits the polymerization reaction. 42 g of urea was dissolved in the degassed solution, which was then made up to a volume of 99 ml with deionized distilled water. Finally, 50 ul of TEMED and 1 ml of freshly prepared 10% ammonium persulphate solution were added, and after mixing, the solution was poured between the gel plates. A slot forming comb was placed in position, and the gel was allowed to polymerize for 60 minutes. After this time, the comb and base spacer were removed, and the tank reservoirs were filled with TBE buffer. A Pasteur pipette was used to flush out each well with buffer; this was done in order to remove accumulated urea, which can prevent a sample from entering the well. 0.1 volumes of 'stop' buffer (0.025% w/v bromophenol blue, 0.025% w/v xylene cyanol, 0.5 M EDTA, 30% w/v Ficoll) was added to each sample, prior to loading onto the gel with a Gilson micropipette. Electrophoresis was performed at 200 mA, until the tracking dyes had travelled the required distance. The gel was removed from between the glass plates, and covered with Saran Wrap. Results were determined by autoradiography, as described in section 3.4.9.

3.4.5 Gel Filtration.

The methods used for labelling nucleic acids with a radioisotope involve using an excess of the labelled nucleotide. Gel filtration was used to remove nucleotides which had not been incorporated into the DNA molecule being labelled. A siliconized glass Pasteur pipette was plugged with siliconized glass wool, and the pipette was filled to within 1 cm of the top with medium grade Sephadex G-50 (pre-swollen in 3 x SSC; 1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.6). Samples were applied to the top of the column, in a volume of approximately 50 ul, and allowed to soak into the gel matrix. 100 ul aliquots of 3 x SSC, 0.1% w/v SDS were applied sequentially to the top of the column, and fractions of the same volume were collected in 1.5 ml Eppendorf tubes as they emerged from the bottom. Fractions were constantly monitored for the presence of radioactivity using a series 900 mini-monitor (Mini-Instruments Ltd., Burnham-on-Croach, Essex, England). The collected fractions were counted by the Cerenkov method, in an LKB model 1214 Rackbeta liquid scintillation counter (Berger & Krug, 1985). Fractions containing the peak of excluded radiolabelled DNA were pooled, and the specific activity of the probe was calculated.

3.4.6 Spin Column Chromatography.

In circumstances where several different DNA probes were labelled at once, the gel filtration procedure described above became too time-consuming. Spin column chromatography is an alternative method for separating unincorporated nucleotides from labelled DNA, which can be applied to several samples at once. The tip of a disposable 2 ml syringe was plugged with siliconized glass wool, and the syringe was filled to the top with Sephadex G-50, pre-swollen in 3 x SSC. The syringe was then placed in a 15 ml tube, and centrifuged for 4 minutes

in a Sorvall RC5B superspeed centrifuge (GSA rotor, 4,000 rpm [1,638 x g_{av}]). The addition of Sephadex G-50, followed by centrifugation, was repeated until the gel matrix was packed to within 0.5 cm of the top of the syringe. 100 ul of 3 x SSC, 0.1% w/v SDS was added to the top of the column, and the centrifugation procedure was repeated. A 1.5 ml Eppendorf tube was placed in the bottom of the centrifuge tube, beneath the tip of the syringe. Samples were made up to a volume of 100 ul with 3 x SSC, 0.1% w/v SDS, and applied to the top of the column. After a final centrifugation step, the Eppendorf tube was removed, and the contents mixed by vortexing thoroughly. 10 ul of the effluent was transferred to a clean 1.5 ml tube, and made up to 100 ul with 3 x SSC, 0.1% w/v SDS. After counting by the Cerenkov method in an LKB model 1214 Rackbeta liquid scintillation counter (Berger & Krug, 1985), the specific activity of the labelled probe was calculated.

3.4.7 Affinity Chromatography.

Nensorb 20 cartridges were used to separate DNA from protein, salts, unincorporated radioactive nucleotides and other low molecular weight contaminants, in circumstances where high purity of the DNA sample was essential. The cartridges function by reversibly binding nucleic acid, and irreversibly binding protein; all other contaminants are flushed out by washing with the appropriate buffer. Pure DNA may then be eluted in 50% methanol. Nensorb 20 cartridges were used according to the instructions of the manufacturer.

3.4.8 Quantitation Of Radioactivity.

The radioactivity emitted by the isotope ^{32}P was quantitated by Cerenkov counting, at an efficiency of approximately 30%, in an LKB model 1214 Rackbeta liquid scintillation counter (Berger & Krug, 1985).

In the calculation of specific activity of a radiolabelled nucleic acid probe, results were expressed in units of disintegrations per minute (dpm) per fmole of DNA.

3.4.9 Autoradiography.

All autoradiography was performed at -70°C, using intensifying screens, and pre-flashed X-ray film. Pre-flashing was achieved using a filtered electronic flash gun, to an $O.D._{540\ nm}$ of 0.1-0.2.

3.5 Specific Methods.

3.5.1 Large-scale Preparation Of Recombinant Plasmid DNA.

Plasmid DNA was prepared by density gradient centrifugation, using a modification of the method used by Ish-Horowicz and Burke (1981). All manipulations involving live bacterial cultures were performed using safe microbiological procedures.

Fresh colonies of E.coli containing the required plasmid were grown immediately prior to each preparation. These were produced by dipping a platinum loop into a thawed bacterial glycerol stock, and streaking onto L-agar/ampicillin plates (50 ug/ml ampicillin, 5 g/litre yeast extract, 10 g/litre tryptone, 5 g/litre NaCl, 15 g/litre agar, pH 7.2). After incubation at 37°C for 24 hours, a single colony was picked using a sterile platinum loop, and inoculated into 10 ml of L-broth/ampicillin (50 ug/ml ampicillin, 5 g/litre yeast extract, 10 g/litre tryptone, 5 g/litre NaCl, pH 7.2). This starter culture was incubated at 37°C, with shaking, until an $O.D._{550\ nm}$ of 0.4 was reached; 1 ml was then used to inoculate 500 ml of fresh L-broth/ampicillin. The total volume of culture grown varied from 1 to 4 litres, depending on the yield of plasmid required; all volumes quoted below refer to an

initial culture volume of 1 litre. After incubation at 37°C overnight, with shaking, bacterial cells were pelleted in 250 ml GSA bottles by centrifugation in the Sorvall RC5B (GSA rotor, 5,000 rpm [2,560 x g_{av}]) for 10 minutes at 4°C. The supernatant was discarded into a suitable anti-bacterial detergent, and each pellet was resuspended in 4 ml of solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0). Resuspended cells harbouring the same plasmid were pooled into a single GSA bottle, giving a total volume of 16 ml, and lysozyme was added to a concentration of 5 mg/ml. After a 10 minute incubation at room temperature, 40 ml of freshly prepared solution II (0.2 M NaOH, 1% w/v SDS) was added, and after gentle shaking, the contents of the GSA bottle were cooled on ice for 10 minutes. At this stage, the cells were fully lysed; cellular protein, and high molecular weight nucleic acid (but not plasmid) were precipitated by addition of 20 ml of solution III (3 M potassium acetate [KOAc], pH 4.8). After vortexing vigorously, and incubation for 30 minutes on ice, the unwanted cell debris was pelleted by centrifugation (GSA rotor, 10,000 rpm [10,240 x g_{av}]) for 10 minutes at 4°C. The supernatant was decanted into a clean GSA bottle, and 0.6 volumes of propan-2-ol were added in order to precipitate the remaining nucleic acid. After 10 minutes at room temperature, the precipitate was pelleted by centrifugation (GSA rotor, 10,000 rpm [10,240 x g_{av}]) for 10 minutes at 4°C. After removing the supernatant, the pellet was resuspended in 20 ml of TE buffer, pH 8.0, by gentle shaking for 30 minutes. The DNA solution was transferred to a polypropylene 30 ml centrifuge tube, and 21.5 g of solid caesium chloride was added, together with ethidium bromide (to a concentration of 0.5 mg/ml). Debris was cleared by centrifugation (HB-4 rotor, 12,000 rpm [15,570 x g_{av}]) for 30 minutes at 19°C. The specific gravity of the supernatant was adjusted to 1.56 by addition of solid caesium chloride,

which was dissolved by gentle mixing. The solution was transferred to a 35 ml Sorvall Ultracrimp centrifuge tube, which was subsequently sealed according to the instructions of the manufacturer. Tubes were spun in a Sorvall OTD 65B ultracentrifuge (TFT 50.38 rotor, 42,000 rpm [159,600 x g_{av}]) for 36 hours at 19°C. The plasmid band was visualized by illumination with long wave u.v. light (generated by a model UVSL-58 Mineralight Lamp, supplied by Ultra-Violet Products, Inc., CA, U.S.A.), and harvested using a 5 ml syringe and needle. Ethidium bromide was extracted from the preparation with TE/caesium chloride saturated propan-2-ol. DNA was dialysed for 2 hours at 4°C against 2 litres of TE buffer, pH 8.0, and again overnight with fresh buffer. The DNA, now free of ethidium bromide and caesium chloride, was extracted twice with phenol/chloroform, twice with water saturated ether, and ethanol precipitated. The pellet was resuspended in an appropriate volume of TE buffer, pH 8.0, immediately prior to use.

3.5.2 Small-scale Preparation Of Recombinant Plasmid DNA.

In circumstances where several different plasmids needed to be prepared at one time, and high yield was not essential (for example, in the analysis of recombinants produced after 'shot-gun' sub-cloning), an alkaline lysis 'miniprep' technique was used, resulting in the production of 1 to 5 ug of DNA (Birnboim & Doly, 1979).

5 ml of L-broth containing ampicillin (50 ug/ml) was inoculated with a single bacterial colony, and incubated at 37°C overnight, with vigorous shaking. 1.2 ml of the overnight culture was transferred to a sterile 1.5 ml Eppendorf tube, and the remainder was stored at 4°C until required. The cells were pelleted by centrifugation (MSE Microcentaur, 13,000 rpm [13,400 x g_{av}]) for 1 minute at room temperature. The supernatant was removed by aspiration, and the pellet

was resuspended by vortexing in 100 ul of ice-cold solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0). After incubation for 5 minutes at room temperature, 200 ul of freshly prepared solution II (0.2 M NaOH, 1% w/v SDS) was added to the tube. The contents were mixed by gently inverting, prior to incubation on ice for 5 minutes. 150 ul of ice-cold solution III (3 M KOAc, pH 4.8) was added, and the tube was vortexed for 10 seconds. After a further 5 minutes on ice, the bacterial cell debris was pelleted by centrifugation (MSE Microcentaur, 13,000 rpm [13,400 x g_{av}]) for 5 minutes at 4^{o}C. The supernatant was transferred to a clean Eppendorf tube, and extracted once with phenol/chloroform, prior to precipitation of the nucleic acid with ethanol. After pelleting the precipitate by centrifugation (MSE Microcentaur, 13,000 rpm [13,400 x g_{av}]) for 5 minutes at room temperature, the supernatant was discarded, and the pellet washed by vortexing in 1 ml of 70% ethanol. After re-centrifugation, and removal of the ethanol, the pellet was briefly dried in a Speed Vac concentrator,and resuspended in an appropriate volume of TE buffer, pH 8.0, containing DNAse-free pancreatic RNAse (20 ug/ml). Plasmid DNA was then analysed by restriction enzyme digestion, and agarose gel electrophoresis.

3.5.3 Purification Of Restriction Fragments.

Restriction fragments were purified from contaminating vector sequences using one of three methods, depending on the amount of material available, and the yield required.

3.5.3.1 Electroelution.

In circumstances requiring the purification of relatively large amounts of a particular restriction fragment (more than 10 ug), electroelution was the preferred technique. Restriction enzyme digested

recombinant plasmids were subjected to agarose gel electrophoresis (see section 3.4.4.1), in order to size fractionate the DNA fragments. The required band was identified by briefly transilluminating the gel with short wave u.v. light, and a slice of agarose containing the DNA fragment of interest was cut from the gel, using a scalpel blade. Care was taken to keep the mass of agarose to a minimum, without leaving any DNA behind in the gel. The gel slice was placed into a piece of dialysis tubing, which had been tied at one end, and filled with TAE buffer. The majority of the buffer was then removed with a Pasteur pipette, leaving the minimum volume to cover the gel slice, and exclude any air bubbles. The tubing was sealed with a dialysis clip, and returned to the electrophoresis tank, such that the longitudinal axis of the gel slice was parallel to the electrodes. 140 V (170 mA) was applied to the tank for 1 hour, allowing the DNA to migrate out of the agarose, into the TAE buffer filling the tubing. The current was reversed for 2 minutes to free any DNA which had attached to the wall of the dialysis membrane, and the buffer surrounding the gel slice was transferred to a clean tube. The membrane was washed out thoroughly with a small volume of TAE buffer, which was subsequently pooled with the fraction already collected. The eluted DNA was extracted three times with water saturated butan-1-ol (in order to remove ethidium bromide), twice with phenol/chloroform, twice with water saturated diethyl ether, and finally ethanol precipitated by centrifugation in the Sorvall RC5B centrifuge (HB-4 rotor, 10,000 rpm [10,815 x g_{av}]) for 30 minutes at room temperature. The DNA pellet was dried briefly in a vacuum desiccator, and resuspended in an appropriate volume of TE buffer, pH 8.0. The purity of the recovered DNA was ascertained by an $O.D._{320\ nm}$-$O.D._{220\ nm}$ scan on a Pye Unicam SP8-400 UV/VIS spectrophotometer. In cases where impurities were identified, an additional

purification step on a Nensorb 20 affinity chromatography column was performed (section 3.4.7).

3.5.3.2 Extraction From Low Melting Point (LMP) Agarose.

In circumstances requiring the purification of smaller amounts of DNA (between 1 ug and 10 ug), extraction from LMP agarose was the preferred technique. Size-fractionation by electrophoresis in LMP agarose was performed exactly as for normal agarose, except that gels were poured and electrophoresed at 4°C. The required band was identified, and cut out as described above (section 3.5.3.1). The volume of the gel slice was estimated, and an equal volume of extraction buffer (0.1 M Tris-acetate, pH 7.5, 5 mM EDTA, 0.5 M NaCl) was added. The gel slice was macerated with a clean glass rod, and the slurry was heated at 65°C for 10 minutes, with occasional shaking, in order to melt the agarose. An equal volume of TE saturated phenol (at room temperature) was added, and the contents of the tube were mixed gently by inversion for 10 minutes. The phenolic and aqueous phases were separated by centrifugation in a microcentrifuge (MSE Microcentaur, 13,000 rpm [13,400 x g_{av}]) for 10 minutes at room temperature. The upper, aqueous layer was transferred to a clean 1.5 ml Eppendorf tube, and an equal volume of fresh extraction buffer was added to the lower, phenolic layer. After mixing and centrifugation as described above, the aqueous layer was again removed, and pooled with the fraction already collected. This aqueous phase was extracted once more with phenol, twice with chloroform/isoamyl alcohol (24:1), and finally once with water saturated diethyl ether. After ethanol precipitation, the recovered DNA was resuspended in TE buffer, pH 8.0, and the purity determined by spectrophotometry (see section 3.5.3.1).

3.5.3.3 Extraction From Agarose With Nensorb 20 Columns.

For the purification of less than 1 ug of a particular

restriction fragment, extraction from agarose gels using Nensorb 20 affinity columns was the preferred technique. The required fragment was identified by size-fractionation on a normal melting point agarose gel; the section containing the DNA of interest was excised as previously described, and placed in an Eppendorf tube. The agarose slice was macerated using a clean glass rod, and the slurry was dissolved in saturated sodium iodide solution (2 ml per gram of slurry) by incubation for 1 hour at 37^{o}C, with occasional gentle vortexing. An equal volume of Nensorb loading buffer (0.1 M Tris-HCl, pH 7.7, 10 mM triethylamine [TEA], 1 mM EDTA) was mixed gently with the sample. A Nensorb 20 column was pre-wetted with 2 ml of 100% methanol, and the sample was loaded, exactly as described by the manufacturer. After washing with 3 ml of loading buffer, followed by 5 ml of deionized distilled water, pure DNA was eluted in 500 ul of 50% methanol. The sample was dried in a Speedvac concentrator, and the pellet resuspended in an appropriate volume of TE buffer, pH 8.0.

3.5.4 Sub-cloning Of Human Papillomavirus Restriction Fragments.

3.5.4.1 Ligation Of HPV Fragments Into Plasmid Vector.

Individual fragments derived from the genome of the HPV type of interest were purified for sub-cloning by extraction from agarose gels; this was achieved by one of the three methods described in section 3.5.3. In certain circumstances, it was not necessary to isolate individual fragments prior to sub-cloning. Instead, the products of a digest were purified on a Nensorb 20 affinity chromatography column (see section 3.4.7), and ligated by a 'shot-gun' procedure into the vector, pUC8.

Vector DNA was prepared from a glycerol stock of *E.coli* JM101 harbouring the plasmid, by density gradient centrifugation (section

3.5.1). 1 ug of pure vector DNA was completely digested with the appropriate restriction enzyme, and the terminal 5' phosphate groups were removed by treatment with calf intestinal alkaline phosphatase (CIP). This was achieved by addition of 1 unit of CIP, and incubation for 30 minutes at 37^{o}C; a further unit of CIP was then added, and the incubation was repeated. The purpose of removing the 5' phosphate groups was two-fold: firstly, it effectively reduces the number of transformants resulting from re-circularization of a single vector molecule. More importantly, it reduces the frequency with which two (or more) vector molecules ligate together; if this were to occur, the construct formed would be indistinguishable from those containing the required HPV insert, up until the stage where recombinants are characterized by restriction enzyme analysis. Digested, phosphatased vector DNA was purified by affinity chromatography on a Nensorb 20 column, and resuspended in sterile TE buffer, pH 8.0, to a concentration of 100 ng/ul.

An aliquot of both linear, phosphatased vector, and the DNA to be sub-cloned was retained for analysis by agarose gel electrophoresis. Ligation reactions were set up in sterile Eppendorf tubes, using a 3:1 molar excess of insert (Legerski & Robberson, 1985), in ligation buffer (66 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM DTT, 2 mM ATP). After the addition of 10 units of T4 DNA ligase, the contents of the tube were mixed by vortexing gently, and the sample was incubated at 19^{o}C for 16 hours. Two additional control ligations were always included: the first, containing non-phosphatased vector only, was to test the efficiency of ligation. The second control, including phosphatased vector only, was to confirm that removal of the 5' phosphate groups had occurred successfully. After ligation, aliquots were removed from each tube, and subjected to agarose gel electrophoresis.

3.5.4.2 Cell Transformation With recombinant Plasmids.

Competent cells were prepared from E.coli K-12 strains JM101 or JM103, using the calcium chloride method of Mandel & Higa (1970). 10 ml of L-broth (without ampicillin) was inoculated with a single colony of JM101 or JM103, and incubated, with vigorous shaking, overnight at 37°C. 1 ml of the resulting culture was used to inoculate 100 ml of L-broth, and the cells were allowed to grow at 37°C to an $O.D._{550\ nm}$ of 0.4. After chilling on ice for 10 minutes, the cells were pelleted by centrifugation in a Sorvall RC5B superspeed centrifuge (HB-4 rotor, 5,000 rpm [2,705 x g_{av}]) for 5 minutes at 4°C. The supernatant was discarded into a suitable detergent, and the cells were gently resuspended in 50 ml of an ice-cold, sterile solution of 50 mM $CaCl_2$, 10 mM Tris-HCl, pH 8.0. After 15 minutes on ice, the cells were pelleted once again by centrifugation, as described above. The supernatant was discarded, and the cells were resuspended in 3.3 ml of 50 mM $CaCl_2$, 10 mM Tris-HCl, pH 8.0, prior to dispensing in 200 ul aliquots into pre-chilled, sterile Eppendorf tubes. After incubation at 4°C for 4 hours, the cells were ready to use for transformation.

A maximum of 40 ng of DNA (either intact plasmid, or post-ligation sample) was added to each aliquot of cells, and after gently mixing by inversion, the cells were incubated on ice for 30 minutes. After this time, the cells were heat-shocked at 42°C for 2 minutes, and 1 ml of L-broth was added to each tube.

Incubation for 1 hour at 37°C, without shaking, allowed the bacteria to recover, and express antibiotic resistance. 200 ul aliquots from each tube were plated by spreading onto L-agar/ampicillin media, containing 0.004% w/v X-gal and 0.0008% w/v IPTG. After allowing the liquid to soak into the agar, the plates were inverted, and incubated at 37°C overnight. Controls included in the procedure were: (i) non-

phosphatased and phosphatased ligation controls, (ii) a transformation control, in which competent cells were transformed with intact pUC8.

3.5.4.3 Characterization Of Recombinant Plasmids.

Plasmid from white colonies (those containing a recombinant molecule) was purified using the 'miniprep' technique described in section 3.5.2. After digestion with the appropriate restriction enzyme, recombinants were analysed by agarose gel electrophoresis. The identity of the insert was confirmed by three methods: (i) determination of the size of the fragment, by comparison with molecular weight markers, (ii) restriction mapping of the insert, followed by comparison with the available nucleotide sequence, (iii) Southern blotting (see section 3.5.9).

3.5.5 Collection, Indexing And Storage Of Clinical Specimens.

Clinical specimens in the form of cervical scrapes were selected from those obtained from a large cohort of women enrolled for a study of the risk associated with HPV infection of the uterine cervix. The subjects were all well women, attending for routine cervical screening at GP's surgeries or local authority clinics in the Manchester area. At the time of their initial attendance at the clinic, a cervical scrape was taken with an Ayres spatula, for preparation of a routine Papanicolau smear. After preparing the smear, the tip of the spatula was broken off into 10 ml of filter sterilized phosphate buffered saline (PBS), pH 7.2, 0.5% SDS, in a sterile 20 ml Universal container. The two samples were sent to the Department of Cytology, at the Christie Hospital, Manchester, where they were assigned an identification number. The sample in PBS/SDS was then sent to the Department of Cytology at St. Mary's Hospital Medical School, London. Universal containers were vortexed vigorously to dislodge cells from the spatula,

which was subsequently removed and discarded. Samples were stored at -70°C, for future analysis. At intervals, the Manchester laboratory provided the results of the smear tests; each sample was assigned the appropriate cytological code(s): 1, specimen unsatisfactory; 2, normal cells only; 3, atypical cells; 4, Trichomonas vaginalis present; 5, monilia present; 6, herpes/wart virus present; 7, actinomyces present. The stored samples of 46 women, the majority of which showed signs of koilocytosis (a condition indicative of wart virus [HPV] infection) were retrieved for analysis. Cells were pelleted by centrifugation in a Sorvall RC5B (HB4 rotor, 4,000 rpm [1,730 x g_{av}]) for 2 minutes, and the supernatant was removed by aspiration. After resuspension by vortexing in 500 ul of sterile PBS, 0.5% SDS, the cell suspension was transferred to a 1.5 ml Eppendorf tube, and stored at -70°C until required. DNA was extracted from the sample as described in section 3.5.6.

3.5.6 Preparation Of DNA From Human Samples.

Frozen samples consisting of cervical cells suspended in 500 ul of PBS were thawed to room temperature, and the cells were pelleted by centrifugation (MSE Microcentaur, 13,000 rpm [13,400 x g_{av}]) for 5 minutes. The supernatant was removed with a pipette, and the cells were resuspended by vortexing gently in 500 ul of cell digestion buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM EDTA, 0.2% w/v SDS). Proteinase K was then added to a final concentration of 0.1 mg/ml, and the samples were incubated at 37°C overnight. Following digestion, samples were phenol/chloroform extracted twice, and ethanol precipitated as described in section 3.4.1, prior to resuspension in 50 ul of TE buffer, pH 8.0.

3.5.7 Radiolabelling Of DNA.

3.5.7.1 Random Hexanucleotide Priming.

DNA was labelled by random priming essentially as described by Feinberg & Vogelstein (1983,1984). Prior to labelling, DNA fragments were either purified by one of the methods described in section 3.5.3, or were prepared as described below.

The recombinant plasmid containing the insert of interest was digested with an appropriate restriction enzyme, and the resulting fragments were separated by electrophoresis through a 1% w/v LMP agarose gel. The region of agarose containing the required DNA fragment was excised as described in section 3.5.3.2, and placed in a 1.5 ml Eppendorf tube. Distilled water was added to the gel slice (3 ml H_2O/gram of wet gel), and the sample was heated in a boiling water bath for 10 minutes, in order to melt the agarose, and denature the DNA. After removing an aliquot for labelling, the remainder of the sample was stored at -20^{o}C, until required. Prior to subsequent use, frozen samples were placed in a boiling water bath for 3 minutes, and then incubated at 37^{o}C for a further 10 minutes. Pure DNA samples free of LMP agarose were denatured by heating in a boiling water bath for 10 minutes, cooled on ice for 5 minutes, and used immediately.

For labelling, 10-40 ng of DNA in a volume of 20-30 ul was transferred to a 1.5 ml Eppendorf tube, and the following were added in the given order: 10 ul of oligolabelling buffer (OLB) (200 mM HEPES, pH 6.6, 50 mM Tris-HCl, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 20 mM each of dATP, dGTP, dTTP, 150 ug/ml hexadeoxribonucleotide primer, $p(dN)_6$, 400 ug/ml BSA); 20 uCi alpha ^{32}P-dCTP (>3,000 Ci/mmole); distilled water (to make the volume up to 49 ul); 1 ul of Klenow polymerase (2 units/ul). The mixture was incubated at 19^{o}C overnight, and the labelled DNA was separated from unincorporated nucleotides by either

gel filtration (section 3.4.5), spin column chromatography (section 3.4.6) or affinity chromatography (section 3.4.7).

3.5.7.2 5' End-Labelling With T4 Polynucleotide Kinase.

Both restriction fragments and synthetic oligonucleotides were 5' end-labelled using T4 polynucleotide kinase. This enzyme catalyzes the transfer of the gamma-phosphate of a ribonucleoside 5'-triphosphate donor to the 5' hydroxyl group of the 'acceptor' nucleic acid fragment. Oligonucleotides can be labelled directly by this method, because they possess a 5' hydroxyl group; restriction fragments have a phosphate group at their 5' terminus, which must be removed before they can be used as substrates for labelling with polynucleotide kinase. This was achieved by ethanol precipitating 1 ug of DNA, and resuspending in 100 ul of dephosphorylation buffer (50 mM Tris-HCl, pH 9.0, 1 mM $MgCl_2$, 100 uM $ZnCl_2$, 1 mM spermidine). After the addition of 1 unit of CIP, the mixture was incubated at 37°C for 30 minutes. A second unit of CIP was then added, and incubation was continued for a further 30 minutes, after which time the dephosphorylated DNA was purified by affinity chromatography (section 3.4.7), and dried down in an Eppendorf tube.

For labelling, 5 pmole of DNA was resuspended in 50 ul of kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol). 20-40 uCi of gamma ^{32}P-ATP (>3,000 Ci/mmole), and 2 units of T4 polynucleotide kinase were added, prior to incubation at 37°C for 15 minutes. After this time, the reaction was chased by addition of 1 ul of non-labelled ATP solution (1 mM), and incubation at 37°C was continued for a further 15 minutes. Labelled DNA was separated from unincorporated nucleotides by one of the methods described previously (sections 3.4.5, 3.4.6, 3.4.7).

3.5.7.3 Preparation Of Labelled DNA Markers.

Markers for gel electrophoresis were prepared by end-labelling with T4 polynucleotide kinase (as described in section 3.5.7.2), or by end-repair with Klenow polymerase; in the presence of suitable deoxynucleotides, the 5'-3' polymerase activity of the Klenow fragment can be used to fill in from a recessed 3' end produced by restriction endonuclease cleavage, using the corresponding 5' extension as template.

1 ug of lambda or phiX174 DNA was digested in a volume of 10 ul with an appropriate restriction enzyme, as described in section 3.4.3. 10 ul of unlabelled nucleotide buffer (0.1 mM each dATP, dGTP, dTTP) was added to the digested DNA, followed by 20 uCi of alpha ^{32}P-dCTP (>3,000 Ci/mmole). The volume of the mixture was made up to 29 ul with deionized distilled water, and 2 units of Klenow polymerase (2 units/ul) were added, prior to incubation at room temperature for 15 minutes. Unincorporated nucleotides were removed by gel filtration (section 3.4.5). Markers commonly used were lambda/HindIII (fragment sizes: 23,130, 9,416, 6,557, 4,361, 2,322, 2,027, 564 and 125 base pairs), and phiX174/TaqI (fragment sizes: 2,914, 1,175, 404, 327, 234, 141, 87, 54, 33, and 20 base pairs).

3.5.8 Preparation And Detection Of Non-radioactive DNA Probes.

3.5.8.1 Non-radioactive Labelling Of Restriction Fragments.

Cloned restriction fragments were labelled using one of two commercially available kits. BluGENE (Gibco-BRL) uses a biotinylated probe, which is subsequently detected by addition of a streptavidin-alkaline phosphatase conjugate. The signal is developed by addition of a suitable substrate. The non-radioactive DNA labelling and detection kit (Boehringer-Mannheim) uses a probe incorporating digoxigenin-

labelled deoxyuridine triphosphate (Dig-dUTP); after hybridization of the probe to the target sequence, hybrids are detected by addition of polyclonal sheep anti-digoxigenin Fab-fragments, conjugated to alkaline phosphatase (<Dig> AP-conjugate). The signal is developed by addition of a suitable substrate. Restriction fragments were labelled using one or other of these kits, exactly as described by the manufacturer.

3.5.8.2 Non-radioactive Labelling Of Oligonucleotides.

Oligonucleotides incorporating a 5' primary amine group were synthesized as described in section 3.5.18. 40 ug of purified oligonucleotide was dissolved in 100 ul of 0.1 M sodium phosphate, pH 7.5. After the addition of 100 ul of dimethylformamide (DMF) containing 1 mg of sulfosuccinimidyl 6-(biotinamido) hexanoate (NHS-LC-biotin), the mixture was incubated at room temperature for 18 hours. Unincorporated biotin molecules were separated from the biotinylated oligonucleotide by gel filtration, as described in section 3.4.5.

3.5.8.3 Detection Of Non-radioactively Labelled Probes.

After hybridization, as described in section 3.5.14, biotin labelled probes were detected by reaction with streptavidin-horse radish peroxidase (HRP) or streptavidin-alkaline phosphatase (AP). Following washing as described in section 3.5.14, samples were re-washed in a similar manner in 1 ml of PBS (0.1 M sodium phosphate, pH 7.5, 0.15 M NaCl), before incubating at 55^{o}C for 20 minutes in 1 ml of PBS, 3% w/v BSA. The resin was pelleted by centrifugation (MSE Microcentaur, 13,000 rpm [13,400 x g_{av}]) for 2 minutes, and the supernatant was discarded. The pellet was resuspended in 100 ul of streptavidin-enzyme conjugate/PBS, 3% w/v BSA (1:75), and the mixture was incubated for 45 minutes at room temperature on a rotating Voss wheel. Excess conjugate was removed by 3 x 10 minute washes in 1 ml of PBS, at room temperature. Samples were assayed for HRP or AP activity

as described below:

(i) For assaying HRP activity only, 400 ul of o-phenylene-diamine (OPD) buffer (0.025 M citric acid, pH 5.0, 0.05 M Na_2HPO_4, 0.045% w/v OPD, 0.012% w/v H_2O_2) was added to each sample. After incubation at 37°C for 10 minutes, the colour developing reaction was terminated by addition of 200 ul of 2 M sulphuric acid. The resin was pelleted by centrifugation, and the $O.D._{492\ nm}$ of the supernatant was determined on the spectrophotometer.

(ii) For assaying AP activity only, 400 ul of nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) buffer (0.33 mg/ml NBT, 0.17 mg/ml BCIP, 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$) was added to each sample, and the mixture was incubated for 1 hour at room temperature, by rotation on a Voss wheel. The resin was pelleted by centrifugation, as described in the preceeding paragraph, and the sample was resuspended in 400 ul of ethanol. After a second 1 hour incubation, the $O.D._{650\ nm}$ of the supernatant was determined on the spectrophotometer.

3.5.9 Southern Transfer And Hybridization.

Southern transfer was performed using a modification of the technique described by Southern (1975). DNA fragments were size-fractionated by electrophoresis through 1%-2% w/v agarose gels (see section 3.4.4.1). DNA markers (HindIII digested lambda DNA, and TaqI digested phiX174) were end-labelled (as described in section 3.5.7.3), and electrophoresed alongside experimental samples. The resulting gel was photographed, and the DNA was denatured by soaking for 2 hours in 0.5 M NaOH, 1.5 M NaCl. After rinsing in 2 x SSC for 5 minutes, thorough neutralization was achieved by soaking the gel in 1 M Tris-HCl, pH 8.0, 1.5 M NaCl for 1 hour. DNA fragments were transferred

to Hybond-N (a nylon blotting membrane obtained from Amersham International) by capillary action, using the apparatus described by Southern (1975). After transfer, the gel was stained for 30 minutes in TAE buffer containing EtBr at a concentration of 0.5 ug/ml, in order to confirm that transfer of DNA from gel to membrane was complete. The membrane was then washed for 5 minutes in 2 x SSC, to remove any adhering agarose, and allowed to dry partially at room temperature for 10 minutes. The membrane was then covered with Saran Wrap, and DNA was covalently immobilized by exposure for 5 minutes to short wave u.v. light (generated by a Fotodyne model 3-3002 transilluminator). The resulting replica of the fragment pattern produced by electrophoresis was either hybridized immediately, or stored in a moist chamber at $4^{o}C$, for up to 5 days.

Membranes bearing immobilized DNA were pre-hybridized in sandwich boxes for 1-2 hours at $65^{o}C$, with shaking, in 6 x SSC, 0.5% w/v SDS, 100 ug/ml sonicated, denatured salmon sperm DNA, 5 x Denhardt's solution (1 x Denhardt's solution is 0.02% w/v Ficoll, 0.02% w/v polyvinyl pyrrolidone, 0.02% w/v BSA [Denhardt, 1966]). Moist membranes were then placed in plastic bags, which were filled with pre-hybridization buffer (approximately 5 ml per 100 cm^2 of membrane), together with single-stranded, ^{32}P-labelled DNA probe. The bags were sealed, taking care to exclude air bubbles, and incubated at $65^{o}C$, with shaking, overnight in a sandwich box filled with water. Following hybridization, excess probe was removed by washing for 3 x 1 hour at $65^{o}C$, in 0.1 x SSC, 0.1% w/v SDS; this procedure was monitored using a Mini-Instruments series 900 mini-monitor. Membranes were allowed to dry at room temperature, and were covered in Saran Wrap, prior to autoradiography as described in section 3.4.9.

3.5.10 Slot-blotting And Hybridization.

Slot-blots were prepared by a modification of the method described by Wickenden et al. (1987 a), using a 72 well suction manifold supplied by Schleicher & Schuell GmbH, Dassel, W. Germany. A Hybond-N nylon hybridization membrane was moistened with 0.5 x SSC, and placed in position in the manifold, exactly as recommended by the manufacturer. After application of vacuum suction, each of the 72 slots was washed with 200 ul of 0.5 x SSC, prior to loading of samples (made up to a volume of 200 ul in 0.5 x SSC). As the liquid is drawn by the vacuum through the Hybond-N, any DNA present is deposited on the surface of the membrane in a limited area defined by the well. Once the liquid had disappeared from each well, the membrane was removed from the manifold, and the captured DNA was denatured by immersion for 30 seconds in 0.5 M NaOH, 1.5 M NaCl. After rinsing for 30 seconds in 0.5 x SSC, neutralization was achieved by immersion for 30 seconds in 0.5 M Tris-HCl, pH 7.5, 1 M NaCl. After a final rinse for 30 seconds in 2 x SSC, the membrane was covered with Saran Wrap, and the DNA was covalently immobilized onto the surface by irradiation with short wave u.v. light (generated by a transilluminator) for 5 minutes. Membranes were then pre-hybridized and hybridized exactly as described in section 3.5.9, except that the buffer used consisted of 0.08 M Tris-HCl, pH 7.8, 0.75 M NaCl, 0.05% w/v SDS, 0.004 M EDTA, 5 x Denhardt's solution, 100 ug/ml denatured, sonicated salmon sperm DNA.

3.5.11 Immobilization Of DNA Onto Diazotized Resins.

The immobilization of DNA to diazotized resins, described by Bunemann et al. (1982) consists of two stages: firstly, the covalent attachment of an aromatic amino compound (2-aminothiophenol) to the resin, forming a 2-aminophenylthioether (APTE) derivative. This is

followed by diazotization of the amino function, forming a diazophenylthioether (DPTE) derivative. This can covalently bond to denatured DNA, via the bases. The chemistry of this procedure is illustrated in figure 9.

3.5.11.1 Preparation Of 2-aminophenylthioether (APTE) Derivative Of Resin.

All manipulations involving 1,4-butanediol diglycidyl ether and 2-aminothiophenol were performed in a fume hood. 20 g batches of resin were washed with distilled water, and transferred to a 50 ml sterile Falcon tube. 20 ml of 1 M NaOH and 1.7 ml of 1,4-butanediol diglycidyl ether were added, and the contents of the tube were mixed by overnight rotation on a Voss wheel, at room temperature. The slurry was transferred to a Buchner funnel, and the liquid was removed as thoroughly as possible, without allowing the resin to dry completely. The moist cake was placed in a clean 50 ml Falcon tube. 23 ml of acetone and 2.86 ml of 2-aminothiophenol were added, and the contents of the tube were mixed once again by rotation overnight at room temperature. Finally, the derivatized resin was transferred to a Buchner funnel, and washed successively with 200 ml each of acetone, 0.1 M HCl, water, 0.1 M HCl, and water again. This 2-aminophenylthioether (APTE) derivative was suspended in sterile distilled water, and stored at $4^{o}C$ until required, or for a maximum of one year.

3.5.11.2 Preparation Of Diazophenylthioether (DPTE) Derivative Of Resin.

1 g of APTE-resin was washed in a sintered glass funnel with 30 ml of distilled water, and resuspended in 1 ml of water. 3.33 ml of 1.8 M HCl were added, and the mixture was incubated on ice for 30 minutes. A fresh solution of 10 mg/ml sodium nitrite was prepared, and a 33 ul aliquot was added to the APTE-resin once every seven minutes. After

each addition, the mixture was gently shaken, and returned to the ice bath. The progress of the reaction was monitored by removing 50 ul from the Falcon tube, and pipetting onto starch iodide paper. Addition of sodium nitrite was continued until the starch iodide paper test became positive (the paper changed from white to dark blue). The slurry was then quickly transferred to an ice-cold sintered glass filter, and washed immediately with 50 ml of ice-cold distilled water, followed by 5 ml of an ice-cold mixture of 25 mM sodium phosphate, pH 6.0 and dimethyl sulphoxide (DMSO) (20:80 v/v). The majority of the moisture was removed from the DPTE-resin (without allowing it to dry completely), and 500 mg was transferred to a 1.5 ml Eppendorf tube.

3.5.11.3 Coupling Of DNA To DPTE Derivative Of Resin.

The DNA to be immobilized was dissolved in 50 ul of 25 mM sodium phosphate, pH 8.0. Immediately prior to immobilization, 200 ul of DMSO was added to the DNA solution, which was then added to the DPTE-resin. DNA denaturation occurs instantaneously on addition of DMSO, so it is not necessary to boil the DNA sample prior to immobilization. The reaction mixture was incubated at room temperature for 48 hours, with constant rotation on a Voss wheel. The DNA-resin was washed in a sintered glass funnel with 50 ml of distilled water, followed by 50 ml of 0.4 M NaOH, at 40^{o}C. Following a final wash with 100 ml of TE buffer at room temperature, the DNA-resin was transferred to a 1.5 ml Eppendorf tube, and stored at 4^{o}C until required, or for a maximum of three months.

3.5.12 Immobilization Of DNA Onto Carbodiimide Activated Resins.

DNA was immobilized via the 5' end using 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene sulfonate (carbodiimide). The chemistry of this procedure is illustrated in

figure 30. The DNA to be immobilized was resuspended in 100 ul of 40 mM sodium MES buffer, pH 6.0, and denatured by heating in a boiling water bath for 10 minutes. 100 mg aliquots of resin were washed in 40 mM MES, pH 6.0, and pelleted by centrifugation (MSE Microcentaur, 13,000 rpm [13,400 x g_{av}]) for 2 minutes. DNA samples were then added to the resin in 1.5 ml screw cap Eppendorf tubes, and the mixture was dried for 2 hours in a Speed Vac concentrator, prior to activation with carbodiimide. 28 ul of freshly prepared carbodiimide solution (80 mg/ml of water) was added to the dried resin, and the mixture was heated in a boiling water bath for 7 minutes, prior to cooling for 5 minutes in a dry ice/ethanol bath. A further 28 ul of 80 mg/ml carbodiimide solution (this time dissolved in 40 mM MES, pH 6.0) was added, and the heating/rapid cooling procedure was repeated. Finally, 140 ul of swelling buffer (10 mM sodium phosphate, pH 7.0, 1 mM EDTA, 0.1 M NaCl) was added to the sample, which was incubated at 4°C for 16 hours. Following incubation, the suspension was transferred to a 15 ml polypropylene centrifuge tube, and the resin was pelleted by centrifugation in the Sorvall RC5B (GSA rotor, 10,000 rpm [10,240 x g_{av}]) for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 4 ml of 0.2 M $NaHCO_3$, pH 10.2. Following mixing by rotation on a Voss wheel for 16 hours, the resin was pelleted as described above, and washed by rotation for 3 x 1 hour in 1 ml of swelling buffer. DNA-resin was stored in TE, pH 8.0 at 4°C, for up to 6 months.

3.5.13 Quantitation Of Immobilized DNA.

The amount of DNA covalently coupled to the resin was determined by two methods.

3.5.13.1 Radiolabelled Tracer.

Prior to immobilization, a sample of DNA was ^{32}P end-labelled with T4 polynucleotide kinase, as described in section 3.5.7.2, to an activity of around 5 x 10^2 dpm/fmole. At the time of setting up the immobilization reaction, a control tube was included which involved addition of 1-2 fmole of radiolabelled tracer. The radioactivity of the control sample was ascertained by Cerenkov counting (Berger & Krug, 1985), prior to washing as described in section 3.5.11.3. After unbound DNA had been removed, the sample was recounted, and the percentage of the input DNA molecules immobilized was determined.

3.5.13.2 Micrococcal Nuclease Assay.

Nuclease assays were carried out essentially as described by Bunemann et al. (1982), using micrococcal nuclease isolated from Staphylococcus aureus. 50 mg aliquots of DNA-resin were washed in a sintered glass funnel with 20 ml of 0.1 M sodium borate, pH 8.8, and transferred to 1.5 ml Eppendorf tubes. Each aliquot was resuspended in 400 ul of 0.05 M sodium borate, pH 8.8, and 15 ul of 0.1 M $CaCl_2$. S.aureus nuclease was added to a concentration of 300 units/ml, and the mixture was incubated at 37°C overnight. After centrifugation (MSE Microcentaur, 13,000 rpm [13,400 x g_{av}]) for 5 minutes, the amount of immobilized DNA was calculated from the absorbance of the supernatant at 260 nm. Nuclease digested DNA has an absorbance at this wavelength approximately 1.6 times that of intact DNA (Bunemann et al., 1982); results were adjusted accordingly.

3.5.14 Hybridization Of Immobilized DNA.

Two types of hybridization assay were routinely performed: the one-step assay, for determining the availability of immobilized DNA for hybridization, and the two-step (or sandwich) assay, for detecting the presence or absence of the 'target' nucleic acid. The conditions

described are for the use of labelled restriction fragments as probes; modifications for the use of oligonucleotide probes are listed at the end of each section.

3.5.14.1 One-step Assay.

One-step hybridizations were carried out in 1.5 ml screw cap Eppendorf tubes, which were constantly rotated on a Voss wheel, in a fixed temperature fan controlled Gallenkamp incubator. DNA-resin corresponding to 0.1-0.5 pmole of immobilized DNA was aliquoted into Eppendorf tubes, using a Gilson pipette. Most reproducible manipulation of small volumes of resin was achieved using yellow Gilson tips, which had approximately 3 mm cut from the narrow end with a sharp scalpel blade. DNA-resin was pre-hybridized for 2 hours at 65^{o}C, in 1 ml of a buffer consisting of 40 mM PIPES pH 6.5, 0.6 M NaCl, 1 mM EDTA, 0.1% w/v SDS and 250 ug/ml denatured, sonicated salmon sperm DNA. The resin was then pelleted by centrifugation (MSE Microcentaur, 13,000 rpm [13,400 x g_{av}]) for 2 minutes, and the supernatant was removed by aspiration. Labelled probe DNA was denatured by heating in a boiling water bath for 10 minutes, and cooled on ice for a further 10 minutes. 5-50 fmole of probe was then added to each Eppendorf tube, together with 50 ul of pre-hybridization buffer. After incubation at 65^{o}C for 1 hour, the resin was pelleted as described above, and washed for 3 x 10 minutes at 65^{o}C in 1 ml of pre-hybridization buffer (without sonicated, denatured salmon sperm DNA). All washes were changed by pelleting the resin by centrifugation, and removing the supernatant by aspiration. The radioactivity of samples was determined, using the Cerenkov method, in an LKB model 1214 Rackbeta liquid scintillation counter (Berger & Krug, 1985). Two controls were always included in each batch of assays: (i) a control in which DNA-resin is replaced with DPTE-resin; (ii) a control in which the labelled probe is non-specific (sonicated calf

thymus DNA, for example).

One-step assays with oligonucleotides were performed in a similar manner, except for the following modifications: (i) pre-hybridization and hybridization were performed at 65°C, in a buffer consisting of 6 x SSC, 0.1% w/v SDS, 250 ug/ml denatured, sonicated salmon sperm DNA; (ii) denaturation of probe prior to hybridization was not necessary; (iii) samples were washed at 65°C in 6 x SSC, 0.1% w/v SDS.

3.5.14.2 Two-step Assay.

Two-step hybridizations were performed as described for one-step assays, except for the following modifications: (i) the amount of DNA-resin used per assay corresponded to 0.1-0.2 pmole of immobilized DNA; (ii) denatured 'target' (or sample) DNA was added immediately prior to addition of the labelled probe; (iii) hybridizations were carried out for 12-16 hours (for labelled restriction fragment probes), of for 8 hours (oligonucleotide probes).

3.5.15 Oligonucleotide Synthesis.

Oligonucleotides were manufactured on an Applied Biosystems Model 381A DNA synthesizer, on a 0.2 micromole scale, using beta-cyanoethyl phosphoramidites. The chemistry of the procedure is shown in figure 11. At the end of each synthesis, the dimethoxytrityl group was left in place at the 5' end of the oligonucleotide.

3.5.16 Oligonucleotide Purification.

Manual oligonucleotide deprotection, and cleavage from the controlled pore glass (CPG) support was achieved by treatment with a fresh solution of concentrated ammonium hydroxide. The cartridge containing the CPG resin and completed oligonucleotide was removed from

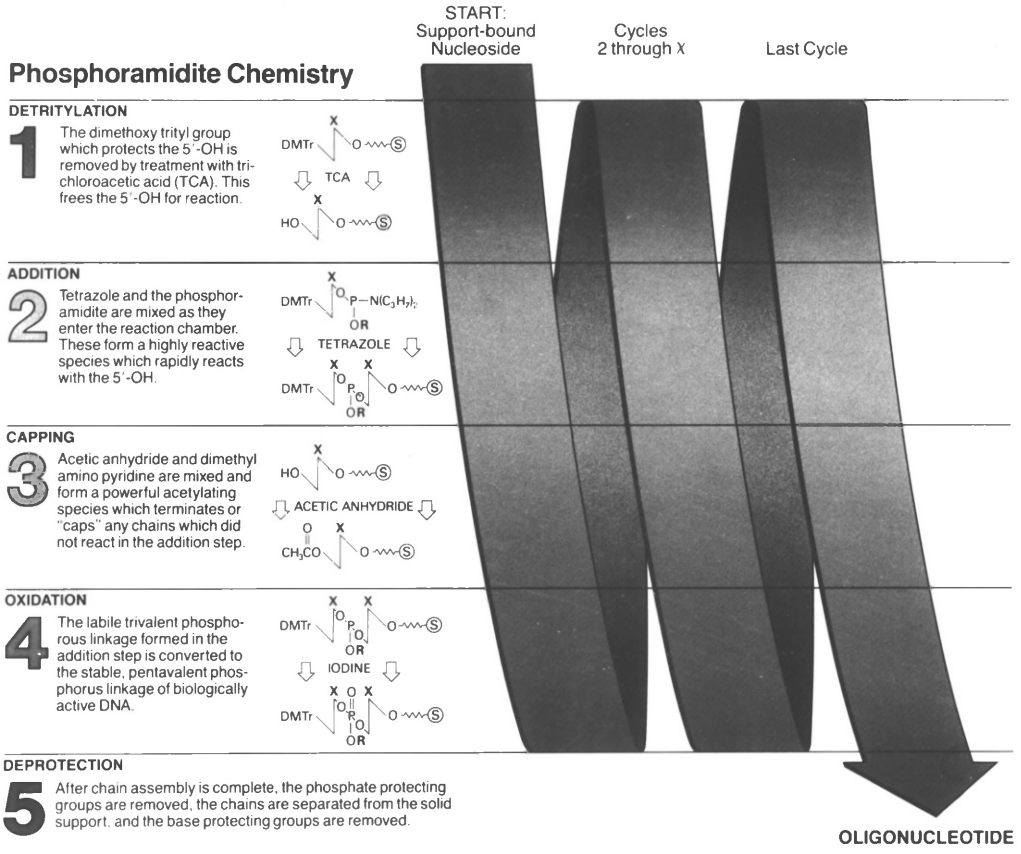


FIGURE 11

THE PRINCIPLE OF PHOSPHORAMIDITE CHEMISTRY FOR THE SYNTHESIS OF OLIGONUCLEOTIDES.
(Reproduced with permission from Applied Biosystems Ltd., Cheshire, U.K.).

the DNA synthesizer. A 5 ml syringe (A) was filled with 2 ml of ammonium hydroxide, and the tip was inserted into the top of the cartridge. A second syringe (B), with the plunger fully depressed, was placed in a similar position, at the bottom of the cartridge. Over the course of 1 hour, the plunger of syringe 'A' was very slowly depressed, while the plunger of syringe 'B' was withdrawn at the same rate. The slow stream of fresh ammonium hydroxide results in cleavage of the base-labile ester linkage between the CPG support and the 3' hydroxyl group of the initial nucleoside; it also results in removal of beta-cyanoethyl phosphate protecting groups. The eluted DNA was collected in syringe B, and transferred to a glass vial with a Teflon-lined cap, prior to incubation at 55^{o}C for 15 hours. This final incubation results in removal of protecting groups from the exocyclic amines of nucleotides A, G and C.

After deprotection and cleavage was complete, the ammonium hydroxide solution containing the oligonucleotide was cooled to room temperature, and full length synthetic DNA strands were purified and desalted, as described by the manufacturer, on an Applied Biosystems oligonucleotide purification cartridge (OPC). 20 ug fractions of pure oligonucleotide in 20% acetonitrile solution were aliquoted into 1.5 ml Eppendorf tubes, evaporated to dryness, and stored at -20^{o}C until required.

3.5.17 The Polymerase Chain Reaction (PCR).

Specific target sequences were enzymatically amplified using a modification of the technique described by Saiki *et al.* (1985 a). Appropriate oligonucleotide primers, each 20 nucleotides in length, were synthesized and purified as described in sections 3.5.15 and 3.5.16. Sample DNA, in a volume of approximately 5 ul of TE buffer, pH

8.0, was placed into a 1.5 ml screw cap Eppendorf tube, and diluted with 50 ul of a buffer consisting of 10 mM Tris-HCl pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 200 uM each of dATP, dCTP, dGTP and dTTP, 1 uM of each oligonucleotide primer, 100 ug/ml gelatin and 1 unit/25 ul of Thermus aquaticus (Taq) DNA polymerase. 50 ul of light mineral oil (obtained from Sigma) was used to overlay samples, which were loaded into an intelligent heating block (supplied by Hybaid Ltd., Sussex, England). Sample DNA was denatured by heating to 95°C for 7 minutes, and the oligonucleotide primers were allowed to anneal to their target sequences by cooling to 55°C, followed by incubation at this temperature for a further 2 minutes. Polymerase-mediated elongation of the annealed primers was achieved by heating the sample to 70°C, and maintaining this temperature for a further 4 minutes. For subsequent cycles, denaturation, primer annealing and polymerase-mediated elongation were performed at the temperatures indicated above, for 1, 0.5 and 4 minutes respectively. The number of cycles performed varied from 10 to 50. After amplification, samples were analyzed by electrophoresis on NuSieve GTG agarose gels (section 3.4.4.1), and/or by sandwich hybridization (section 3.5.14.2).

3.5.18 Amplified Signal Affinity Capture (ASAC).

Oligonucleotide pairs for amplified signal affinity capture (ASAC) were synthesized as described in section 3.5.15, except that a 'tail' of four 'A' residues, followed by a single Aminolink-2 residue, was added to the 5' end during synthesis. The chemical structure of Aminolink-2, a methyl phosphoramidite analogue, is shown in figure 12. After deprotection and cleavage in concentrated ammonium hydroxide, ASAC oligonucleotides were purified as described in section 3.5.16. The resulting oligonucleotides are identical to those synthesized using

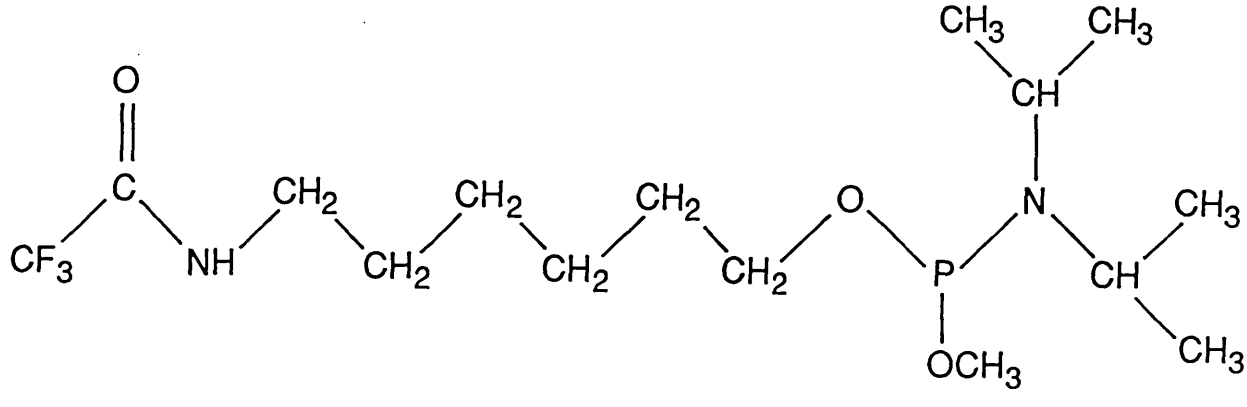


FIGURE 12

THE CHEMICAL STRUCTURE OF AMINOLINK 2.
(Reproduced with permission from Applied Biosystems, Ltd., Cheshire, U.K.).

conventional phosphoramidites, except for the presence of a $-(CH_2)_6-NH_2$ group at the 5' terminus; the primary amine can be used to couple molecules such as biotin, or other labels involved in non-radioactive nucleic acid detection systems. Aminolink-modified oligonucleotides were coupled to sulfosuccinimidyl 6-(biotinamido) hexanoate (NHS-LC-biotin), as described in section 3.5.8.2, prior to removal of unincorporated biotin by gel filtration (section 3.4.5). Biotinylated oligonucleotides were then used in PCR amplification (section 3.5.17), to produce products which were biotinylated at the 5' end.

After the final cycle of PCR, 5 ul of the reaction volume was removed, and transferred to a clean 1.5 ml screw cap Eppendorf tube, prior to denaturing by heating for 10 minutes in a boiling water bath. After cooling on ice for 5 minutes, the sample was diluted by addition of 15 ul of hybridization buffer (8 x SSC, 0.13% w/v SDS, 0.13 mg/ml BSA). 50 fmole of kinase end-labelled probe oligonucleotide (approximately 5 x 10^2 dpm/fmole) was added to the sample, and the mixture was hybridized at 65^{o}C, for 30 minutes. After this time, 100 ul of buffer 1 (10 mM sodium phosphate, pH 7.5, 1 mM EDTA, 1 M NaCl) was added, followed by 50 ul of a 50% v/v suspension of streptavidin-agarose in buffer 1. Biotinylated PCR product/probe hybrids were captured by incubation at 37^{o}C for 15 minutes, with constant mixing on a Voss rotating wheel. The resin was pelleted by centrifugation (MSE Microcentaur, 13,000 rpm [13,400 x g_{av}]) for 30 seconds, and the supernatant was discarded. After resuspending in 1 ml of buffer 1, the streptavidin-agarose was washed by rotation on the Voss wheel at 37^{o}C for 5 minutes. After two further washes in the same buffer, the resin was pelleted, the supernatant was removed, and the radioactivity of the sample was determined by Cerenkov counting in an LKB model 1214 Rackbeta liquid scintillation counter (Berger & Krug, 1985). A

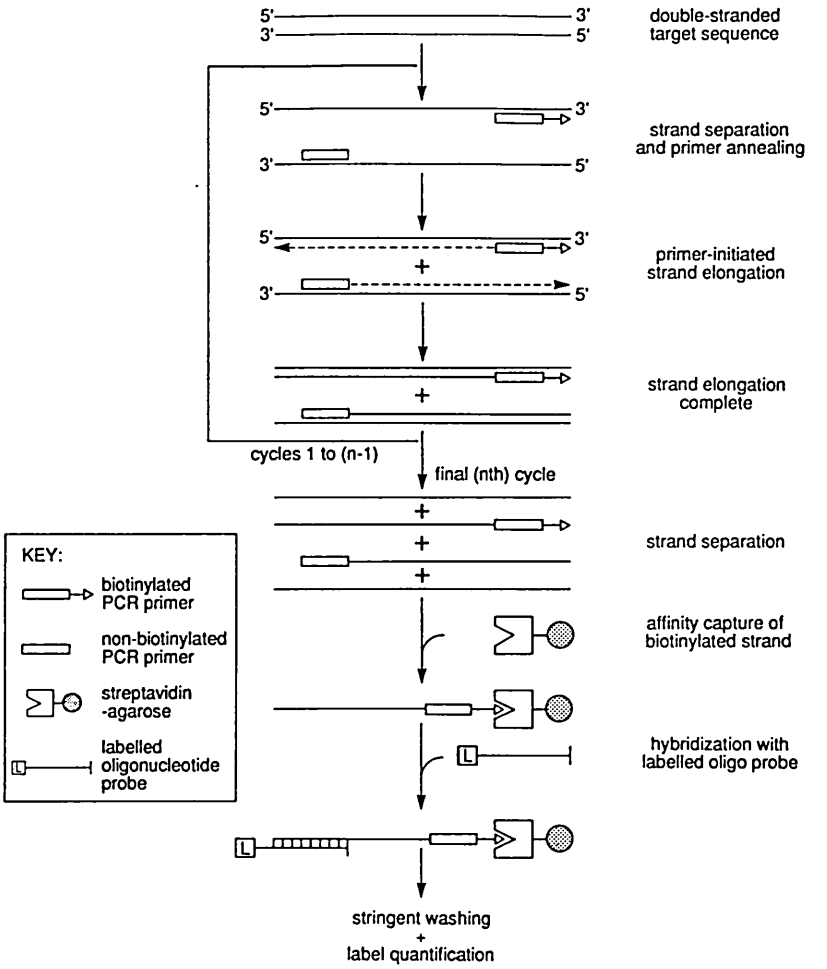


FIGURE 13

PRINCIPLE OF AMPLIFIED SIGNAL AFFINITY CAPTURE (ASAC).

diagrammatic representation of ASAC is shown in figure 13.

4. RESULTS.

During the course of this investigation, there were a number of stages at which it became obvious that a particular technique or component associated with the sandwich assay was superior to the others tested. In such circumstances, the inferior options were discarded, and only the most suitable were retained.

4.1 Restriction Enzyme Analysis Of HPV Types 6b, 11, 16 And 18.

The initial aim of this study was to develop a nucleic acid-based diagnostic assay for the detection of HPV infection in clinical samples, using the sandwich hybridization technique. Although HPV types 6, 11, 16, 18, 31, 33, 35 and 39 have all been associated with lesions of the cervix, the first four types listed above were chosen for this study for the following reasons: i) Current evidence suggests that HPV 6 and HPV 11 are predominantly associated with low-grade lesions, whereas all of the other types listed have been connected with malignant carcinomas (Wickenden et al., 1987 c). For this reason, it could be of diagnostic importance to be able to distinguish between infections involving types 6 and 11, and those involving different types of the virus; ii) The DNA detected in approximately 50% of tumours harbouring HPV nucleic acid is that of type 16, with HPV 18 being found in the majority of the remainder (zur Hausen, 1987); iii) The genomes of virus types 6b, 11, 16 and 18 are readily available as clones in the plasmid pBR322; iv) Full sequence data, and extensive analysis of genomic structure are available for these four virus types.

As discussed in section 1.3, a fundamental difference between sandwich hybridization and conventional blotting procedures is that the former requires two nucleic acid probes derived from the genome of the organism of interest, whereas the latter utilizes a single probe. Many

of the studies designed for examining the prevalence of cervical HPV infection have used either Southern blotting, or one of the rapid hybridization techniques discussed in section 1.1.2. Consequently, it is common to find that the complete genome of the HPV type of interest is used as a hybridization probe, rather than a specific sub-genomic fragment. Clearly, in order to develop a sandwich assay, it was essential to isolate at least two non-overlapping fragments from the genome of HPV types 6b, 11, 16 and 18. One of these fragments (A) could then be immobilized, and the other (B) suitably labelled for use as a hybridization probe.

The genomes of HPV types 11 (7,931 bp), 16 (7,904 bp) and 18 (7,857 bp) were obtained as complete genomes in plasmid pBR322. A 5,368 bp EcoRI/BamHI sub-genomic fragment of type 6b was also obtained as a clone in pBR322. All four constructs were the kind gift of Colin Wickenden, and were initially obtained from Professor Harald zur Hausen, of the Institut Fur Virologie, Heidelberg, W. Germany.

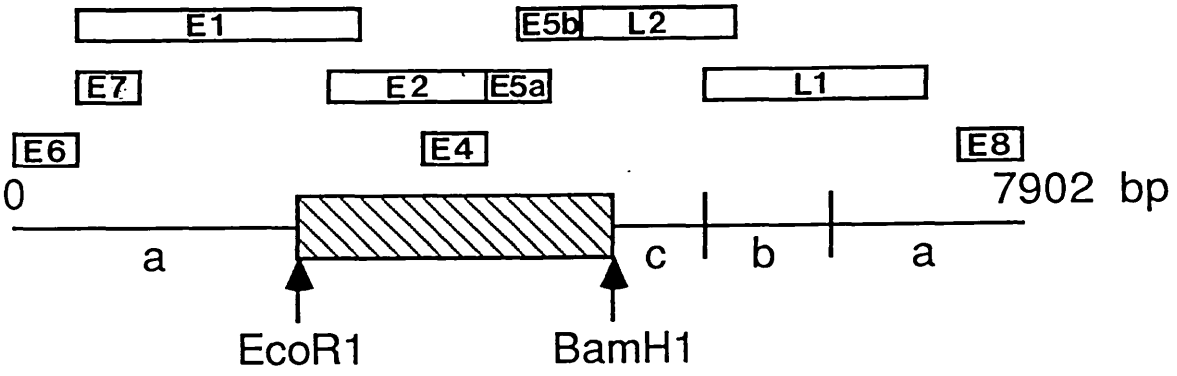
Full genomic sequences for HPV types 6b (Schwarz et al., 1983), 11 (Dartmann et al., 1986), 16 (Seedorf et al., 1985 a) and 18 (Cole & Danos, 1987) were retrieved from the European Molecular Biology Laboratory (EMBL) database; the data obtained included the nucleotide positions flanking the putative open reading frames. The retrieved sequences were then analysed with 'CUTSIT', a program designed to identify recognition sites for the most common restriction enzymes. One of the considerations in the choice of fragments for use in the sandwich assay was one of restriction enzyme cost. Since an aim of this study was to develop an assay which could be used for the analysis of a large number of samples, it was reasonable to assume that the large scale preparation of the appropriate restriction fragments would eventually be a necessity. For this reason, the fragment sizes produced

by digestion with the cheapest restriction enzymes were examined to find suitable fragment pairs for use in sandwich hybridization. The genomes of HPV types 11 and 16 contain a single recognition site for BamHI, and the covalently closed, circular molecules were originally linearized at this point, prior to cloning into the BamHI site of pBR322. Similarly, the HPV 18 genome contains a single site for EcoRI, and was consequently cloned into the EcoRI site of pBR322. HPV 6b contains a single recognition site for both BamHI and EcoRI, and digestion with both of these enzymes produces fragments 5,368 bp and 2,534 bp in length. The larger of the fragments was cloned into BamHI/EcoRI digested pBR322. Because of the infrequent occurrence of recognition sites, these two enzymes were not capable of producing a variety of genomic sub-fragments suitable for use in the sandwich assay. The next enzyme to be considered was PstI; on examination of the positions of the recognition sites for this enzyme in the genome of the four HPV types of interest, it was immediately apparent that a variety of fragment sizes ideal for development of the assay were produced. The fragment sizes produced by PstI digestion of the HPV 6b 5,368 fragment are shown in Figure 14. Digestion of the genome of virus types 11, 16 and 18 generates the fragment sizes listed in Figures 15, 16 and 17 respectively.

Each of the plasmids containing an HPV-derived insert was used to transform E.coli K-12 HB101 cells, as described in section 3.5.4.2. This was followed by large scale plasmid preparation and purification, as described in section 3.5.1. HPV insert was subsequently purified from vector sequences by electroelution, as described in section 3.5.3.1, with a yield of approximately 30% (see section 4.2). After digestion of 1 ug of purified DNA with PstI (section 3.4.3), the resulting fragments were size fractionated by electrophoresis through

FIGURE 14

THE GENOMIC ORGANIZATION OF HPV TYPE 6b



= Restriction fragment not included in clone.

GENOME SIZE:	7902 bp
SIZE OF CLONE IN pBR322:	5368 bp
ORIGIN OF CLONE IN pBR322, NUCLEOTIDES:	4722-2188
NUCLEOTIDE POSITION OF BamHI SITE:	4722
NUCLEOTIDE POSITION OF EcoRI SITE:	2188
NUCLEOTIDE POSITIONS OF PstI SITES:	3912, 5408, 6489.
SIZES OF PstI RESTRICTION FRAGMENTS:	a. 3601 bp b. 1081 bp c. 686 bp

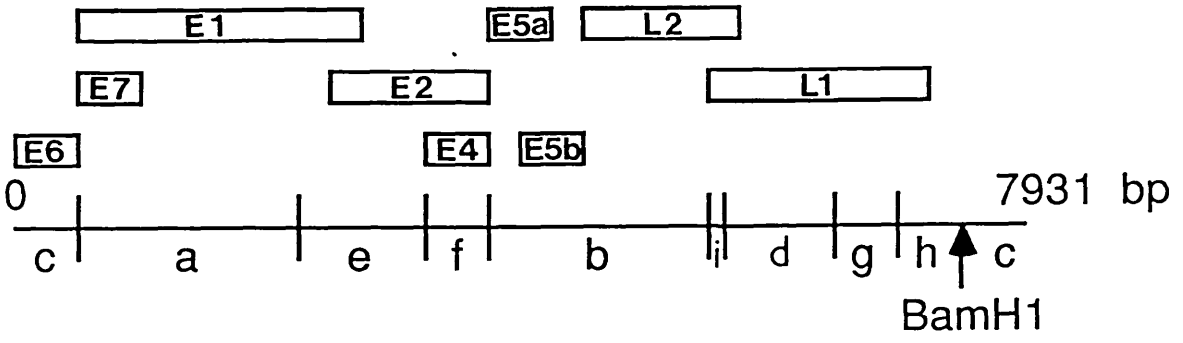
NUCLEOTIDE POSITIONS AND SIZE OF OPEN READING FRAMES (ORFs):

ORF	NUCLEOTIDES	SIZE (bp)
E6	30- 551	522
E7	440- 823	384
E1	715-2778	2064
E2	2696-3826	1131
E4	3240-3581	342
E5a	3872-4159	288
E5b	4003-4374	372
L2	4378-5799	1422
L1	5678-7288	1611
E8	7611- 2	294

Nucleotide position "1" determined by alignment with the "G" residue of the HpaI restriction site (GTTAAC) of bovine papillomavirus type 1.

FIGURE 15

THE GENOMIC ORGANIZATION OF HPV TYPE 11



GENOME SIZE:	7931 bp
SIZE OF CLONE IN pBR322:	7931 bp
NUCLEOTIDE POSITION OF BamHI SITE:	7072
NUCLEOTIDE POSITIONS OF PstI SITES:	572, 2346, 3137, 3896, 5399, 5432, 6474, 6909.
SIZES OF PstI RESTRICTION FRAGMENTS:	a. 1774 bp b. 1503 bp c. 1431 bp d. 1042 bp e. 791 bp f. 759 bp g. 435 bp h. 163 bp i. 33 bp

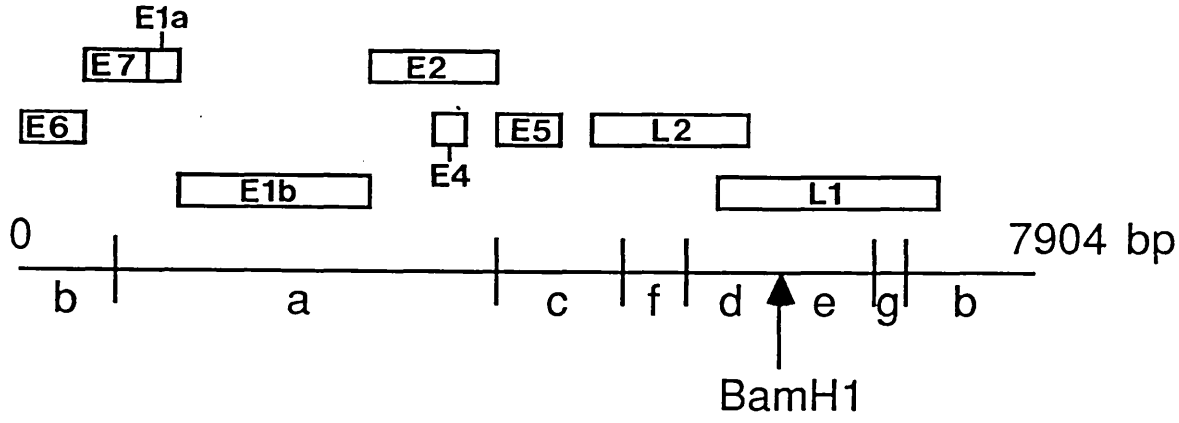
NUCLEOTIDE POSITIONS AND SIZE OF OPEN READING FRAMES (ORFs):

ORF	NUCLEOTIDES	SIZE (bp)
E6	18- 552	534
E7	494- 824	330
E1	715-2779	2064
E2	2696-3824	1128
E4	3231-3579	348
E5a	3862-4144	282
E5b	3987-4368	381
L2	4408-5782	1374
L1	5660-7274	1614

Nucleotide position "1" determined by alignment with the "G" residue of the HpaI restriction site (GTTAAC) of bovine papillomavirus type 1.

FIGURE 16

THE GENOMIC ORGANIZATION OF HPV TYPE 16.



GENOME SIZE: 7904 bp

SIZE OF CLONE IN pBR322: 7904 bp

NUCLEOTIDE POSITION OF BamHI SITE: 6150

NUCLEOTIDE POSITIONS OF PstI SITES: 875, 3692, 4755, 5238, 6787, 7003.

SIZES OF PstI RESTRICTION FRAGMENTS:

a. 2817 bp
b. 1776 bp
c. 1063 bp
d. 912 bp
e. 637 bp
f. 483 bp
g. 216 bp

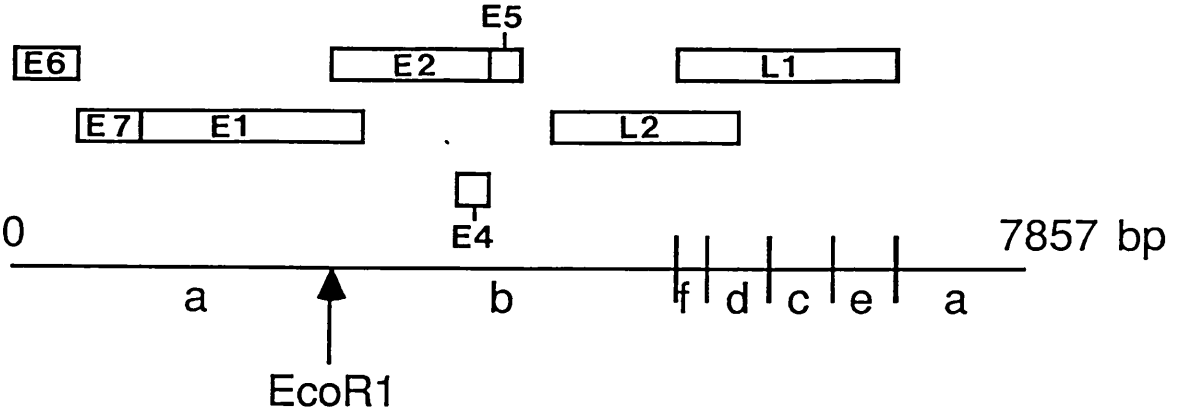
NUCLEOTIDE POSITIONS AND SIZE OF OPEN READING FRAMES (ORFs):

ORF	NUCLEOTIDES	SIZE (bp)
E6	65- 556	39
E7	544- 855	312
E1a	859-1167	309
E1b	1104-2810	1707
E2	2725-3849	1124
E4	3332-3616	285
E5	3863-4096	234
L2	4133-5653	1521
L1	5526-7151	1626

Nucleotide position "1" determined by alignment with the "G" residue of the HpaI restriction site (GTTAAC) of bovine papillomavirus type 1.

FIGURE 17

THE GENOMIC ORGANIZATION OF HPV TYPE 18.



GENOME SIZE:	7857 bp
SIZE OF CLONE IN pBR322:	7857 bp
NUCLEOTIDE POSITION OF EcoRI SITE:	2440
NUCLEOTIDE POSITIONS OF PstI SITES:	5246, 5313, 5770, 6325, 6766.
SIZES OF PstI RESTRICTION FRAGMENTS:	a. 3531 bp b. 2806 bp c. 555 bp d. 457 bp e. 441 bp f. 67 bp

NUCLEOTIDE POSITIONS AND SIZE OF OPEN READING FRAMES (ORFs):

ORF	NUCLEOTIDES	SIZE (bp)
E6	87- 578	492
E7	509- 904	396
E1	908-2884	1977
E2	2796-3911	1116
E4	3405-3681	277
E5	3915-4154	239
L2	4166-5629	1464
L1	5418-7133	1716

Nucleotide position "1" determined by alignment with the "G" residue of the HpaI restriction site (GTTAAC) of bovine papillomavirus type 1.

a 1% agarose gel (section 3.4.4.1). The results of the electrophoresis (shown in Figure 18) clearly demonstrate that digestion of the HPV DNA results in the production of fragments of the predicted size. The faint band of around 4.4 kb present in lanes 3 and 4 could be due to: (i) contaminating vector sequences present in the purified HPV DNA; (ii) incomplete digestion of the HPV DNA.

4.2 Purification Of HPV Restriction Fragments.

It was necessary to purify restriction fragments for two reasons: (i) for use as immobilized capture probe (fragment A) or labelled probe (fragment B) in a sandwich assay; (ii) for sub-cloning the appropriate sub-genomic fragments derived from the HPV types of interest.

Three methods for the purification of specific nucleic acid fragments from contaminating sequences were investigated: (i) electro-elution; (ii) recovery by phenol extraction from low melting point (LMP) agarose; (iii) recovery from normal agarose, using Nensorb 20 affinity chromatography columns. Results obtained with restriction fragments varying in length from around 0.1 to 8 kb suggested that, for the purification of relatively large amounts of DNA (that is, more than 10 ug), electroelution resulted in the highest yield, usually in the range of 30%-50%. Yields at the higher end of this range were regularly achieved when care was taken to rinse the inner surfaces of the dialysis tubing with TE buffer at the end of the procedure. Occasion-ally, contaminants in the recovered material became apparent as sharp peaks at around 280 nm on scanning from 320 nm to 220 nm in the u.v. spectrophotometer. Contaminants from agarose have been reported to inhibit the activity of restriction enzymes and T4 DNA ligase (although the effect is much less apparent on Klenow polymerase [Feinberg &

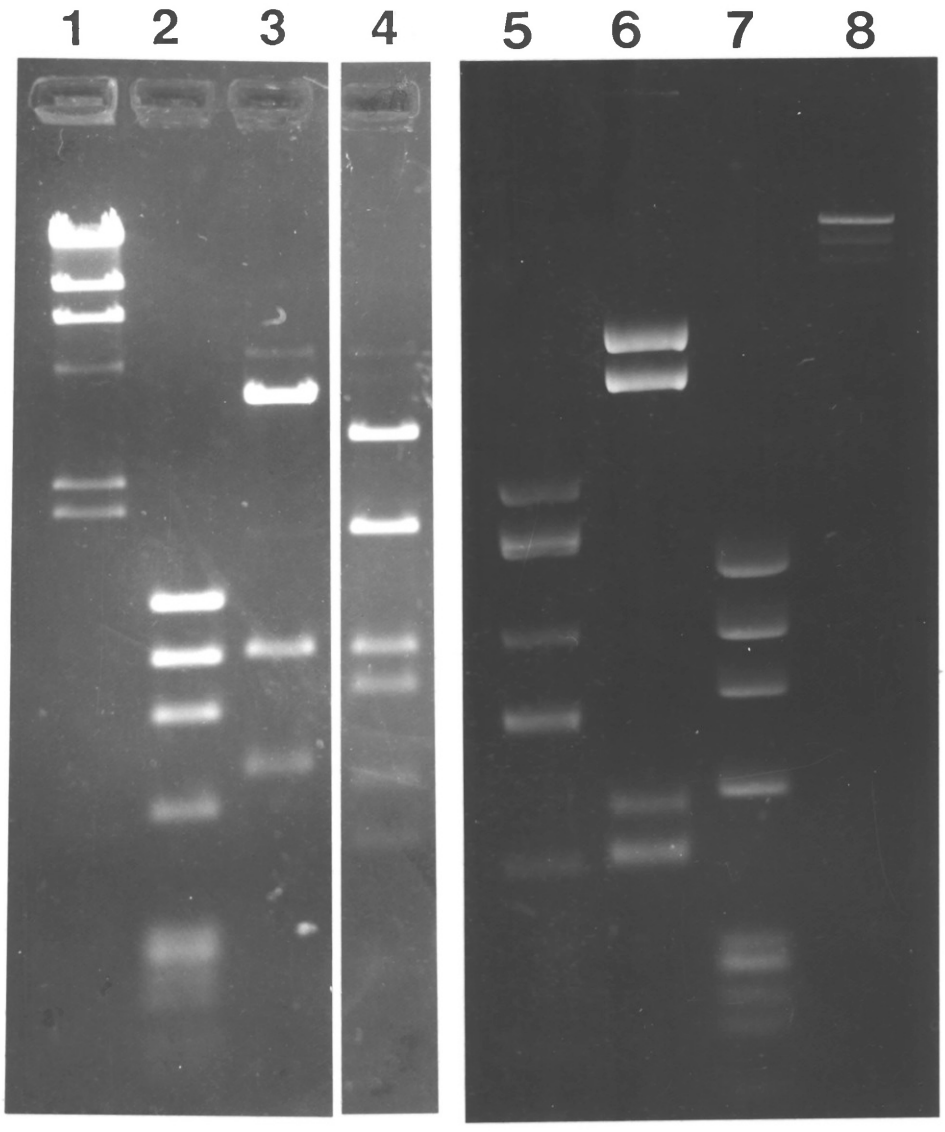


FIGURE 18

Restriction enzyme analysis of cloned DNA originally isolated from HPV types 6b, 11, 16 and 18.

Lanes 1 and 8 contain 1 µg of lambda-HindIII markers (fragment sizes 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.6 and 0.1 kb). Lanes 2 and 7 contain 1 µg of øX174-HaeIII markers (fragment sizes 1,353, 1,078, 872, 605, 310, 281, 271, 234, 194, 118 and 72 bp). Lane 3 contains 1 µg of HPV 6b 5,368 bp EcoRI-BamHI fragment, digested with the enzyme PstI (fragment sizes 3,601, 1,081 and 686 bp). Lane 4 contains 1 µg of HPV 16 DNA (7,904 bp) digested with PstI (fragment sizes 2,817, 1,776, 1,063, 912, 637, 483 and 216 bp). Lane 5 contains 1 µg of HPV 11 DNA (7,931 bp) digested with PstI (fragment sizes 1,774, 1,503, 1,431, 1,042, 791, 759, 435, 163 and 33 bp). Lane 6 contains 1 µg of HPV 18 DNA (7,857 bp) digested with PstI (fragment sizes 3,531, 2,806, 555, 457, 441 and 67 bp). Lanes 1-4 and 5-8 represent results from two separate agarose gels.

The results are discussed fully in the text.

Vogelstein, 1983]). Consequently, contaminated eluted material was further purified by phenol/chloroform extraction and ethanol precipitation prior to use, resulting in a further reduction in final yield (typically around 25%-30%). This problem was markedly less pronounced when using 'nucleic acid' grade (NA) agarose (Pharmacia), although a disadvantage is that this product is significantly more expensive than normal grade agarose. The purity of eluted DNA fragments was routinely ascertained by agarose gel electrophoresis; a typical example of such a gel is shown in figure 19.

Highest percentage recoveries and purities were achieved using Nensorb 20 columns (typically 75%-85% recovery); the disadvantage of this method is that the capacity of each column is 20 ug of nucleic acid or protein (so in the purification of a restriction digest, for example, the mass of the enzyme must be taken into consideration). Nensorb columns were thus unsuitable for large-scale preparation of DNA fragments prior to their use in immobilization studies, because the expense involved became prohibitive. In view of the fact that an aim of this investigation was to produce an economically viable procedure, efforts were made to keep the cost of preparation of assay components to a minimum, without compromising on reliability or sensitivity. Nevertheless, Nensorb columns were routinely used for the preparation of vector and insert fragments prior to sub-cloning. Since proteins bind irreversibly to the column, restriction enzymes and phosphatase are efficiently removed from DNA, eliminating the requirement for phenol extraction. In order to ensure that the enzyme was completely removed from a pre-ligation sample, 1 ug of PstI digested HPV 16 DNA was purified on a Nensorb column; a further 1 ug of undigested DNA was added to the sample, and the mixture was incubated overnight under conditions suitable for enzyme activity. Subsequent

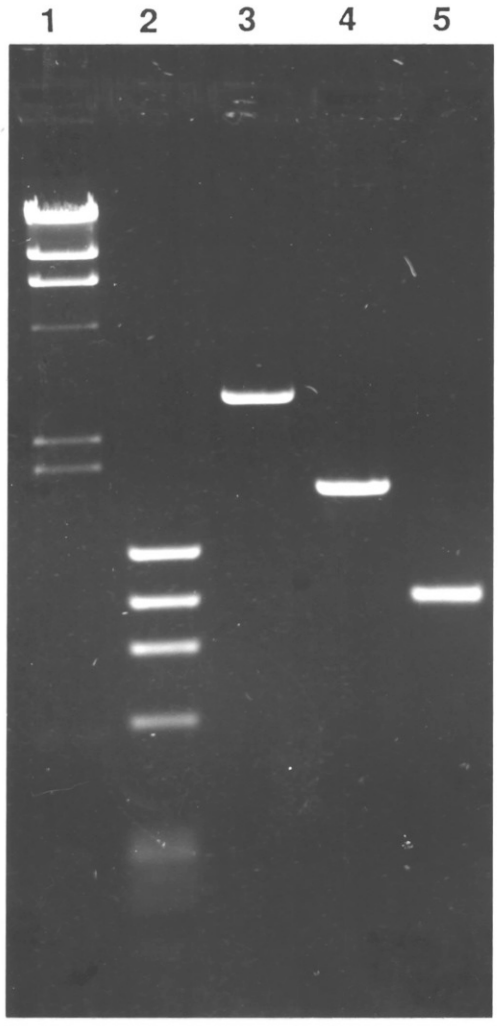


FIGURE 19

Agarose gel of purified HPV 16 PstI restriction fragments.

Lane 1 contains 0.5 µg of lambda-HindIII markers (fragment sizes 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.6 and 0.1 kb). Lane 2 contains 0.5 µg of øX174-HaeIII markers (fragment sizes 1,353, 1,078, 872, 605, 310, 281, 271, 234, 194, 118 and 72 bp). Lane 3 contains 100 ng of HPV 16 2,817 bp PstI fragment. Lane 4 contains 100 ng of HPV 16 1,776 bp PstI fragment. Lane 5 contains 100 ng of HPV 16 1,063 bp PstI fragment. DNA in lanes 3, 4 and 5 was purified by electroelution, exactly as described in section 3.5.3.1, prior to loading. After electrophoresis through a 1% agarose gel, (section 3.4.4.1), DNA was stained with ethidium bromide, and the bands were photographed on FP4 film, as described in section 3.4.4.1.

agarose gel electrophoresis demonstrated that the undigested DNA remained intact, indicating that no active enzyme existed in the purified sample (data not shown).

4.3 Sub-cloning Of HPV Restriction Fragments.

As already stated, the majority of nucleic acid-based assays for the detection of HPV infection rely on Southern blotting (Durst et al., 1987), or on one of the rapid blotting techniques (Wickenden et al., 1987 c). The consequence of this is that full-length genomic probes tend to be used, rather than specific sub-genomic fragments. None of the studies published to date utilize synthetic oligodeoxyribonucleotide probes, mainly because of the 10- to 100-fold reduction in sensitivity associated with such probes, when compared to those produced by nick translation or random priming. Hybridization conditions allowing discrimination between HPV types 6b, 11, 16 and 18 have been reported (Wickenden et al., 1987 a). An example of a slot-blot prepared using these conditions is shown in figure 20. Using full-length genomic probes in the case of HPV types 11, 16 and 18, and a 5,368 bp EcoRI-BamHI sub-fragment in the case of type 6b, it is clear that, using the appropriate stringency, it is readily possible to distinguish between the four virus types. The observed hybridization between types 6b and 11 is not unexpected, because these isolates share 82% homology at the nucleic acid level (Schwarz et al., 1983).

The results of a Southern blot for the identification of type specific probes for the detection of HPV types 16 and 18 are shown in figure 22. The hybridization and washing stringencies used to produce the blot are listed in section 3.5.9. The results demonstrate that both the HPV 16 and HPV 18 probes hybridize strongly to the appropriate fragments in the digests, but not to fragments derived from a different

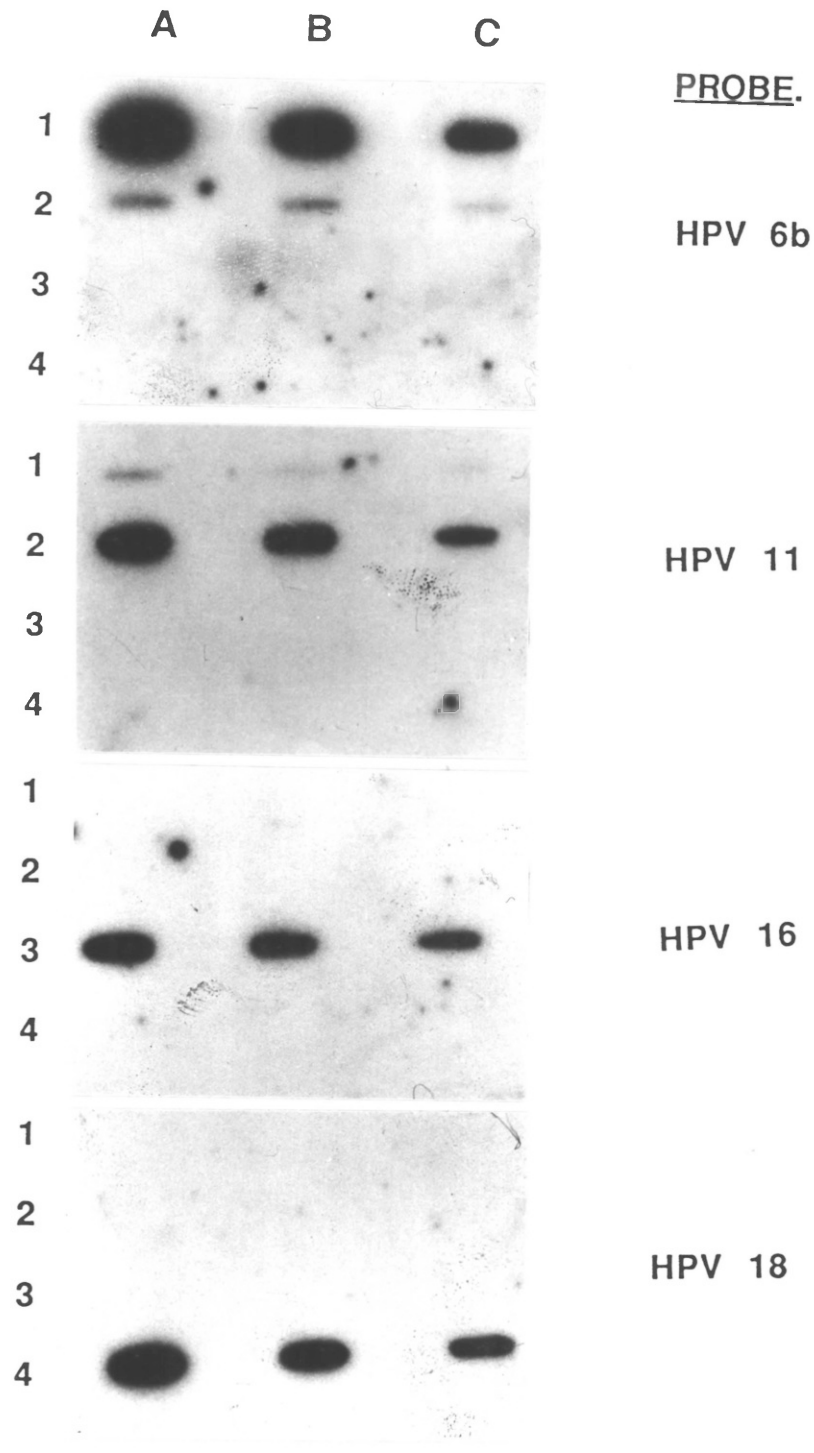


FIGURE 20

Results of a slot-blot to demonstrate specific hybridization for discriminating between HPV types 6b, 11, 16 and 18.

Slots A, B and C contain 20 amole, 10 amole and 5 amole of DNA respectively. DNA from HPV types was loaded as follows: 1=HPV 6b, 2=HPV 11, 3=HPV 16, 4=HPV 18. Four identical filters were prepared, and individually hybridized with the single HPV probe indicated, exactly as described in section 3.5.10. DNA from HPV types 6b, 11, 16 and 18 was labelled with ^{32}P by random priming to an activity of approximately 1×10^7 dpm/fmole. After hybridization, filters were washed for 3 x 1 hour at 65°C in 0.1 x SSC, 0.1% w/v SDS. Results were determined by 72 hours of autoradiography (with intensifying screens), on pre-flashed X-OMAT XAR-5 film.

Although a slight cross-hybridization between HPV 6b and HPV 11 is apparent, HPV type-specific hybridization is obvious.

HPV type (the faint band of around 3,000 bp in lane 7 is due to vector contamination in both the HPV 18 digest, and the HPV 16 probe; it does not correspond with the predicted size of any HPV 18 fragment). Hybridization of the HPV 16 probe with the 483 bp and 216 bp HPV 16 PstI fragments is not evident, because DNA of this size is frequently inefficiently transferred from gel to membrane during the blotting process; however, all of the HPV 18-derived fragments have transferred and hybridized (with the exception of the 163 bp fragment). It is interesting to note that the HPV 6b probe does not hybridize with any of the products of the digests of HPV types 16 and 18, and the HPV 11 probe hybridizes very weakly with the HPV 16 4,146 bp fragment; since this fragment contains a region derived from pBR322, contamination of the HPV 11 probe with vector sequences could be the reason for this.

Although, at high stringency, it should be possible to use any of the HPV 16 or HPV 18 restriction fragments listed in the legend to figure 22 as type-specific probes, it may not be possible to apply the same stringency to sandwich assays. In a further attempt to identify the region of a given HPV genome least likely to cross-hybridize with the analogous region from another virus type, the nucleotide sequences of HPV types 6b, 11, 16 and 18 were compared using the program 'DIAGON'. This program produces a 'dot matrix' diagram containing a representation of all of the nucleotide matches between a pair of sequences; any similarities which may be present are identified by eye. The results of DIAGON plots comparing HPV types 6b, 11, 16 and 18 are shown in figure 21. Consider, for example, the case of the HPV 6b nucleotide sequence compared with that of type 11; the computer was instructed to take the first nine nucleotides of the 6b sequence, and compare these with the entire genome of type 11. Each time an exact match was found, a dot was placed in the relevant position on the plot.

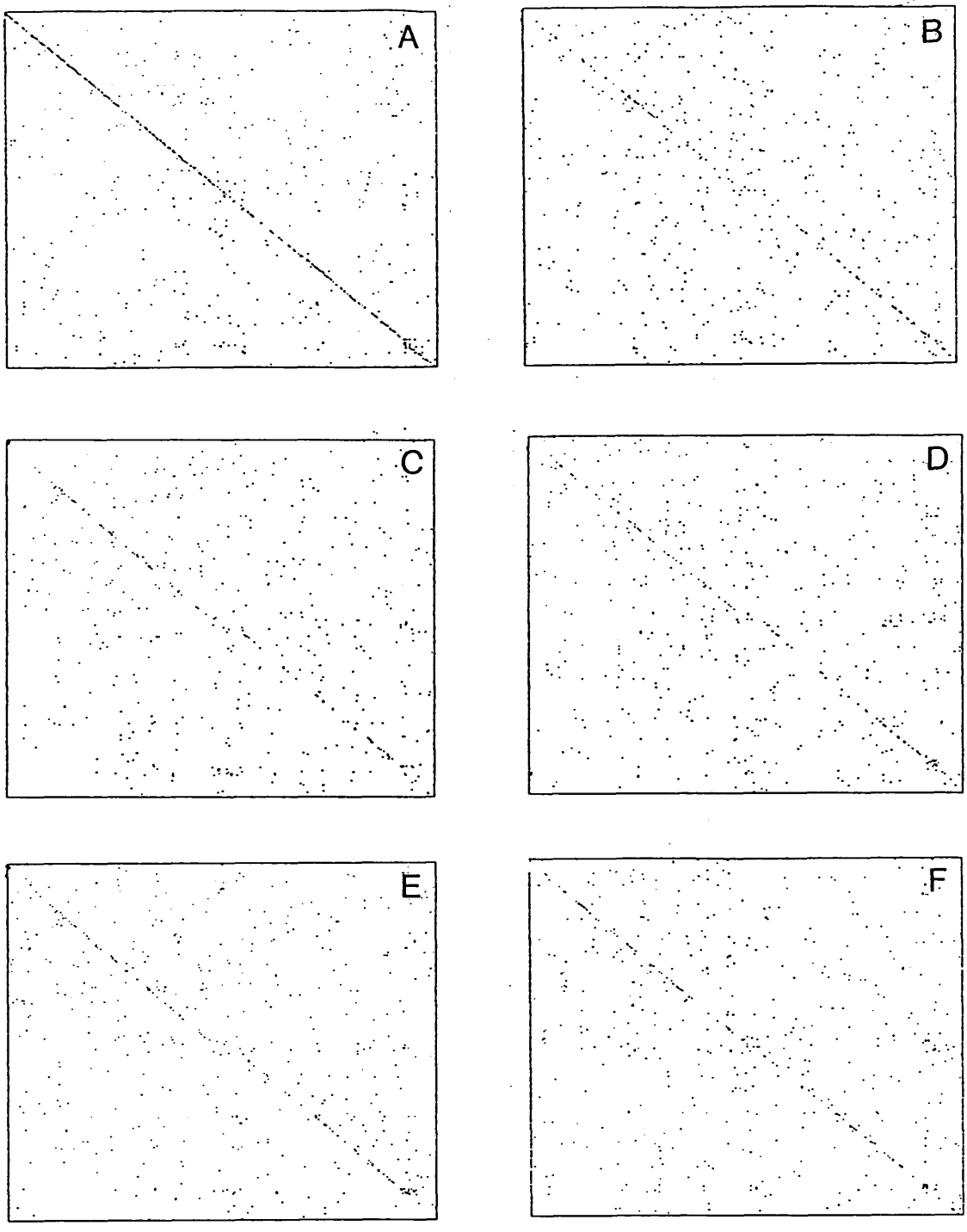


FIGURE 21

'DIAGON' PLOTS TO QUALITATIVELY DEMONSTRATE HOMOLOGY BETWEEN HPV TYPES 6b, 11, 16 AND 18.

All plots were prepared using a scan length of nine, and a score of nine (nucleotide stretches nine residues in length from the 'vertical' sequence were compared with the total 'horizontal' sequence; a dot was plotted on the diagram only at the position of an exact match). The diagrams above are comparisons of the following HPV types ('vertical' sequence first): **A**, HPV 6b vs. HPV 11; **B**, HPV 6b vs. HPV 16; **C**, HPV 6b vs. HPV 18; **D**, HPV 11 vs. HPV 16; **E**, HPV 11 vs. HPV 18; **F**, HPV 16 vs. HPV 18.

The results are discussed fully in section 4.3.

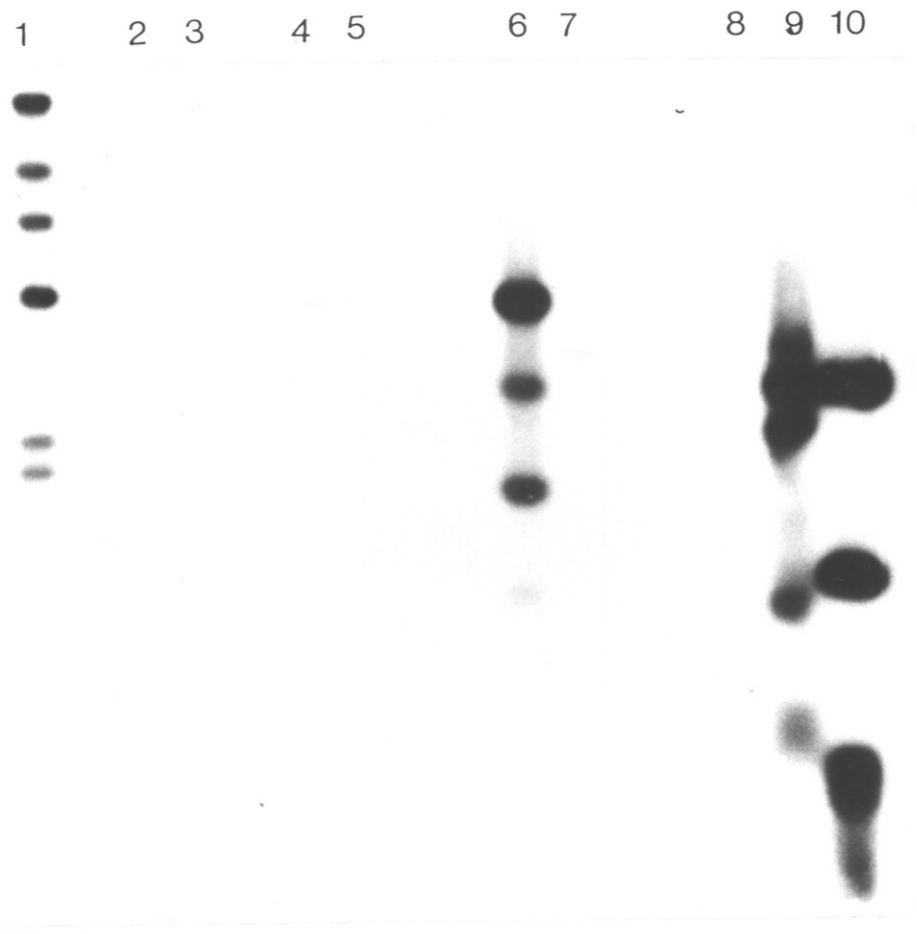


FIGURE 22

Southern blot to identify sub-genomic HPV 16 and HPV 18 fragments which do not hybridize with other HPV types.

Lane 1 contains lambda-HindIII fragments (sizes 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.6 and 0.1 kb), labelled by the Klenow-mediated end-repair method (section 3.5.7.3). Lanes 2, 4, 6 and 8 contain 100 ng of PstI digested pBR322-HPV 16 (fragment sizes 4,146, 2,817, 1,776, 1,766, 1,063, 483 and 216 bp). Lanes 3, 5, 7 and 9 contain 100 ng of BamHI/PstI digested, EcoRI linearized HPV 18 (fragment sizes 2,806, 2,321, 1,047, 555, 524, 441 and 163 bp). Lane 10 contains øX174-TaqI fragments (sizes of the five largest fragments are 2,914, 1,175, 404, 327 and 231 bp), labelled by Klenow-mediated end-repair. After electrophoresis through a 1% agarose gel (section 3.4.4.1), the separated fragments were transferred to a Hybond-N nylon membrane (section 3.5.9). The filter was cut into strips, separating lanes 1-3, 4-5, 6-7 and 8-10, and each strip was hybridized with the appropriate probe, labelled with ^{32}P by random priming to an activity of around 5 x 10^6 dpm/fmole. The probes used were as follows: lanes 1-3, HPV 6b; lanes 4-5, HPV 11; lanes 6-7, HPV 16; lanes 8-10, HPV 18. After washing for 3 x 1 hour at 65^{o}C in 0.1 x SSC, 0.1% w/v SDS, results were determined by overnight autoradiography on X-OMAT XAR-5 film.

The results are discussed fully in the text.

This process was repeated, moving along a single nucleotide at a time, until the entire genome of HPV 6b was compared with that of type 11. On the plot, the two sequences are aligned with their first nucleotide in the top left corner, so any homology is seen as a diagonal line running from top left to bottom right. It is important to remember that DIAGON plots only provide a qualitative representation of homology between two sequences; clearly, in the case described above, if the size of the DNA segment used to 'probe' the HPV 11 sequence were shorter than nine nucleotides, a high 'background' of dots would appear. Conversely, if the sequence were much longer than nine nucleotides, the homology between HPV 6b and HPV 11 would no longer be apparent. The results of the DIAGON study of HPV types 6b, 11, 16 and 18 demonstrate that: (i) types 6b and 11 share the greatest homology; (ii) comparison of type 6b or 11 with type 16 or 18 results in a staggered diagonal line; this is usually indicative of an insertion or deletion in one or other sequence (which presumably occured after evolutionary divergence); (iii) there is no obvious genomic region which has a sequence unique to a particular HPV type. Despite their lack of success in identifying HPV type-specific restriction fragments, DIAGON plots were used do demonstrate the lack of homology between sub-genomic HPV fragments chosen as immobilized and labelled probes in the sandwich assay (see Appendix A).

Having demonstrated that HPV type-specific hybridization is possible using a target sequence immobilized on a Hybond-N nylon membrane, the next step was to identify the parameters which would need to be adjusted in order to develop a sandwich hybridization assay for achieving the same result; the first requirement was the isolation of suitable sub-genomic fragments, for use as immobilized (fragment A) and free, labelled (fragment B) probes. In theory, a positive result

should be obtained only if there is a nucleic acid fragment in the sample capable of forming a linking bridge between A and B, thus resulting in the attachment of probe to the solid support. If A were to hybridize directly to B, regardless of the presence or absence of target, a false positive result would be obtained. To complicate matters, A and B were to be used in a vast molar excess over the target DNA, in an attempt to produce favourable hybridization kinetics. If, for instance, A and B were in fifty-fold excess over the target DNA, just 2% cross-hybridization between A and B would be sufficient to generate a signal equivalent to that resulting directly from the presence of the target. This artificial signal would be apparent regardless of whether or not the clinical sample were infected. Initial studies in which two restriction fragments were isolated by electro-elution from a single PstI digest of HPV DNA were unsuccessful, because purified fragments were frequently contaminated with significant amounts of the other PstI restriction fragments. Although this problem was markedly reduced by subjecting electroeluted fragments to a second round of purification in an agarose gel, the final yield of pure DNA was low (around 5%-10%). Although this is not a significant problem in the isolation of a fragment for use as a probe (a single microgram of DNA is sufficient for several labelling reactions), such a low yield is inconvenient with respect to the purification of suitable quantities of DNA for immobilization studies. In order to circumvent the problem of cross-contamination, sub-genomic HPV fragments were cloned into the vector pUC8; this vector was chosen because it has a high copy number, and thus provides excellent yields of recombinant DNA after a large-scale plasmid preparation (see section 3.5.1).

With respect to the choice of fragments A and B, the following points were considered important:

(a) The fragments chosen as A and B must not cross-hybridize, for the reasons described above.

(b) In order to minimise the risk of obtaining a false negative result due to sample degradation, the fragments chosen as A and B should be derived from adjacent positions on the viral genome.

(c) A variety of fragment sizes should be available, in order to optimize the assay with respect to the size of immobilized and labelled probes.

(d) The genomic location of restriction fragments should be considered carefully, before selection of A and B. When HPV DNA integrates within the host cell chromosome, a specific region of the viral genome appears to be frequently deleted (Choo et al., 1987). This region comprises the E1/E2 ORFs, so nucleic acid probes derived from this location should be avoided, in order to reduce the possibility of obtaining a false negative signal as a result of deletion of the 'target' region of the viral genome. Deletions have also been observed in the long control region (LCR) of HPV 16 (G.C.N. Parry, personal communication).

Prior to sub-cloning, individual restriction fragments were purified using Nensorb 20 affinity columns (section 3.5.3.3). Alternatively, all of the fragments produced on digestion of a particular HPV genome were purified on a Nensorb 20 column, and sub-cloned using a 'shot-gun' procedure, as described in section 3.5.4. The results obtained using DNA purified in this way were invariably superior to those obtained using DNA purified by phenol/chloroform extraction, or by electroelution. This may be because residual phenol and contaminants present in agarose inhibit the activity of T4 DNA ligase. Alternatively, Nensorb purification may reduce the activity of naturally

occuring nucleases, which result in the destruction of the 'sticky ends' of digested insert and vector sequences. In fact, recombinants prepared using DNA purified by phenol extraction were frequently found to have lost the restriction enzyme recognition site used for the insertion of the HPV DNA fragment; this may be a result of 'sticky end' damage. This phenomenon was rarely observed with Nensorb purified DNA fragments.

After sub-cloning, recombinants were grown as described in section 3.5.2, and analysed by three methods: (i) determination of the size of the insert, by comparison with molecular weight standards; (ii) restriction enzyme analysis of the insert, followed by comparison with the expected fragment sizes (generated by application of the 'CUTSIT' programme to the appropriate nucleotide sequence); (iii) Southern blotting. An example of a typical Southern blot designed to confirm the size and source of the insert in recombinants is shown in figure 23. In this case, the plasmids were generated by 'shot-gun' sub-cloning of a PstI digest of HPV 18 DNA into the vector pUC8; the sizes of the fragments produced by this digest are listed in figure 17. It is clear from the blot that all of the recombinants derived from HPV 18 contain an insert in the size range 400 to 600 bp. There are three HPV 18 PstI fragments in this range, of sizes 555, 457 and 441 bp. All three fragments comprise part of the L1 ORF; the 457 bp fragment also overlaps the 3' end of the L2 ORF (see figure 17). Probes were prepared by random priming of the inserts of the recombinants in lanes 3 and 4 (figure 23), and these were hybridized back to a Southern blot of PstI digests of the same recombinants (figure 24). The purpose of this was to demonstrate that: (i) the inserts in the two recombinants were not the same, (ii) the inserts in the two recombinants did not cross-hybridize. The results of the blot demonstrate this clearly. Subsequent

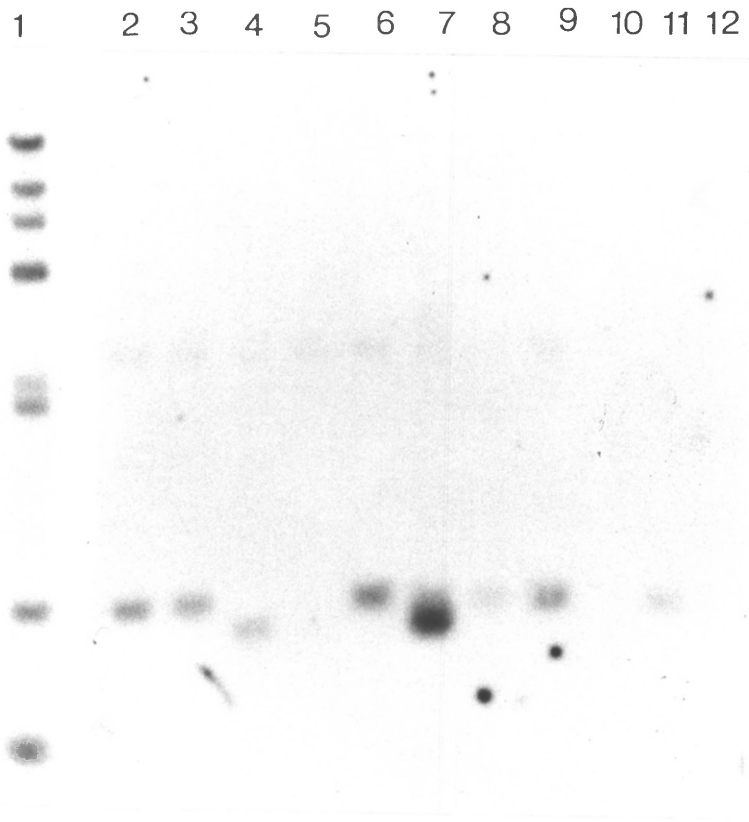


FIGURE 23

Results of a Southern blot to confirm the source of the insert in sub-clones derived from HPV 18.

Lane 1 contains lambda-HindIII fragments (sizes 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.6 and 0.1 kb), labelled by the Klenow-mediated end-repair method (section 3.5.7.3). Lanes 2 to 12 contain approximately 50 ng of PstI digested plasmid DNA, isolated by the 'mini-prep' technique from eleven different sub-clones generated by 'shot-gun' cloning from PstI digested, EcoRI-linearized HPV 18 (section 3.5.4). After electrophoresis through a 1% agarose gel, exactly as described in section 3.4.4.1, the separated fragments were transferred to a Hybond-N nylon membrane (section 3.5.9), and hybridized with an HPV 18 probe, labelled with ^{32}P by random priming to an activity of approximately 5 x 10^6 dpm/fmole. After washing for 3 x 1 hour at 65°C in 0.1 x SSC, 0.1% w/v SDS, results were determined by autoradiography for 4 hours on X-OMAT XAR-5 film.

The results clearly demonstrate that at least eight of the sub-clones contain an insert derived from HPV 18. The size of the inserts is around 400-600 bp.

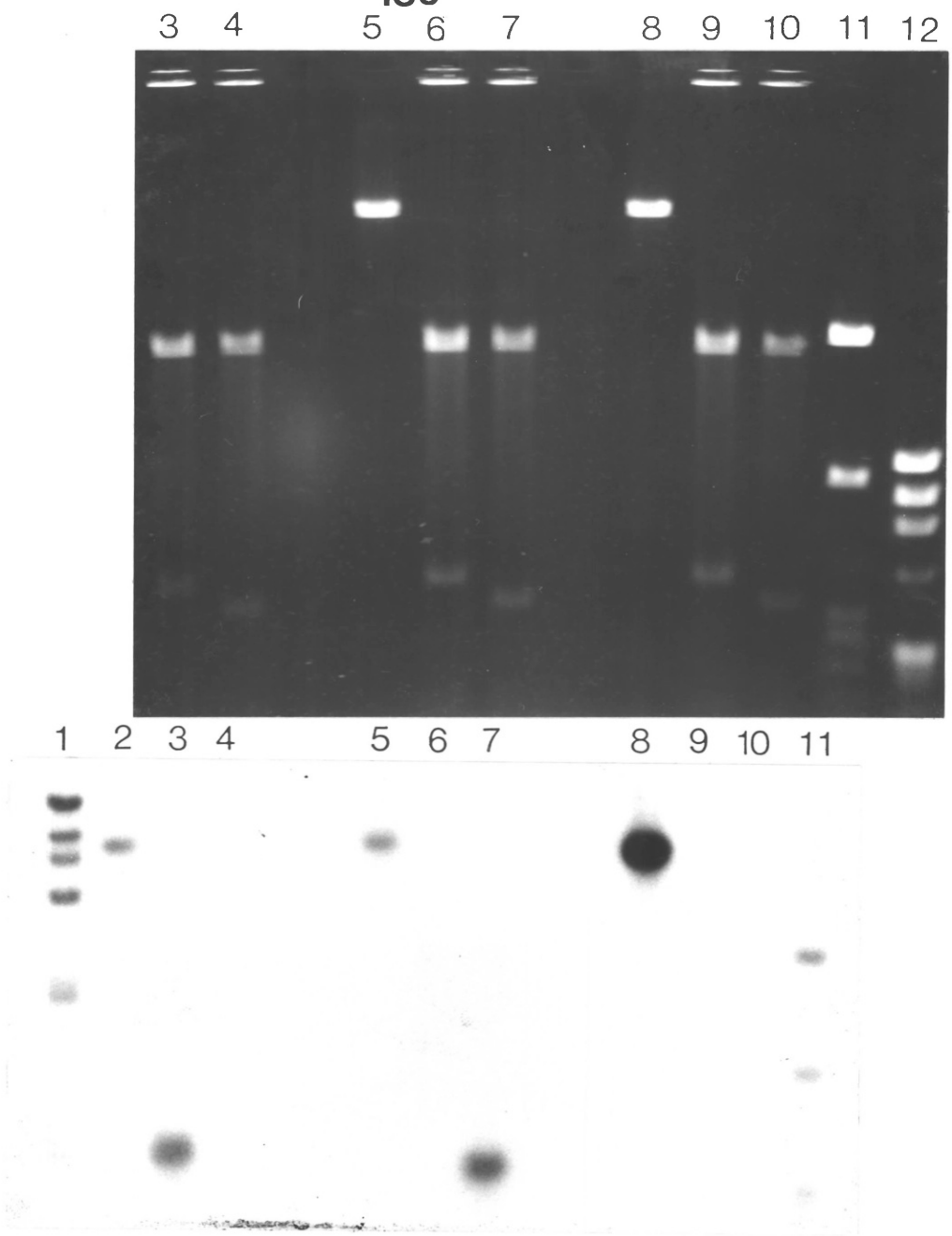


FIGURE 24

Results of a Southern blot to confirm the identity of inserts in sub-clones derived from HPV 18.

Lane 1 contains lambda-HindIII fragments (sizes 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.6 and 0.1 kb), labelled by the Klenow-mediated end-repair method (see section 3.5.7.3). Lanes 2, 5 and 8 contain 50 ng of linear HPV 18 (7,857 bp). Lanes 3, 6 and 9 contain 50 ng of PstI digested pJN4 (555 bp HPV 18 PstI fragment in pUC8), and lanes 4, 7 and 10 contain 50 ng of PstI digested pJN5 (441 bp HPV 18 PstI fragment in pUC8). Lane 11 contains øX174-TaqI fragments (sizes of the five largest fragments are 2,914, 1,175, 404, 327 and 231 bp), labelled by Klenow-mediated end-repair. Lane 12 contains 1 µg of unlabelled øX174-HaeIII (fragment sizes 1,353, 1,078, 872, 605, 310, 281, 271, 234, 194, 118 and 72 bp). After electrophoresis through a 1% agarose gel [TOP] (section 3.4.4.1), the separated fragments were transferred to a Hybond-N nylon membrane (section 3.5.9). The filter was cut into strips, separating lanes 1-4, 5-7 and 8-12, and each strip was hybridized with the appropriate probe, labelled with ^{32}P by random priming to an activity of around 5 x 10^5 dpm/fmole. The probes used (all derived from HPV 18) were as follows: lanes 1-4, 555 bp PstI fragment; lanes 5-7, 441 bp PstI fragment; lanes 8-12, 3,531 bp EcoRI/PstI fragment. After washing for 3 x 1 hour at 65°C in 0.1 x SSC, 0.1% w/v SDS, results were determined by overnight autoradiography on X-OMAT XAR-5 film [BOTTOM].

The results are discussed fully in the text.

restriction enzyme analysis with the enzymes AccI and KpnI was used to confirm that the insert in the recombinant in lane 3 was the 555 bp fragment, and that in lane 4 was the 441 bp fragment (data not shown). Glycerol stocks of these two constructs, named pJN4 and pJN5 respectively, were prepared, and both plasmids were produced in high yield, as described in section 3.5.1. These two fragments are ideal candidates for development of a sandwich assay, because: (i) they do not cross-hybridize, (ii) they occupy adjacent positions on the genome; (iii) they are derived from a suitable location (the L1 ORF).

The results of a similar study, for the identification of inserts derived from HPV type 16, are shown in figure 25. In this case, the recombinants in lanes 2, 3 and 4 contain 1,776, 483 and 216 bp HPV 16 inserts respectively. The identity of each insert was subsequently confirmed by restriction enzyme analysis (data not shown). The 1,776 bp fragment comprises the 3' end of the L1 ORF, the entire LCR and E6/E7 ORFs, together with the 5' end of E1a. The 483 bp fragment lies fully within the L2 ORF, and the 216 bp fragment entirely within L1 (see figure 16). The constructs containing the 1,776, 483 and 216 bp HPV 16 fragments were named pJN1, pJN2 and pJN3 respectively. In order to identify a suitable pair of fragments which did not cross-hybridize, PstI digests of pBR322-HPV 16 were Southern blotted, and individually hybridized with the inserts of pUC8 recombinants, containing the HPV 16 derived fragments indicated in the legend to figure 26. The following points are apparent from studying the blot: (i) the 216 bp fragment does not give a strong signal; this could be because it does not transfer efficiently out of the gel (possibly due to diffusion), or because it is not efficiently immobilized by the u.v. cross-linking procedure used with Hybond-N (section 3.5.9); (ii) the 2,817 bp probe hybridizes to the appropriate complementary sequence, and also to the

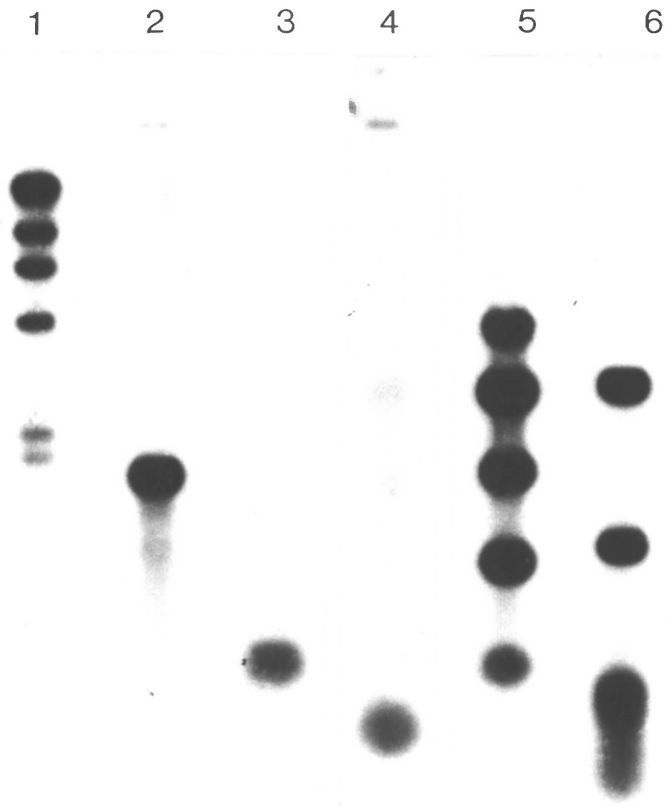


FIGURE 25

Results of a Southern blot to confirm the identity of inserts in sub-clones derived from HPV 16.

Lane 1 contains lambda-HindIII fragments (sizes 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.6 and 0.1 kb), labelled by the Klenow-mediated end-repair method (see section 3.5.7.3). Lanes 2, 3 and 4 contain approximately 50 ng of PstI digested pJN1, pJN2 and pJN3 respectively. Lane 5 contains 500 ng of PstI digested pBR322-HPV 16 (fragment sizes 4,146, 2,817, 1,776, 1,766, 1,063, 483 and 216 bp). Lane 6 contains øX174-TaqI fragments (sizes of the five largest fragments are 2,914, 1,175, 404, 327 and 231 bp), labelled by Klenow-mediated end-repair. After electrophoresis through a 1% agarose gel, exactly as described in section 3.4.4.1, the separated fragments were transferred to a Hybond-N nylon membrane (see section 3.5.9), and hybridized with an HPV 16 probe, labelled with ^{32}P by random priming to an activity of approximately 6.4 x 10^6 dpm/fmole. After washing for 3 x 1 hour at 65°C in 0.1 x SSC, 0.1% w/v SDS, results were determined by overnight autoradiography on X-OMAT XAR-5 film.

The results demonstrate that the inserts in the sub-clones are derived from HPV 16, and the sizes of the inserts suggest that the constructs contain the following HPV 16 PstI fragments: pJN1, 1,776 bp; pJN2, 483 bp; pJN3, 216 bp. Insert identity was subsequently confirmed by restriction enzyme analysis.

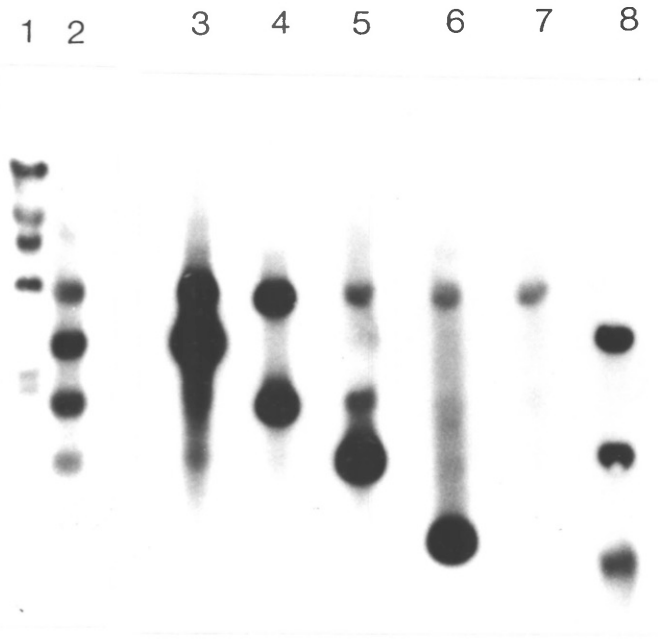


FIGURE 26

Southern blot to determine the extent of cross-hybridization between HPV 16 PstI fragments.

Lane 1 contains lambda-HindIII fragments (sizes 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.6 and 0.1 kb), labelled by the Klenow-mediated end-repair method (section 3.5.7.3). Lanes 2 to 7 contain approximately 50 ng of PstI digested pBR322-HPV 16 (fragment sizes 4,146, 2,817, 1,776, 1,766, 1,063, 483, and 216 bp). Lane 8 contains øX174-TaqI fragments (sizes of the five largest fragments are 2,914, 1,175, 404, 327 and 231 bp), labelled by Klenow-mediated end-repair. After electrophoresis through a 1% agarose gel (section 3.4.4.1), the separated fragments were transferred to a Hybond-N nylon membrane (section 3.5.9). The filter was cut into strips, separating lanes 2 to 7, and each strip was hybridized with the appropriate probe, labelled with ^{32}P by random priming to an activity of around 3 x 10^5 dpm/fmole. The probes used (all derived from HPV 16) were as follows: lane 2, full-length HPV 16; lane 3, 2,817 bp PstI fragment; lane 4, 1,776 bp PstI fragment; lane 5, 1,063 bp PstI fragment; lane 6, 483 bp PstI fragment; lane 7, 216 bp PstI fragment. After washing for 3 x 1 hour at 65°C in 0.1 x SSC, 0.1% w/v SDS, results were determined by overnight autoradiography on X-OMAT XAR-5 film.

The results are discussed fully in the text.

1,776 bp fragment; (iii) the 1,063 bp probe hybridizes to the appropriate complementary sequence, and also to the 1,776 bp fragment; (iv) the 1,776 bp probe hybridizes to the appropriate complementary sequence, but not to the 2,817 bp or 1,063 bp fragments; (v) the 483 bp probe hybridizes just to the appropriate complementary sequence; (vi) the dark band around 4 kb in lanes 3, 4, 5 and 6 is in fact a pBR322 fragment (3,234 bp), with a flanking 912 bp HPV 16 fragment (total size, 4,146 bp). This fragment has been detected by the probe, because DNA labelled was isolated from plasmid prepared by the 'mini-prep' technique (section 3.5.2) after a single round of electrophoresis through LMP agarose. Consequently, each probe is contaminated with pUC8 vector sequences, and pUC8 is derived from pBR322, so it is not unexpected that the two vectors should cross-hybridize. The remaining pBR322 fragment in each digest (1,129 bp) is flanked by a 637 bp HPV 16 fragment (total size, 1,766 bp); the 1,129 bp region of pBR322 has no homology with pUC8, so it is not detected by the 'contaminated' probe. It is interesting to note that the contamination of probe with pUC8 vector is most pronounced when the size of the HPV 16 insert is closest to the size of pUC8 (2,678 bp). This illustrates perfectly a problem which must be avoided at all costs in preparation of the restriction fragments for use as A and B in the sandwich assay; it is essential that at least one of the two fragments (and preferably both) is completely free from contaminating vector sequences. This will avoid false positive signals resulting from hybridization between contaminating vector sequences in both immobilized and labelled fragments.

The slot-blot shown in figure 27 was prepared in an attempt to verify the cross-hybridization between the HPV 16 2,817 bp and 1,776 bp fragments observed in figure 26. The results appear to confirm the cross-hybridization, and also suggest that these two fragments

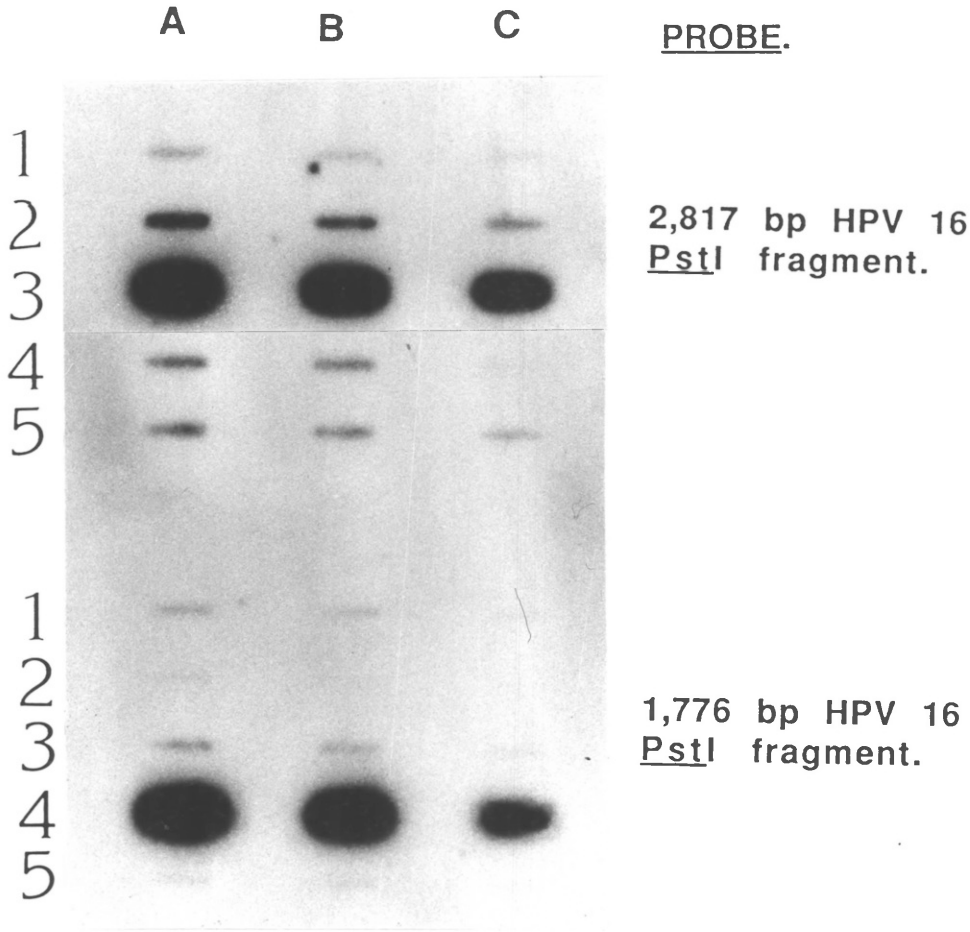


FIGURE 27

Slot-blot to demonstrate cross-hybridization between HPV 16 2,817 bp and 1,776 bp PstI fragments.

Slots A, B and C contain 20 amole, 10 amole, 5 amole of DNA respectively. DNA from HPV types was loaded as follows: 1=HPV 6b, 2=HPV 11, 3=HPV 16 2,817 bp PstI fragment; 4=HPV 16 1,776 bp PstI fragment; 5=HPV 18. Two identical filters were prepared, and individually hybridized with the single HPV 16 sub-genomic fragment indicated (section 3.5.10). Probes were labelled with ^{32}P by random priming to an activity of 3-5 x 10^6 dpm/fmole. After hybridization, filters were washed for 3 x 1 hour at 65°C in a buffer consisting of 0.1 x SSC, 0.1% w/v SDS. Results were determined by 72 hours of autoradiography (with intensifying screens), on pre-flashed X-OMAT XAR-5 film.

The results demonstrate that, under these stringent conditions, the 2,817 bp and 1,776 bp HPV 16 PstI fragments cross-hybridize with DNA from HPV types 6b, 11 and 18. More importantly, the fragments also hybridize with each other; this makes them unsuitable for use in the sandwich assay.

hybridize weakly to DNA derived from HPV types 6b, 11 and 18. However, in retrospect, the experiment was not well designed; despite the fact that the 2,817 and 1,776 bp fragments were purified by two rounds of electroelution, it is possible that the preparations were contaminated with a small amount of pUC8 plasmid DNA. If this were the case, plasmid contaminating both fragments may be the source of the apparent cross-hybridization. Immobilized pUC8 DNA should have been included in the blot, in order to confirm that the observed cross-hybridization was not due to contamination. Slot-blots were found to be extremely useful for checking that the two fragments chosen as probes for the sandwich assay did not cross-hybridize; for example, figure 28 demonstrates that the preparations of HPV 16 1,776 bp and 483 bp PstI fragments did not cross-hybridize (and were therefore not both contaminated with pUC8 DNA). Slot-blots were also found to be useful for the identification of sub-genomic fragments which hybridized specifically to the virus type from which they were derived, and not to any other type. A typical example of such a blot is shown in figure 29; the results clearly demonstrate that the three HPV 6b fragments tested hybridize weakly with HPV 11; the 1,081 bp fragment also hybridizes weakly with HPV type 18. It is interesting to note that, in both figures 28 and 29, the signal obtained for hybridization to 20 amole of target DNA is smaller than the signal obtained for 10 amole of target. The reason for this is unknown; it is possible that a greater proportion of the DNA at a higher concentration had a chance to re-anneal prior to immobilization, and was thus not cross-linked to the membrane by irradiation with u.v. light.

Having sub-cloned fragments suitable for use as immobilized and labelled probes in a sandwich assay, available hybridization supports and DNA immobilization chemistries were examined in detail.

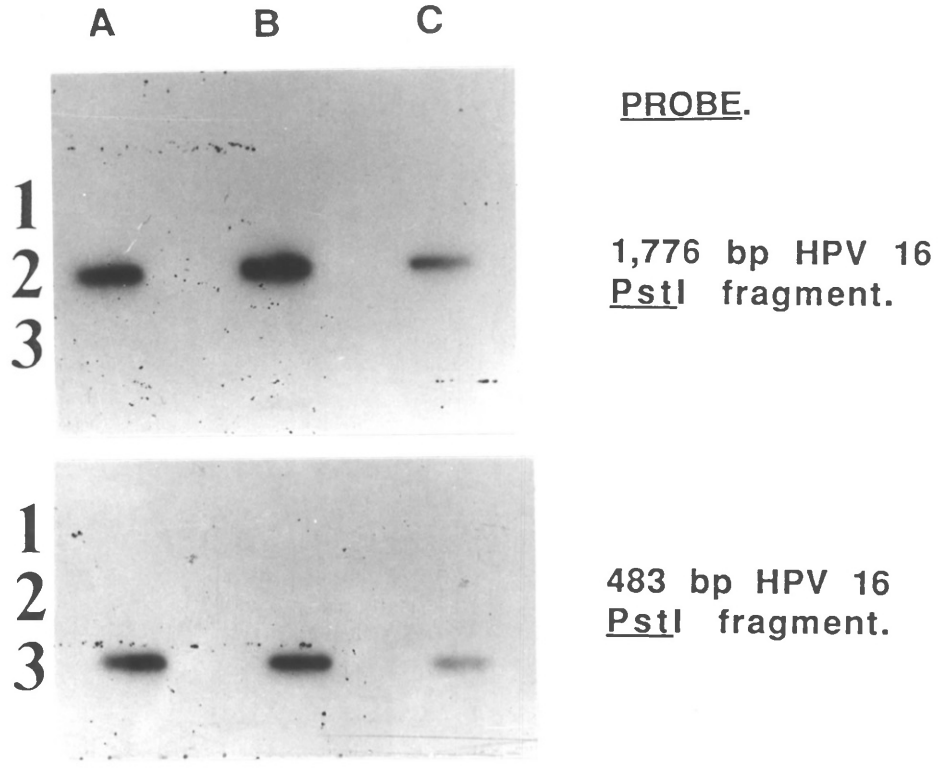


FIGURE 28

Slot-blot to demonstrate HPV 16 sub-genomic fragments chosen for use in a sandwich assay are HPV type-specific, and do not cross-hybridize.

Slots 1A, 1B and 1C contain 20 amole of HPV 6b, HPV 11 and HPV 18 DNA respectively. Slots 2A, 2B and 2C contain 20 amole, 10 amole, 5 amole of HPV 16 1,776 bp PstI fragment (insert from pJN1) respectively. Slots 3A, 3B and 3C contain 20 amole, 10 amole and 5 amole of HPV 16 483 bp PstI fragment (insert from pJN2) respectively. Two identical filters were prepared, and individually hybridized with the HPV probe indicated, exactly as described in section 3.5.10. Probes were labelled with ^{32}P by random priming to an activity of around 5×10^5 dpm/fmole. After hybridization, filters were washed for 3 x 1 hour at 65°C in 0.1 x SSC, 0.1% w/v SDS. Results were determined by 72 hours of autoradiography (with intensifying screens), on pre-flashed X-OMAT XAR-5 film.

The results clearly demonstrate that, at this high stringency: (i) The HPV 16 1,776 bp and 483 bp fragments do not hybridize with each other; (ii) Neither of these two fragments cross-hybridizes with HPV 6b, HPV 11 or HPV 18.

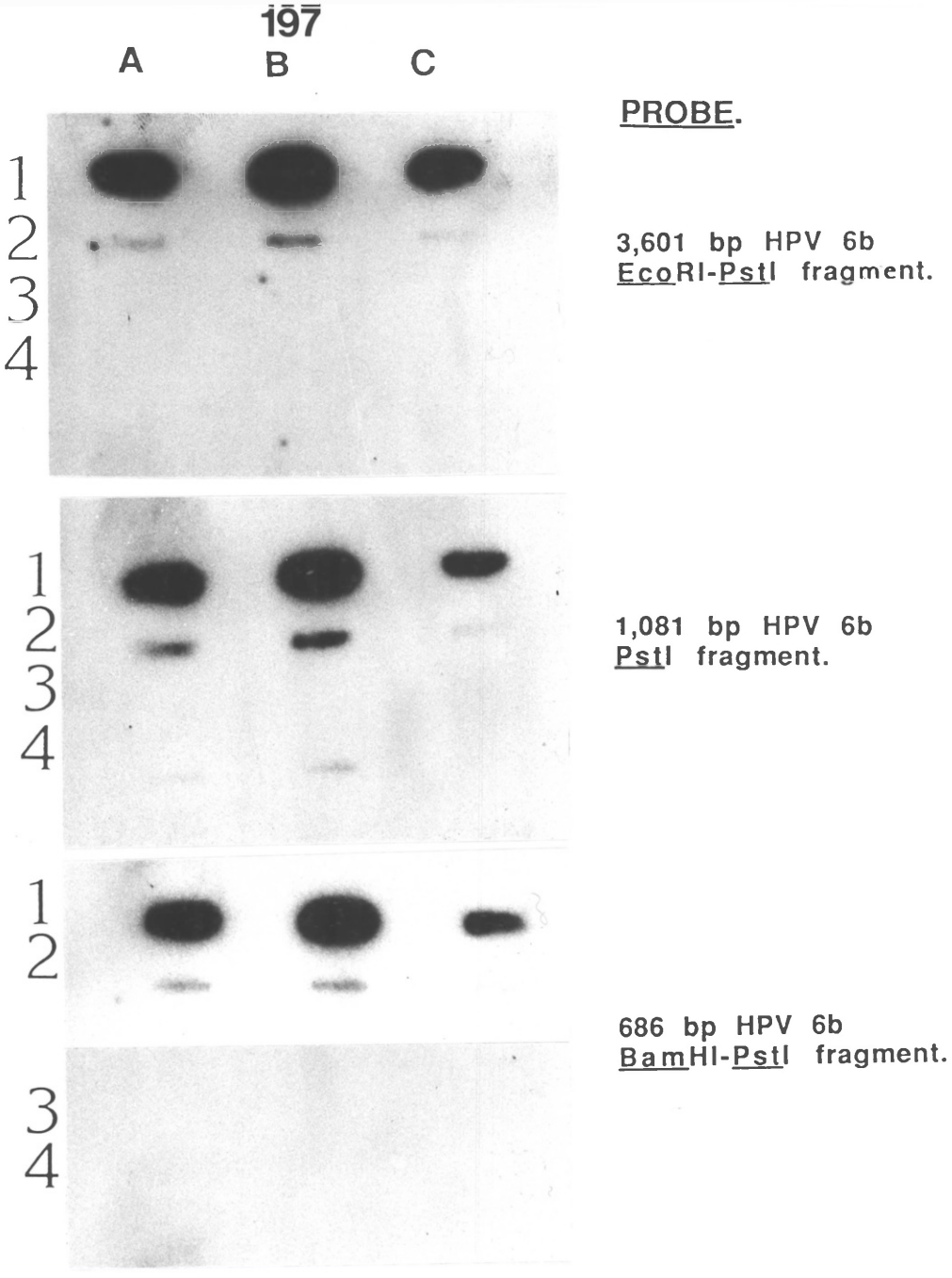


FIGURE 29

Slot-blot to identify cross-hybridization of HPV 6b sub-genomic fragments with HPV types 11, 16 and 18.

Slots A, B and C contain 20 amole, 10 amole, 5 amole DNA respectively. DNA from HPV types was loaded as follows: 1=HPV 6b, 2=HPV 11, 3=HPV 16, 4=HPV 18. Three identical filters were prepared, and individually hybridized with the single HPV 6b sub-genomic probe indicated (section 3.5.10). Probes were labelled with ^{32}P by random priming to an activity of approximately 1-3 x 10^6 dpm/fmole. After hybridization, filters were washed for 3 x 1 hour at 65^{o}C in a buffer consisting of 0.1 x SSC, 0.1% w/v SDS. Results were determined by 72 hours of autoradiography (with intensifying screens), on pre-flashed X-OMAT XAR-5 film.

The results demonstrate that the 3,601 bp and 686 bp probes cross-hybridize with HPV 11 DNA, and that the 1,081 bp probe cross-hybridizes with HPV 11, and also weakly with HPV 18.

4.4 Choice of Solid Support And Chemistry Of Immobilization.

The majority of the nucleic acid-based diagnostic assays described to date (including the sandwich assays reviewed in section 1.3) fail in their aim to be suitable for routine use, predominantly because of procedural complexities. Since an aim of this study was to produce an assay format which was simple, and amenable to automation, a variety of solid supports, the majority with a macroporous particulate structure, were considered as an alternative to the customary nitrocellulose or nylon membrane. There are several advantages inherent in the use of a macroporous particle rather than a membrane as the support: firstly, such a particle has a large surface area to volume ratio, thus providing an ideal surface for nucleic acid immobilization, and subsequent hybridization. Secondly, by keeping the solid support in homogeneous suspension during the hybridization process, the kinetics of the reaction should approximate to those resulting from a similar hybridization performed entirely in solution (Lund et al., 1988). Finally, the pre- and post-hybridization washing steps would be simple, because the bead could be pelleted by centrifugation, prior to removal of the supernatant (containing non-hybridized probe) by aspiration. The centrifugation step could be dispensed with entirely if the bead were to be magnetic, because a magnet could be used to retain the support in a tube whilst the supernatant was decanted.

Previously reported studies have shown that macroporous (rather than 'solid') beads are most suitable for coupling, and subsequent hybridization of nucleic acids (Bunemann, 1982). Sephacryls S-500 and S-1000 in particular have a high capacity for binding DNA, and a large percentage of the bound material is available for hybridization (see section 1.6) (Bunemann, 1982; Langdale & Malcolm, 1985). For this reason, Sephacryl S-1000 was chosen for the initial study, to determine

the most appropriate chemistry for the covalent coupling of the HPV restriction fragments chosen as the 'catching reagent' (fragment A) in the sandwich hybridization assay. On examination of the published studies describing nucleic acid immobilization, it became clear that the most successful methods (in terms of immobilization efficiency, and availability of immobilized molecules for hybridization) involve covalent coupling, either directly to the support, or via a suitable spacer molecule. Physical coupling procedures (passive adsorption [Alberts & Herrick, 1971] and entrapment [Bolton & McCarthy, 1962]) are unsuitable, because of the instabilty of the immobilized molecules, and extreme variability in their hybridization properties. In addition, immobilization protocols involving the enzyme T4 DNA ligase (Goldkorn & Prockop, 1986; Kremsky et al., 1987) were considered unsuitable, because a major aim in the development of a sandwich assay for the detection of HPV infection was that the technique should be suitable for screening very large numbers of samples; the cost and instability of the enzyme could result in a significant increase in the unit cost of the assay, and would certainly be prohibitively expensive during the developmental stages. For these reasons, purely chemical immobilization procedures were considered; since it was initially not clear whether it would be advantageous to immobilize fragment A via one or other end, rather than randomly via the bases, it was decided to investigate two coupling procedures, one resulting in end-attachment, and the other resulting in multiple internal linkages. Of the methods designed for the end-attachment of nucleic acids to solid supports, 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate (carbodiimide)-mediated immobilization via the 5' phosphate group has been reported to be the most efficient procedure (Langdale & Malcolm, 1985; Gingeras et al., 1987). Although several methods for multiple-attach-

ment have been used successfully, the diazotization procedure involving a 1,4-butanediol diglycidyl ether spacer described by Bunemann in 1982 was chosen, because of the simplicity and efficiency of the procedure, when compared to alternatives involving extremely toxic chemicals like cyanogen bromide (Poonian et al., 1971). In addition, DNA immobilized onto Sephacryl S-500 by this method has been shown to exhibit excellent hybridization properties (Bunemann, 1982; see section 1.6.3).

4.4.1 Immobilization Of DNA By Coupling To Carbodiimide Activated Resins.

The chemistry of the coupling procedure involving water soluble carbodiimide is shown in figure 30. Trial immobilization studies were performed using the 483 bp HPV 16 PstI fragment (fragment 'f' in figure 16). DNA was immobilized onto Sephacryl S-1000, exactly as described in the legend to table 2. Input DNA concentrations were varied between 140 and 560 pmole per gram of resin, in order to determine whether this has any influence on the immobilization efficiency. The results of this investigation are shown in table 2. The amount of non-specifically bound DNA was calculated as follows:

$$\%\ \text{Non-covalently Bound} = \frac{\text{DNA coupled in absence of activating agent.}}{\text{DNA coupled in presence of activating agent.}} \times 100$$

Each result in the table is adjusted, so that the figures quoted refer specifically to covalently immobilized DNA. Of the two methods used to determine the immobilization efficiency, the radiolabelled tracer assay was consistently found to give higher results than the micrococcal (S7) nuclease assay. The latter involves the hydrolysis of immobilized single-stranded DNA molecules, with the release of oligo- and mononucleotides containing terminal 3' phosphate groups into the surrounding buffer; it is possible that a proportion of the immobilized molecules are spatially orientated such that approach of the nuclease

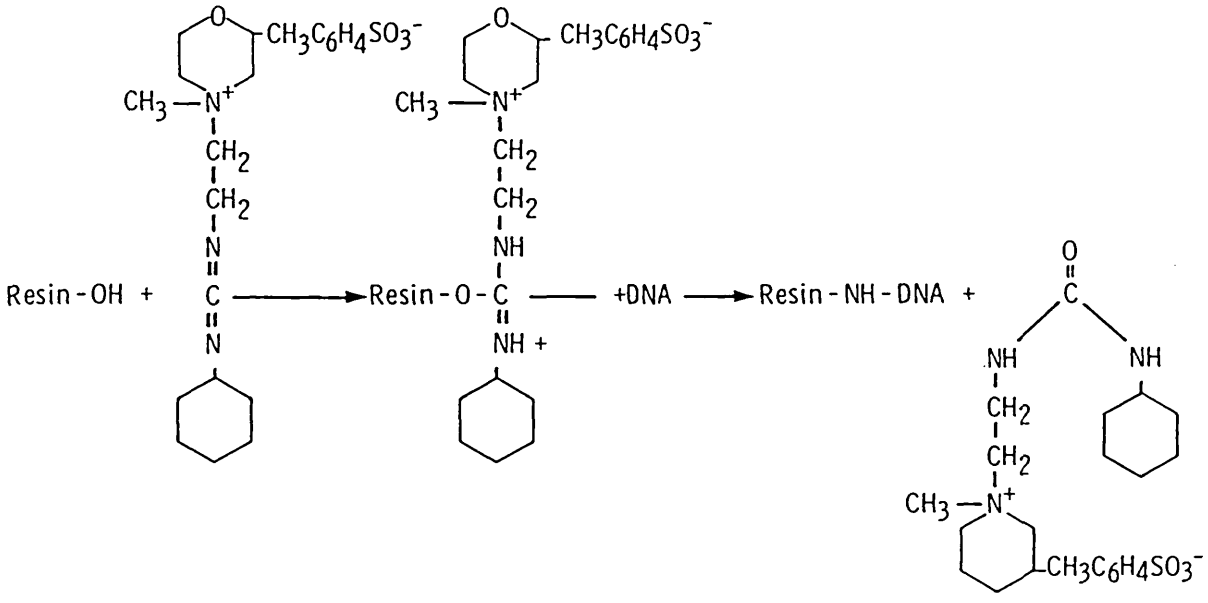


FIGURE 30

CHEMISTRY OF DNA IMMOBILIZATION TO CARBODIIMIDE ACTIVATED RESINS.

Input DNA (pmole/g	^{32}P Assay. Bound DNA (pmole/g)	^{32}P Assay. % Bound	Nuclease Assay. Bound DNA (pmole/g)	Nuclease Assay. % Bound
140	22 ± 8	16	ND	ND
280	50 ± 16	18	38 ± 19	14
420	63 ± 18	15	46 ± 21	11
560	50 ± 21	9	41 ± 17	7

TABLE 2

Effect of input DNA concentration on the efficiency of DNA immobilization on to Sephacryl S-1000, using a carbodiimide chemistry.

HPV 16 483 bp PstI fragment was immobilized by a carbodiimide chemistry, exactly as described in section 3.5.12. The efficiency of immobilization was determined by radiolabelled tracer (^{32}P) and micrococcal nuclease assays, as described in section 3.5.13. Results for the ^{32}P assay take into account non-specifically bound DNA. Nuclease assays were performed on 100 mg batches of resin, in a volume of 400 µl. Nuclease assay results were not determined (ND) for the lowest input DNA concentration, because too little DNA was immobilized to be detected reliably by spectrophotometry. Errors refer to standard deviation from the mean, and each result is the average of three determinations.

The results demonstrate an immobilization efficiency significantly lower than previously reported values (Langdale & Malcolm, 1985).

molecule is not possible, and are thus resistant to hydrolysis with this enzyme. If this hypothesis is correct, it would be reasonable to assume that this effect would be more pronounced for a strand attached at multiple positions, than it would be for a similar molecule immobilized specifically via the 5' end, because every nucleotide involved in a covalent bond with the support would not be released into solution. Over a range of input DNA concentrations, the average amount of DNA immobilized by the carbodiimide procedure according to the nuclease assay is 74% of the average value obtained for the radiolabelled tracer assay. The corresponding figure for DNA immobilized by a diazotization reaction is 72% (see table 3). Surprisingly, it appears that DNA immobilized randomly via the bases is not significantly less accessible to micrococcal nuclease than that immobilized via the 5' phosphate group; clearly, the accessibility of DNA to nuclease is not governed solely by the degree of covalent attachment via the bases.

The maximum immobilization efficiency (18% according to the radiolabelled tracer assay, and 14% according to the nuclease assay) occurs at an input DNA concentration of 280 pmole per gram of resin. As the input DNA concentration increases, the immobilization efficiency decreases, possibly due to competition between DNA molecules for binding sites. The most striking observation resulting from these results is that the immobilization efficiency obtained using this procedure is considerably less than that reported by other workers; for example, SV40 DNA (5,243 bp) was immobilized onto cellulose particles with an efficiency of 30%-45% (Shih & Martin, 1974), and DNA fragments of sizes 2,700, 879, 341 and 201 bp have been coupled to Sephacryl S-500 with respective efficiencies of 25%, 43%, 41% and 39% (Langdale & Malcolm, 1985). The efficiency with which fragments are immobilized by the carbodiimide method decreases with increasing fragment size

Input DNA (pmole/g)	Predicted * Immobilized (pmole/g)	^{32}P Assay. Bound DNA (pmole/g)	^{32}P Assay. % Bound	Nuclease Assay. Bound DNA (pmole/g)	Nuclease Assay. % Bound
140	100	84 ± 20	60	62 ± 24	44
280	200	162 ± 36	58	112 ± 38	40
420	300	248 ± 51	59	176 ± 43	42
560	400	269 ± 60	48	202 ± 58	36

TABLE 3

Effect of input DNA concentration on the efficiency of DNA immobilization on to Sephacryl S-1000, using a diazotization chemistry.

HPV 16 483 bp PstI fragment was immobilized by a diazotization chemistry, exactly as described in section 3.5.11. The efficiency of immobilization was determined by radiolabelled tracer (^{32}P) and micrococcal nuclease assays, as described in section 3.5.13. Results for the ^{32}P assay take into account non-specifically bound DNA. Nuclease assays were performed on 50 mg batches of resin, in a volume of 400 µl. Predicted immobilized DNA refers to the expected amount of immobilized DNA, according to a previously reported average immobilization efficiency of 71% (Bunemann et al., 1982). Errors refer to standard deviation from the mean, and each result is the average of three determinations.

(Gilham, 1968), so although a range of fragment sizes were not tested in this investigation, an immobilization efficiency of around 40% was predicted for a strand of DNA 483 bp long. In fact, the figures shown in table 2 reflect the best of the results obtained using this coupling method; several similar attempts were totally unsuccessful (data not shown), and efficiencies greater than 18% were never observed. Because of the unpredictable nature of the carbodiimide-mediated coupling procedure, an alternative method of immobilization was examined.

4.4.2 Immobilization Of DNA By Coupling To DPTE-resins.

The chemistry of the coupling procedure involving a diazonium ion is shown in figure 9. Initial studies were once again performed using the 483 bp HPV 16 PstI fragment, using DPTE-activated Sephacryl S-1000 as the solid support. Input DNA concentrations were varied in the range 140-560 pmole per gram of resin, and immobilization efficiencies were determined using radiolabelled tracer and nuclease assays; the results are shown in table 3. A maximum immobilization efficiency of 60% (tracer assay) was observed at an input concentration of 140 pmole per gram of resin, with a significant decrease in efficiency at an input concentration of 560 pmole/g. The average immobilization efficiency according to the nuclease assay was 72% of the average efficiency attained according to the tracer assay.

In order to test the effect of DNA fragment size on the efficiency of immobilization, a series of restriction fragments of sizes ranging from 3,601 bp down to 216 bp, together with a synthetic oligonucleotide 20 residues in length, were coupled to Sephacryl S-1000 at an input concentration of 140 pmole/g. The results of this investigation (see table 4) demonstrate that the 2,817 bp fragment is immobilized most efficiently according to tracer (71%) and nuclease (59%)

Fragment Size (bp)	^{32}P Assay. Bound DNA (pmole/g)	^{32}P Assay. % Bound	Nuclease Assay. Bound DNA (pmole/g)	Nuclease Assay. % Bound
3601	87 ± 18	62	64 ± 13	46
2817	99 ± 19	71	83 ± 17	59
1776	94 ± 24	67	70 ± 17	50
1063	78 ± 17	56	67 ± 16	48
555	97 ± 20	69	77 ± 20	55
483	88 ± 20	63	70 ± 19	50
216	74 ± 19	53	59 ± 16	42
20*	56 ± 5	40	ND	ND

TABLE 4

Effect of fragment size on the efficiency of DNA immobilization on to Sephacryl S-1000, using a diazotization chemistry.

DNA fragments (input concentration 140 pmole/g of resin) were immobilized by a diazotization chemistry, exactly as described in section 3.5.11 The efficiency of immobilization was determined by radiolabelled tracer (^{32}P) and micrococcal nuclease assays, as described in section 3.5.13. Results for the ^{32}P assay take into account non-specifically bound DNA. Nuclease assays were performed on 50 mg batches of resin (100 mg in the case of the 216 bp fragment), in a volume of 400 µl. Nuclease assay results were not determined (ND) for the immobilized oligonucleotide PCO3 (*). The PstI restriction fragments were derived from the following sources: 2,817, 1,776, 1,063, 483 and 216 bp, HPV 16; 555 bp, HPV 18. The 3,601 bp EcoRI/PstI fragment was from HPV 6b. Oligonucleotide PCO3 is a PCR primer, derived from the human ß-globin gene. Errors refer to the standard deviation from the mean, and each result is the average of three determinations.

assays, and that the oligonucleotide is coupled least efficiently (40%, according to the tracer assay). Amongst the restriction fragments, there is no obvious correlation between size and immobilization efficiency; in the case of nucleic acids immobilized to DBM activated cellulose, the observed binding efficiencies of homopolymers indicate that negligible binding occurs with A and C residues, and G residues bind twice as efficiently as T residues. If such a bias also occurs in the case of DPTE activated supports, it is possible that the variation in coupling efficiency observed in table 4 is a reflection of the G/C:A/T content of the fragments. Although the 20-mer oligonucleotide is immobilized least effectively, 40% is still covalently coupled to the Sephacryl; although it is not possible to calculate the frequency with which cross-links occur between the activated support and a nucleic acid strand using these results, at least 40% of the 20-mer oligonucleotide sequences are involved in at least one (and possibly several) cross-links. Hybridization studies involving the immobilized oligo were totally unsuccessful, indicating that it is not free to form a stable duplex with a complementary sequence (see section 4.5.1).

In order to determine the most suitable solid support for the sandwich assay, in terms of the efficiency of both covalent DNA immobilization, and subsequent hybridization, three macroporous resins were chosen for study: these were Sephacryls S-500 and S-1000, and Sepharose CL4B. In addition, non-porous Dynospheres M450 were investigated, because of their magnetic properties. Ordinary Sepharose was avoided, because of its instability at temperatures above 40^{o}C, and in organic solvents; Sephadex was also avoided, because of reports of instability in acetone, and high levels (90%) of non-specific binding in the absence of the activating agent (sodium nitrite) (Langdale & Malcolm, 1985). DPTE derivatives of the four chosen supports (all initially

possessing hydroxyl groups on their surface) were prepared as described in sections 3.5.11.1 and 3.5.11.2. Due to limited availability in the case of the Dynospheres, the reaction was scaled down so that a total of 50 mg of DPTE derivative was prepared. In each case, the DNA fragment used was the 483 bp HPV 16 PstI fragment, at an input concentration of 140 pmole/g of resin. The results, shown in table 5, indicate that Sephacryls S-500 and S-1000 covalently bind 58% and 60% of the input DNA respectively (according to the tracer assay); the corresponding figures for the nuclease assay are 36% and 44%. It is interesting to note that the nuclease assay suggests an immobilization efficiency 62% of that obtained for the tracer assay in the case of S-500; the corresponding value is 73% in the case of S-1000. This may be a reflection of the difference in pore size between these two resins; according to the manufacturer (Pharmacia-LKB), although the wet bead diameter in both cases is 40-105 um, the molecular weight fractionation range for polysaccharides is 4×10^4-2×10^7 in the case of S-500, and 5×10^5->10^8 for S-1000. The pore size in S-1000 is such that particles greater than 300-400 nm in diameter are excluded. Calculation has suggested that the exclusion limits for nucleic acids are 1.078 kb in the case of S-500, and around 20 kb in the case of S-1000 (Langdale & Malcolm, 1985). Since the DNA fragment immobilized was just 0.483 kb, it is reasonable to assume that strands are immobilized within the network of pores, as well as to the outside surface of the particles. The smaller pore size in the case of S-500 may result in a greater proportion of the immobilized molecules becoming inaccessible to approach of the nuclease molecule.

The immobilization results obtained for Dynospheres M450 and Sepharose CL4B were poor; after subtraction of the value obtained for non-covalent binding, efficiencies of 6% and 3% respectively were

Support	^{32}P Assay.			Nuclease Assay.		
	Input DNA (pmole/g)	Bound DNA (pmole/g)	% Bound	Input DNA (pmole/g)	Bound DNA (pmole/g)	% Bound
Sephacryl S-500	140	81 ± 18	58	140	51 ± 26	36
Sephacryl S-1000	140	84 ± 20	60	140	62 ± 24	44
Dynospheres M450	140	8 ± 2	6	140	ND	ND
Sepharose CL4B	140	4 ± 1	3	140	ND	ND

ND, not determined.
Each result represents the mean of five determinations. Errors refer to maximum variation from the mean.

TABLE 5

Efficiency of covalent immobilization of 483 bp HPV 16 PstI fragment to four different solid supports.

DNA was immobilized by a diazotization chemistry, exactly as described in section 3.5.11. The efficiency of immobilization was determined by radiolabelled tracer and micrococcal nuclease assays, as described in section 3.5.13. It was not possible to perform the nuclease assay on Dynospheres M450 or Sepharose CL4B, because too little DNA was immobilized to be detected reliably by spectrophotometry.

The results clearly demonstrate that most efficient binding is achieved using Sephacryl S-1000 as the support.

observed, according to the tracer assay. Due to the low binding, it was not possible to use the nuclease assay, because the $O.D._{260\ nm}$ of the released nucleic acid was too low to be detected reliably by spectrophotometry. The result obtained for the Dynospheres was particularly disappointing, because initial attempts to manipulate the particles with a magnet indicated that it would be perfectly possible to dispense with the centrifugation step during pre- and post-hybridization washes. This could have had a dramatic effect on the ease with which the sandwich assay could be subjected to automation in the future. Soon after this failure to couple DNA to Dynospheres M450 using a diazotization chemistry, a similar study originating from the laboratory of origin of the magnetic particles (Apothekernes Laboratorium A.S., Oslo) reported the same result (Lund et al., 1988). Although no conclusive evidence to explain the coupling failure is presented, the authors suggest that scaling down Bunemann's procedure for preparing the DPTE-Dynospheres derivative may result in less favourable conditions. The expense involved in obtaining larger amounts of Dynospheres precluded studies to investigate this hypothesis; however, initial results using carbodiimide-mediated end-attachment of DNA fragments or oligonucleotides to amino- or carboxyl-modified (rather than hydroxyl-modified) Dynospheres have resulted in immobilization efficiencies of 65% and 60% respectively (Lund et al., 1988).

Immobilization studies involving Sepharose CL4B resulted in a partial breakdown of the physical structure of the gel matrix, even though cross-linked Sepharose is reputably stable in the pH range 3-14, and also in many organic solvents (including acetone). It is possible that exposure to 1,4-butanediol diglycidyl ether, 2-aminothiphenol or 1.38 M HCl results in disruption of the polysaccharide chains. Regardless of the reason for the degradation, Sepharose CL4B was not a suit-

able support for the immobilization of DNA by a diazotization chemistry, because of the poor coupling efficiency observed.

4.4.3 Time-course Of DNA Immobilization On DPTE-resins.

According to the protocol reported by Bunemann, the reaction between DNA and the activated DPTE-resin should be allowed to proceed for 2 days, in order to attain the maximum immobilization efficiency. In order to investigate this statement, the time-course of DNA coupling to activated Sephacryl S-1000 was studied. The DNA fragment used was linearized pJN3 (a pUC8 derivative, containing a 216 bp insert derived from the genome of HPV 16 [fragment 'g' in figure 16]; total size, 2,894 bp), at an input concentration of 140 pmole/g of resin; previous results have shown that DNA fragment size does not have a significant influence on the immobilization efficiency. Immobilization reactions were allowed to proceed for 10 minutes, 1 hour, 24 hours and 48 hours, after which time the resin was washed, and the coupling efficiency determined by the radiolabelled tracer assay (but not by the nuclease assay). The results are shown in figure 31. The control value represents the average of values obtained for each of these time points, in the absence of the activating agent (and is thus an average representation of non-covalent attachment). The error bars refer to the maximum variation from the mean observed in three separate determinations. The results indicate that the immobilization efficiency attained after just ten minutes of reaction (73%) is only 9% lower than the value obtained after a 2 day reaction (82%); these efficiencies are considerably higher than those recorded in earlier studies (see table 4). Since the same batch of APTE-Sephacryl S-1000 was used in both investigations, it is possible that the variation in immobilization efficiency resulted from a more extensive activation on production of

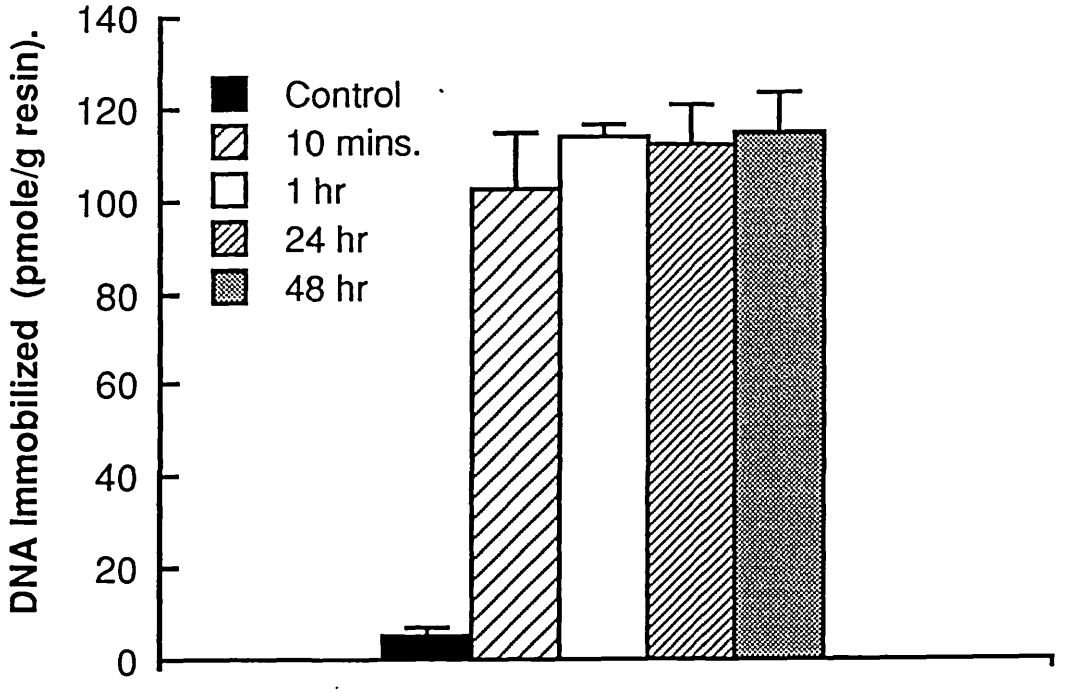


FIGURE 31

Time-course of DNA immobilization by diazotization to DPTE-Sephacryl S-1000.

DNA used was Pst1 linearized pJN3 (pUC8 + 216 bp HPV 16 Pst1 fragment), at an input concentration of 140 pmole/gram of Sephacryl S-1000. The reaction was followed by incorporating 2 fmole of linear pJN3, labelled with ^{32}P by Klenow end-filling to a specific activity of 2.3 x 10^2 dpm/fmole.
Each bar represents the average of three determinations.

the DPTE-derivative in the case of the immobilization time-course study; the presence of a greater number of reactive diazonium ions on the Sephacryl beads could reasonably be predicted to increase the number of cross-links formed with the nucleic acid strands, possibly decreasing the number of DNA molecules which escape without forming a single cross-link. The reason for this postulated activation discrepancy is unclear, since identical protocols were followed in both studies. Although it appears that the immobilization reaction essentially reaches completion after an hour, hybridization studies performed with the coupled DNA suggest that a longer reaction time results in increased stability (see section 4.5.2; figure 34).

It is important to bear in mind at this point that the highest immobilization efficiencies are not necessarily most desirable; reduced coupling yields may be outweighed by excellent availability of immobilized sequences for hybridization (Bunemann & Westhoff, 1983).

4.4.4 Stability Of DNA Immobilized On DPTE-resins.

As discussed in section 1.3.2, in order to be suitable for use as 'fragment A' in a sandwich assay, it is essential that the cross-link(s) between the support and the nucleic acid fragment result in a stable attachment. It has been reported that, in general, DNA covalently coupled to a solid support leaches off in a biphasic manner; this effect appears to be more pronounced in the case of DNA immobilized by diazotization than by CNBr activation (Bunemann, 1982). In order to investigate the rate at which DNA is lost after covalent coupling to Sephacryl S-1000, a batch of DNA-resin was produced exactly as described in section 3.5.11. The DNA immobilized was linearized pJN3, at an input concentration of 140 pmole/g of resin; the immobilization efficiency was initially determined at 66%, according to the nuclease

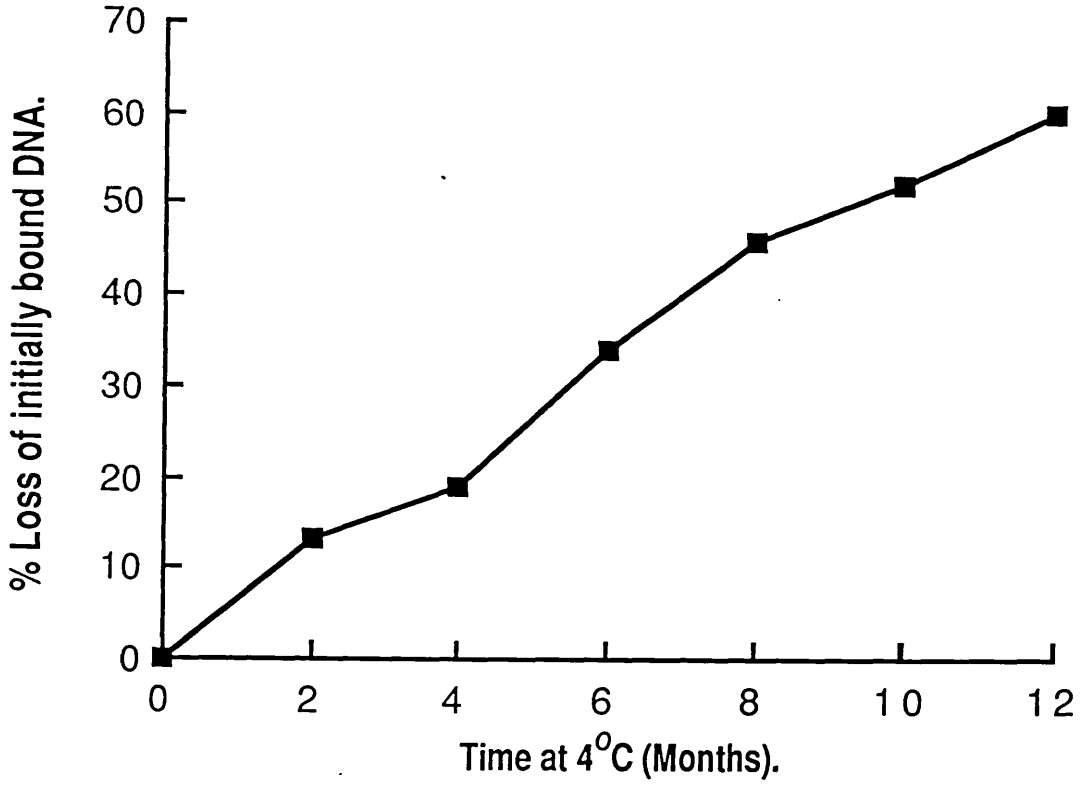


FIGURE 32

Time-course of loss of immobilized DNA from DPTE-Sephacryl S-1000.

Immobilized DNA was linear pJN3 (pUC8 + 216 bp HPV 16 PstI fragment). DNA-resin was stored at 4°C for the indicated period, after which time a 50 mg aliquot was removed, and washed thoroughly (section 3.5.11.3). Following analysis by micrococcal nuclease assay (section 3.5.13.2), the percentage loss of immobilized DNA was calculated. The amount of DNA initially bound was 92.4 pmole/gram of Sephacryl S-1000 (determined by the nuclease assay).
Each point represents a single determination.

assay. The resin was stored at 4°C, and aliquots were removed at two month intervals, for a period of one year, in order to determine the percentage loss of initially bound DNA; the results are shown in figure 32. Great care was taken to wash the aliquots thoroughly prior to assay, in order to remove non-covalently bound DNA. The tracer assay was not suitable for this study, because of the short half-life of the isotope ^{32}P (14.3 days), and the possibility that radiolysis could increase the rate of loss of immobilized DNA. The results demonstrate that, unlike the data reported by Bunemann, DNA is lost at a rate of approximately 5% per month, over the 12 month period. It is possible that this loss is a result of selective breakage of unstable bonds between nucleotides and the solid support; this would be particularly pronounced in the case of DNA strands attached at a single position. Alternatively, immobilized DNA may be attacked by contaminating nucleases, although this is unlikely, since the resin and buffers were pre-sterilized prior to use. Throughout the course of this study, DNA-resin was always used within 4 months of manufacture, so approximately 80% of initially coupled DNA remained covalently bound. Since every batch of resin was pre-hybridized for 2 hours at 65°C prior to use in a sandwich assay, it is reasonable to assume that non-covalently bound DNA was removed prior to hybridization; this prevents a reduction in signal due to a competition solution phase hybridization between uncoupled DNA and target sequences.

4.4.5 Summary Of Immobilization Data.

The results obtained from studies on the covalent immobilization of DNA can be summarized as follows:

(a) Immobilization efficiencies obtained by 5' end-attachment of restriction fragments to Sephacryl S-1000 by carbodiimide activation

were highly variable; the highest efficiency obtained (according to the ^{32}P tracer assay) was 18%. The method was frequently totally unsuccessful.

(b) The following results were obtained for DNA immobilized by diazotization:

(i) Over the range of input DNA concentrations 140-560 pmole/g of resin, the maximum immobilization efficiency (60%) was observed at the lowest input concentration.

(ii) Over the range of fragment sizes 3,601-216 bp, size had no significant effect on immobilization efficiency (typically within the range 53%-85%). An oligonucleotide 20 residues in length was coupled with an efficiency of 40%.

(iii) Of four supports tested, activated Sephacryl S-1000 resulted in the highest binding efficiency, with the lowest levels of non-covalent attachment. 73% of the immobilized strands were accessible to micrococcal nuclease. Dynospheres M450 and Sepharose CL4B were unsuitable, because of extremely low coupling efficiencies.

(iv) Although the immobilization reaction appears to reach completion after approximately one hour, most stable coupling resulted if the reaction was allowed to proceed for 48 hours (see section 4.5.2).

(v) The stability of coupled DNA is such that approximately 5% of the DNA is lost each month, for at least 12 months after immobilization. Unlike previously reported results (Bunemann & Westhoff, 1983), the DNA is lost in a linear, rather than in a biphasic fashion.

(c) Of the two methods used to determine the immobilization efficiency, the ^{32}P tracer assay was found to give higher results than the

nuclease assay; this probably reflects the fact that the former is a measure of the total DNA immobilized, whereas the latter is a representation of the amount of coupled nucleic acid which is accessible to the nuclease molecule. It is possible that the nuclease assay gives a more accurate indication of the availability of DNA for hybridization; DNA not accessible to nuclease may also be inaccessible to a complementary nucleic acid strand, or may be immobilized in such a way that the hybrid formed between the two strands is of significantly reduced stability (see section 4.5.1).

All subsequent investigations were performed using Sephacryl S-1000 as the solid support, because it is macroporous, physically and chemically stable, and binds DNA efficiently, with a low level of non-covalent attachment. S-1000 was chosen in preference to S-500, because immobilization efficiencies were greater, and a higher proportion of the coupled DNA was accessible to micrococcal nuclease, in the case of S-1000. The diazotization coupling procedure was chosen in preference to the carbodiimide procedure, because of unpredictability and low coupling yields in the case of the latter method. In addition, DNA immobilized on Sephacryl by diazotization has been reported to exhibit excellent hybridization properties (Bunemann, 1982; Langdale & Malcolm, 1985). An input DNA concentration of 140 pmole/g was ideal; assuming a coupling efficiency of at least 60%, an immobilized concentration of 84 pmole/g would result. This is equivalent to 84 fmole/mg, so a single milligram of DNA-resin should provide a vast excess of immobilized probe for capture of the target sequence.

Having determined the most suitable solid support and chemistry with respect to DNA immobilization, the hybridization properties of the immobilized DNA were investigated.

4.5 One-step Hybridizations.

The purpose of the one-step hybridization assay was to determine the availability of immobilized DNA fragments for hybridization with a complementary probe free in solution. In order to optimize this procedure, a number of hybridization buffers and washing conditions were tested, and the effects of concentration and size of the immobilized DNA on the hybridization efficiency were examined. The efficiency of hybridization was calculated as a percentage of the maximum theoretical value, according to the formula;

$$\% \text{ Hybridization Efficiency} = \frac{\text{Final Hybridization Value (dpm)} - \text{Non-specific binding (Control) Value (dpm)}}{\text{Initial Input Of Probe (dpm)}} \times 100$$

Two controls were included in each batch of one-step assays, in order to determine the extent of non-specific binding: (i) a control in which DNA-resin was replaced by DNA-free resin (activated and subsequently washed as described in section 3.5.11, without the addition of DNA); (ii) a control in which non-complementary immobilized DNA (sonicated calf thymus DNA, for example) was used in place of immobilized HPV-derived DNA. Occasionally, a third control was included, involving hybridization of a non-complementary probe (labelled calf thymus DNA, for example) with immobilized HPV-derived DNA. All three controls were consistently found to give similar values for the level of non-specific binding, indicating that this binding is due to an interaction between the probe and the solid support, rather than between the probe and the immobilized DNA. In all one- and two-step assays, the radioactive probe was detected in a scintillation counter, using the Cerenkov method; the quenching effect of the Sephacryl beads on the number of counts was measured, and found to be negligible for masses of Sephacryl up to 5 mg (data not shown).

4.5.1 One-step Assays Using A Labelled Restriction Fragment As Probe.

It is important to optimize the efficiency of the one-step hybridization, because the interaction between immobilized sequences and a complementary probe free in solution is analogous to that between the immobilized DNA and the target sequence in a two-step sandwich assay. In order to investigate the effect of hybridization buffer composition on the efficiency of one-step hybridization, three buffers were chosen for study, each of which was based on a previously published protocol. The composition of each of the three buffers (A, B and C) is shown in the legend to table 6; buffer A was tested because it allows closely related HPV types to be readily distinguished in a slot-blot assay (Wickenden et al., 1987 a; see figure 20). In fact, the ability to discriminate between closely related virus types (for instance, HPV 6b and HPV 11, which share 82% homology at the nucleic acid level) results from the extremely stringent post-hybridization washing conditions (see table 6). Buffer B was tested because it has been used successfully in hybridization assays based on DNA immobilized by diazotization onto Sephacryl S-500 (Langdale & Malcolm, 1985). Buffer C was initially used in the first filter-based sandwich assay designed for the detection of adenovirus infection in clinical samples. The results of overnight hybridizations performed with buffers A, B and C are shown in table 6. A fifty-fold excess of immobilized DNA over probe was used, and the results clearly demonstrate that the most efficient washing occurs in buffer B. It is important to bear in mind that the labelled probe used is double-stranded, so re-annealing of labelled strands to their complementary sequences in solution will result in withdrawal of a proportion of the probe from the hybridization equilibrium. The hybridization and washing conditions associated with buffer B result in around 64% of the probe hybridizing specif-

Buffer.		Initial dpm ($x10^3$)	Final dpm ($x10^3$)	% Initial Counts Remaining.	% Hyb.
A	E	1,678	79	4.7	3.4
	C	1,642	21	1.3	
B	E	1,721	1,127	65.5	64.1
	C	1,628	23	1.4	
C	E	1,663	271	16.3	14.7
	C	1,702	28	1.6	

E= Experimental value.
C= Control value.

TABLE 6

Effect of buffer composition on the efficiency of one-step hybridization.

0.5 pmole of HPV 16 483 bp PstI fragment, immobilized by diazotization onto Sephacryl S-1000, was hybridized at 65°C, in 50 µl of the indicated buffer, with 10 fmole of complementary probe, labelled by random priming to an activity of approximately 1.6 x 10^5 dpm/fmole. The hybridization buffers used were as follows: **A**, 0.08 M Tris-HCl, pH 7.8, 0.75 M NaCl, 0.05% w/v SDS, 0.004 M EDTA, 5 x Denhardt's solution, 100 µg/ml denatured, sonicated salmon sperm DNA; **B**, 40 mM PIPES, pH 6.5, 0.6 M NaCl, 1 mM EDTA, 0.1% w/v SDS, 250 µg/ml denatured, sonicated salmon sperm DNA; **C**, 4 x SSC, 0.02% w/v polyvinylpyrrolidone, 0.02% w/v Ficoll, 0.25% w/v SDS, 200 µg/ml denatured, sonicated salmon sperm DNA. Post-hybridization washing was performed as described in section 3.5.14.1, for 3 x 10 minutes at 65°C, in 1 ml of the following buffer: **A**, 0.1 x SSC, 0.1% w/v SDS; **B**, hybridization buffer (minus salmon sperm DNA); **C**, 1 x SSC, 0.1% w/v SDS. All buffers and washing conditions are based on published protocols (**A**, Wickenden et al., 1987 a; **B**, Langdale & Malcolm, 1985; **C**, Ranki et al., 1983 a,b). Control values were obtained by hybridizing probe to sonicated, immobilized calf thymus DNA (average fragment size around 500 bp). The final percentage hybridization value is shown after subtraction of the control value. Each result is the average of three determinations.

The results are discussed fully in the text.

ically with immobilized sequences. The corresponding figures for buffers A and C are much lower (3.4% and 14.7% respectively). The possible reasons for these poor efficiencies are: (i) the buffer composition and hybridization temperature are too stringent to allow formation of the hybrid complex between immobilized and labelled DNA; (ii) the washing conditions (salt concentration and temperature) are too stringent, and result in dissociation of complexes formed between immobilized and labelled sequences. The washing conditions used with buffer A (65^{o}C, 0.1 x SSC, 0.1% w/v SDS) are clearly very stringent; the hybrid complex formed between DNA immobilized on a nitrocellulose membrane and a labelled probe appears to be more stable than that formed between DNA immobilized by diazotization onto Sephacryl S-1000 and the same probe. The significantly lower hybrid stability in the latter case may be due to the frequency and nature of the cross-links formed between the Sephacryl and the immobilized DNA; nucleotides involved in a covalent linkage may be unavailable for base pairing with the complementary DNA strand, and could thus have a destabilizing effect on the hybrid complex similar to that caused by a base pair mismatch. Because very little is known regarding the frequency of cross-linking and the hybridization properties of a nucleotide involved in such a bond, this hypothesis is purely speculative. Nevertheless, it is clear from the results shown in table 6 that buffer B is the most suitable of the three buffers tested, because it results in a high hybridization efficiency (65%) with a low level of non-specific binding (1.4%). It is interesting to note that non-specific binding is relatively constant, regardless of the nature of the hybridization buffer and washing conditions; even after washing under highly stringent conditions (65^{o}C, 0.1 x SSC, 0.1% w/v SDS), the non-specific signal is 81% of that remaining after washing at a lower stringency (65^{o}C, 1 x

SSC, 0.1% w/v SDS). Increasing the number of washes above three had no effect on either the hybridization signal or the non-specific background (data not shown).

The effect of hybridization rate enhancers like dextran sulphate and polyethylene glycol was not investigated, because a number of reports have demonstrated that addition of these chemicals can have a negative influence on sandwich hybridization efficiency (Ranki et al., 1983 a,b; Langdale & Malcolm, 1985). Rate enhancers function by effectively increasing the concentration of nucleic acid in solution, due to the exclusion of DNA or RNA molecules from the volume occupied by the hydrated polymer. This effect can be variable when a probe with a competing strand is being used (or when the target molecule is double-stranded, as is the case with the papillomaviruses), because the polymer can increase the rate of probe or target self-reassociation (potentially decreasing the signal), but can also enhance the formation of networks of labelled molecules at the point of hybridization to the target nucleic acid strand (potentially increasing the signal) (Wahl et al., 1979). In the case of sandwich assays where both probe and target have competing strands in solution, self-reassociation appears to be the predominant effect; for this reason, rate enhancers were not studied in this investigation.

For the reasons described above, buffer B (40 mM PIPES, pH 6.5, 0.6 M NaCl, 1 mM EDTA, 0.1% w/v SDS, 250 ug/ml sonicated, denatured salmon sperm DNA) was chosen for use in all future one- and two-step hybridization reactions, and post-hybridization washes were performed at 65^{o}C for 3 x 10 minutes, in hybridization buffer (minus salmon sperm DNA).

In order to examine the effect of immobilized DNA concentration on the efficiency of the one-step hybridization, four different batches

of Sephacryl S-1000, each with a different concentration of immobilized DNA, were prepared. The concentrations of coupled DNA, according to the nuclease assay, were 62, 112, 176 and 202 pmole/g of resin. 0.5 pmole of immobilized DNA was hybridized overnight with 10 fmole of complementary probe, as described in the legend to table 7. The results clearly show that, although concentration of immobilized DNA has little effect on hybridization efficiency, the highest value obtained (66%) was for 112 pmole of DNA/g of resin. This corresponds well with the results shown in table 6. It is interesting to note that as the volume of resin required (resuspended at a concentration of 1 mg/2 ul) decreases, the non-specific background decreases. Although this effect does not appear to be linear (the background with 5 ul of resin is 40% of that resulting with 16.1 ul, rather than just 31%), it is obvious that background results from interaction between the probe and the solid support; the more Sephacryl present in the reaction, the higher the proportion of the probe which will be non-specifically bound. In order to keep background levels as low as possible, the mass of Sephacryl used in each assay should be kept to a minimum; however, in the case of DNA-resin suspended at a concentration of 1 mg/2 ul, volumes of less than 3 ul were not easy to manipulate accurately. More dilute suspensions were also found to result in pipetting inaccuracies (data not shown); for these reasons, DNA-resin was routinely suspended at a concentration of 1 mg/2 ul, and volumes of 3-5 ul were used for each two-step sandwich assay (although considerably larger volumes were used for one-step assays). Assuming an average immobilization efficiency of 50% (according to the nuclease assay), and an initial input concentration of 140 pmole/g of resin (the amount found to be optimum in table 3), this represents an immobilized DNA concentration of 70 fmole/mg of resin. Thus, 3-5 ul of the DNA-resin suspension is equivalent to

Conc. Of DNA On Resin (pmole/g)	Required Vol. Of Resin (ul)		Initial dpm ($x10^3$)	Final dpm ($x10^3$)	% Hyb.
62	16.1	E	3,216	1,928	57
		C	3,179	81	
112	8.9	E	3,198	2,177	66
		C	3,209	60	
176	5.7	E	3,227	2,038	62
		C	3,294	44	
202	5.0	E	3,186	2,078	64
		C	3,195	32	

TABLE 7

Effect of concentration of immobilized DNA on efficiency of one-step hybridization.

0.5 pmole of HPV 16 483 bp PstI fragment, immobilized by diazotization on to Sephacryl S-1000, was hybridized at 65°C, in 50 µl of buffer, with 10 fmole of complementary probe, exactly as described in section 3.5.14.1. The probe was labelled to an activity of approximately 3.2 x 10^5 dpm/fmole. Experimental (E) and control (C) values were obtained using the volume of resin indicated, resuspended in TE buffer to 1 mg/2 µl. The concentration of DNA on the resin was determined by the micrococcal nuclease assay (section 3.5.13; see table 3). The control values were obtained by hybridizing probe to sonicated, immobilized calf thymus DNA (average fragment size around 500 bp). The final percentage hybridization value is shown after subtraction of the control value. Each result represents the average of five determinations.

The results are discussed fully in the text.

105-175 fmole of coupled DNA; the amount of immobilized DNA used in two-step assays was typically within this range.

The effect of immobilized fragment size on the efficiency of the one-step hybridization was investigated, as described in the legend to table 8. The results show that, in the size range 3,601 to 216 bp, fragment size does not have a significant effect on hybridization efficiency. The best results were obtained for the 555 bp fragment (66% efficiency); the equivalent value for the 3,601 bp fragment was 55%. Although there is variation in hybridization efficiency between these two extremes, there appears to be no correlation with immobilized fragment size. The hybridization efficiency obtained for the coupled 20-mer oligonucleotide is extremely low (3%), although it is important to point out that the probe used was a 341 bp beta-globin probe, rather than an exactly complementary 20-mer oligonucleotide. This low value could be due to: (i) unavailability of the oligonucleotide for hybridization, due to the presence of cross-links on internal (rather than terminal) residues; (ii) over-stringent hybridization and/or washing conditions; (iii) instability of the hybrid complex formed between the 341 bp fragment and the oligo, due to solution-phase competition of the unlabelled 341 bp complementary strand. In retrospect, the experiment with the oligonucleotide was poorly designed; improvements which could have been made are: (i) the hybridization and washing conditions should have been reduced in stringency, to optimize hybridization of the oligo; (ii) the probe used should ideally have been a complementary kinase-labelled 20-mer oligonucleotide. However, subsequent studies involving oligonucleotides immobilized by diazotization onto Sephacryl S-1000 have demonstrated that, even under these favourable conditions, the hybridization efficiency is poor (5%-10%) (U.B. Voss, personal communication).

Immobilized Fragment Size (bp)	Initial dpm In Reaction ($X 10^3$)	Final dpm In Reaction ($X 10^3$)	% Hybridization
3,601	25,461	14,274	55
CONTROL	24,860	190	
2,817	20,056	12,279	60
CONTROL	21,873	178	
1,776	11,950	7,153	59
CONTROL	10,676	119	
1,063	7,552	4,765	62
CONTROL	7,452	112	
555	3,679	2,487	66
CONTROL	3,720	69	
483	3,387	1,989	57
CONTROL	3,485	48	
216	1,638	1,046	62
CONTROL	1,580	26	
20*	2,560	97	3
CONTROL	2,546	29	

TABLE 8

Effect of immobilized fragment size on the efficiency of one-step hybridization.

0.5 pmole of DNA immobilized by diazotization on to Sephacryl S-1000 was hybridized at 65°C, in 50 µl of buffer, with 10 fmole of complementary probe, exactly as described in section 3.5.14.1. Probes were labelled by random priming to the following specific activities (dpm/fmole): 3,601 bp, 2.4×10^6; 2,817 bp, 1.9×10^6; 1,776 bp, 1.2×10^6; 1,063 bp, 7.2×10^5; 555 bp, 3.6×10^5; 483 bp, 3.2×10^5; 216 bp, 1.4×10^5. In each case, the mass of Sephacryl used for experimental and control determinations was constant. The control value was obtained by hybridizing probe to sonicated, immobilized calf thymus DNA (average fragment size around 500 bp). The final percentage hybridization value is shown after subtraction of the control value. The DNA fragments were derived from the sources described in the legend to figure 4. The immobilized oligonucleotide PCO3 (*) was hybridized with a 341 bp ß-globin gene fragment (insert from plasmid pKJ7), labelled by random priming to an activity of 2.5×10^6 dpm/fmole. Each result is the average of five determinations.

The results are discussed fully in the text.

4.5.2 Effect Of Length Of Immobilization Time On The Efficiency Of Hybridization Of Immobilized DNA.

Studies to determine the time-course of the immobilization of DNA onto Sephacryl S-1000 by diazotization suggested that the reaction is essentially complete after one hour (section 4.4.3). In order to examine the effect of reaction time on the hybridization properties of immobilized DNA, one-step assays were performed on four batches of DNA-resin which had been prepared by reaction times of 10 minutes, 1 hour, 24 hours and 48 hours. In all other respects, the overnight hybridization assays were identical. The results (shown in figure 34) clearly demonstrate that hybridization efficiency increases as a function of increased time of immobilization reaction. The possible explanations for this are as follows: (i) short coupling times result in unstable linkages between DNA and the activated support; these linkages are subsequently broken on overnight hybridization at 65^{o}C; (ii) short coupling times result in a lower availability of immobilized DNA than longer coupling times. The second point is very unlikely; in fact, it would be reasonable to predict that a longer coupling time should result in an increase in the frequency with which cross-links form between the support and the DNA strand, effectively reducing the amount of available DNA. It is possible that a more stringent washing step is required immediately after immobilization, in order to detach all but the most firmly coupled DNA; the recommended procedure involves washing with 0.4 M NaOH at 40^{o}C, followed by a rinse in TE buffer at room temperature (see section 3.5.11.3; Bunemann et al., 1982). In this investigation, the ratio of immobilized DNA (A) to labelled probe (B) was 50:1; a further increase in this ratio did not result in an increase in hybridization efficiency, but rather resulted in a higher non-specific background signal, due to the increased mass of resin

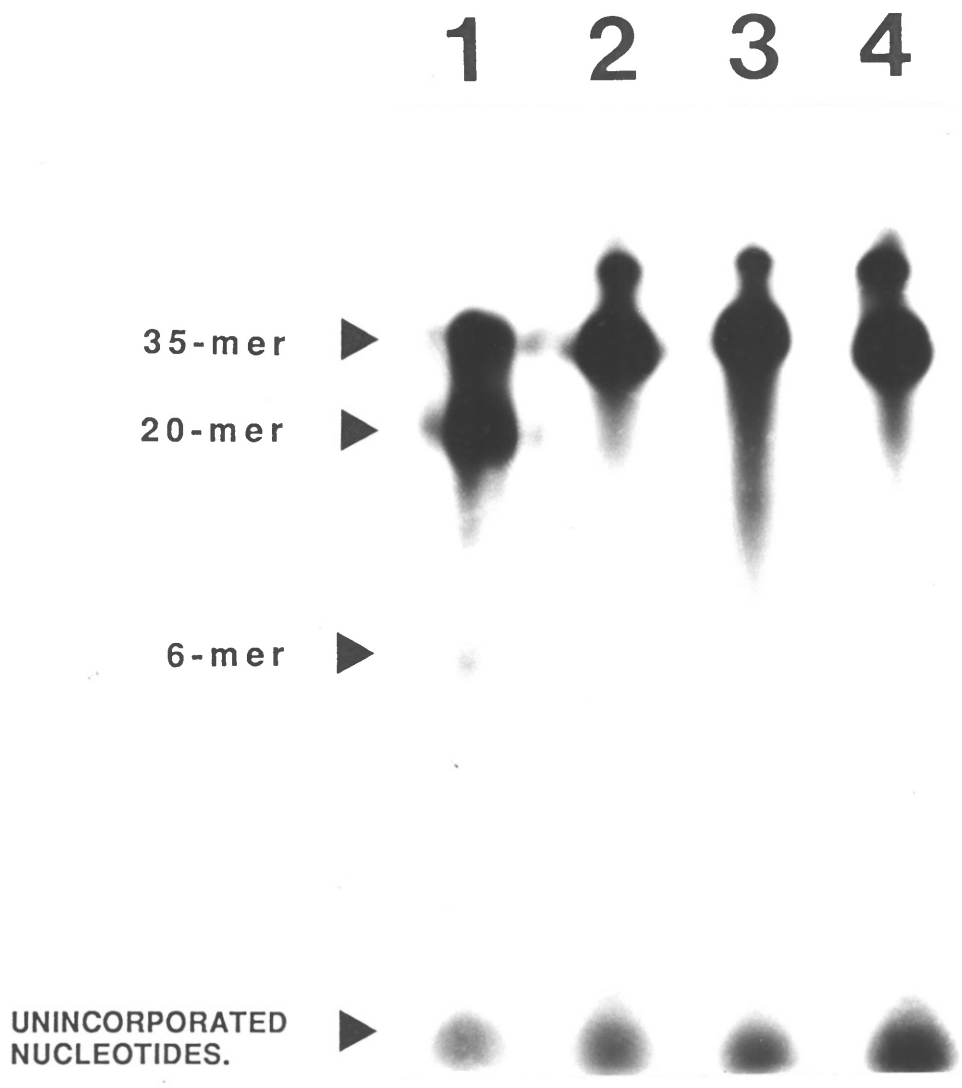


FIGURE 33

Polyacrylamide gel electrophoresis for the characterization and purification of synthetic oligonucleotides.

Lane 1 contains a mixture of oligonucleotides (1 pmole each of a 35-mer, 20-mer and 6-mer), kinase end-labelled with ^{32}P to an activity of approximately 5 x 10^2 dpm/fmole. Lanes 2, 3 and 4 contain 1 pmole of 30-mer oligonucleotides PR11-1, PR16-1 and PR18-1 (kinase end-labelled with ^{32}P to around 5 x 10^2 dpm/fmole) respectively. The labelled oligonucleotides were electrophoresed through a 25% urea polyacrylamide gel, as described in section 3.4.4.2. After drying, the gel was autoradiographed for 1 hour, using X-OMAT XAR-5 film.

The results clearly show that the 30-mers are largely free from low molecular weight contaminants. The higher molecular weight band associated with the 30-mers, but not with the markers in lane 1, is discussed in the text.

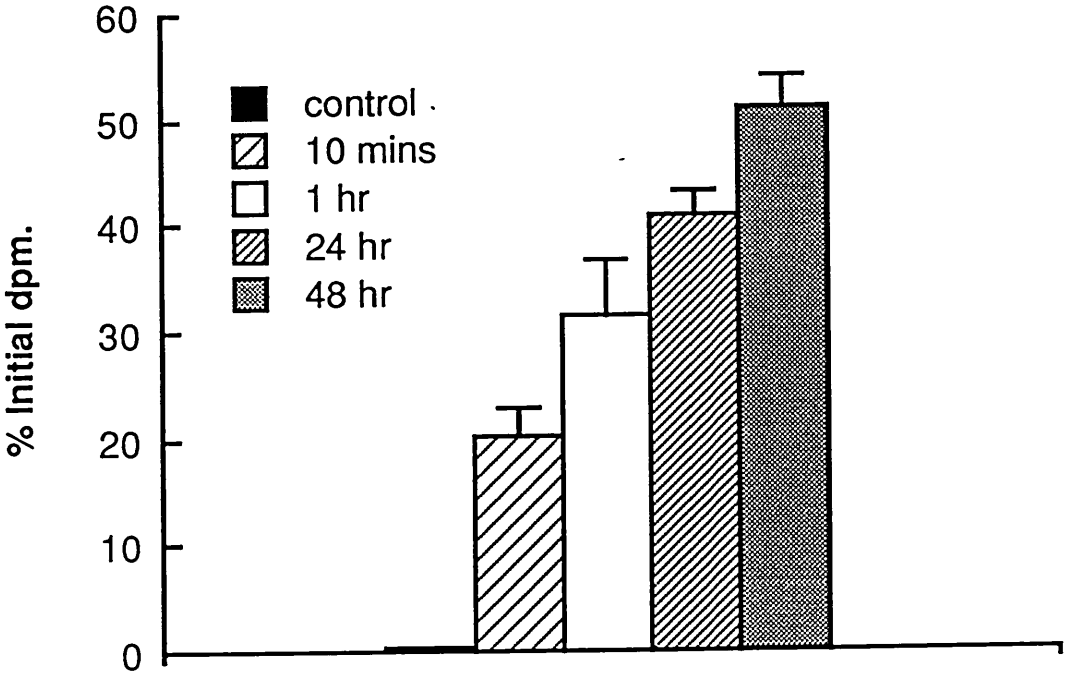


FIGURE 34

Effect of duration of diazotization reaction on hybridization efficiency of DNA immobilized on DPTE-Sephacryl S-1000.

Immobilized DNA used was Pst1 linearized pJN3 (pUC8 + 216 bp HPV 16 Pst1 fragment) (A). 0.5 pmole of A immobilized by diazotization for the time indicated was hybridized at 65°C, in 50 µl of buffer, with 20 fmole of probe (HPV 16 216 bp Pst1 fragment, labelled by random priming to an activity of 3.9×10^5 dpm/fmole).
Each bar represents the average of two determinations.

required (data not shown). In general, throughout the study, an A:B ratio of between 25:1 and 50:1 was found to result in maximum hybridization efficiency, with the highest signal/background (S/B) ratio. It is clear from figure 34 that hybridization with DNA allowed to couple for just 10 minutes results in a hybridization efficiency approximately 40% of the equivalent value for DNA allowed to couple for 48 hours. This discrepancy in efficiency is probably due to a combination of two factors: (i) the lower coupling time results in a higher proportion of the cross-links between DNA and resin being unstable; on hybridization overnight at 65^{o}C, unstable bonds break, releasing previously immobilized DNA into solution. This results in the actual concentration of immobilized DNA being less than the predicted concentration, so the A:B ratio may be less than 25:1, resulting in sub-optimal hybridization efficiencies; (ii) release of previously coupled DNA results in solution phase competition with probe, thus reducing the proportion of labelled molecules free to hybridize with DNA strands coupled to the support.

For the reasons described above, immobilization reactions were always allowed to proceed for 48 hours, and DNA-resin batches were washed overnight at 65^{o}C, in pre-hybridization buffer, prior to nuclease assay, and use in sandwich hybridization.

4.5.3 Time-course Of One-step Hybridizations.

The time-course of the one-step hybridization was determined for restriction fragment probes, and also for kinase end-labelled oligonucleotides, as described in the legends to figures 35 and 36. Labelled oligonucleotides were purified by polyacrylamide gel electrophoresis, as described in section 3.4.4.2; an autoradiograph of a typical preparative gel is shown in figure 33. The cause of the highest

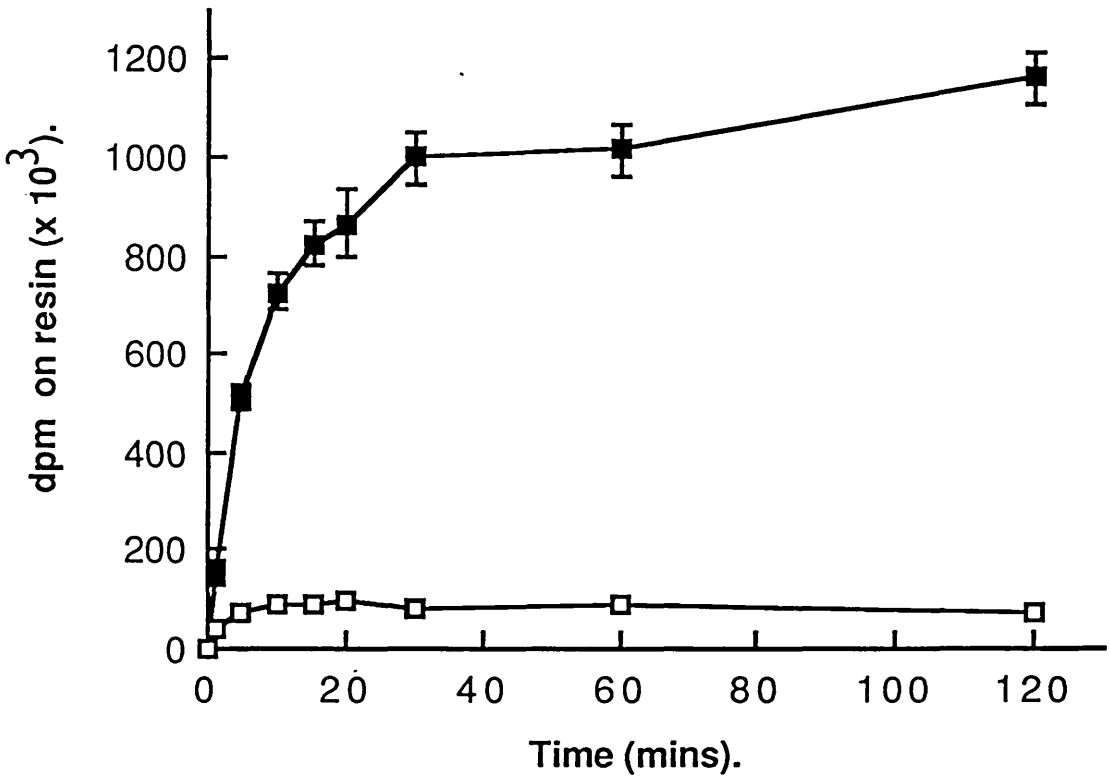


FIGURE 35

Time-course of one-step hybridization of restriction fragments to diazotized DNA-Sephacryl S-1000.

Immobilized DNA used was HPV 16 483 bp Pst1 fragment (A). 0.5 pmole of A immobilized on Sephacryl S-1000 was hybridized at 65°C, in 50 µl of buffer, with 5 fmole of probe (HPV 16 483 bp Pst1 fragment [■], or HPV 16 1063 bp Pst1 fragment [□]; labelled by random priming with ^{32}P to an activity of 3.3 x 10^5 dpm/fmole. Each point represents the average of five determinations.

molecular weight bands present in lanes 2, 3 and 4 is unknown; it is possible that it results from oligonucleotide secondary structure, which leads to retarded migration on passage through the pores in the gel.

The results for the restriction fragment indicate that a hybridization efficiency of approximately 70% is achieved after just 2 hours; this efficiency is significantly higher than previously determined values (see table 8), possibly because a higher A:B ratio (100:1) was used, as opposed to the more commonly used ratio of 50:1. However, concomitant with the higher hybridization efficiency is an increase in the level of non-specific binding.

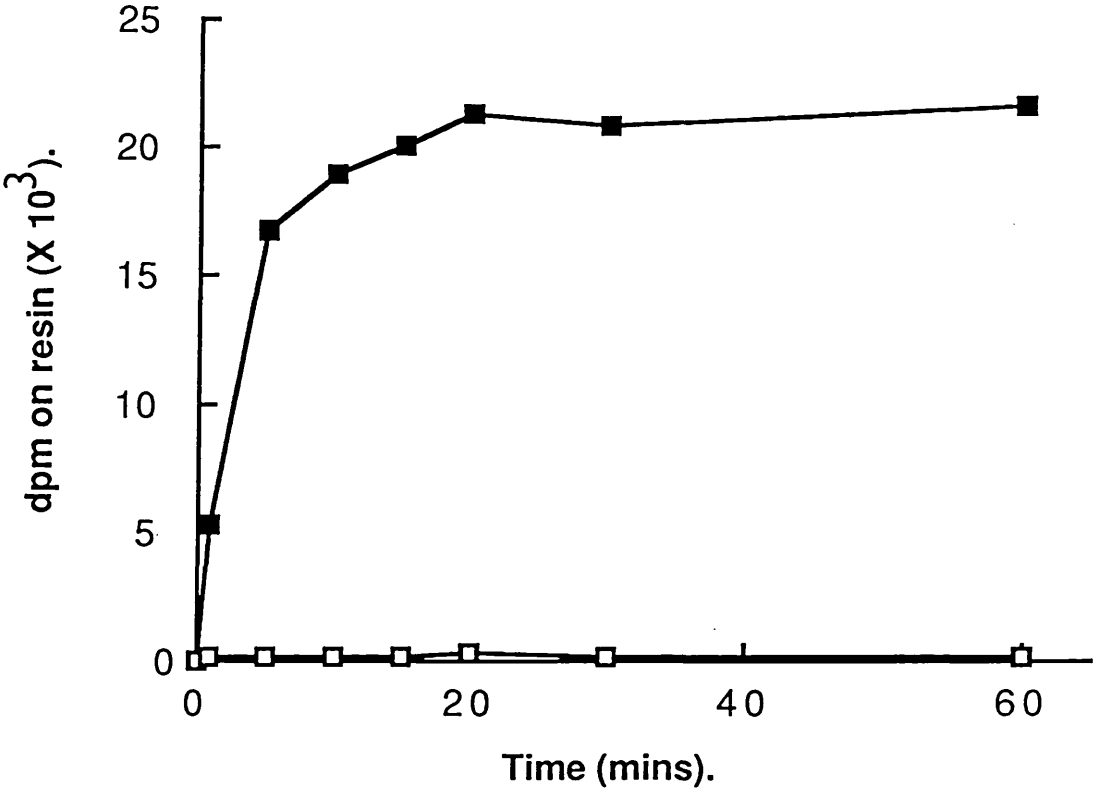


FIGURE 36

Time-course of one-step hybridization of oligonucleotides to diazotized DNA-Sephacryl S-1000.

Immobilized DNA used was HPV 16 483 bp Pst1 fragment (A). 0.5 pmole of A immobilized on Sephacryl S-1000 was hybridized at 65°C in 50 µl buffer with 50 fmole probe (complementary [■] or non-complementary [□] 30-mer oligonucleotide, kinase end-labelled with ^{32}P to an activity of 5.2 x 10^2 dpm/fmole). Each point represents the average of five determinations.

4.5.4 Summary Of One-step Hybridization Data.

The results obtained from studies on the hybridization of a probe free in solution with immobilized DNA (coupled to Sephacryl S-1000 by diazotization) can be summarized as follows:

(a) The one-step hybridization assay provides an indication of the availability of immobilized DNA for hybridization with a complementary sequence free in solution.

(b) Of three buffers tested, the best results were obtained by hybridization in 40 mM PIPES, pH 6.5, 0.6 M NaCl, 1 mM EDTA, 0.1% w/v SDS, 250 ug/ml denatured, sonicated salmon sperm DNA, at 65^{o}C. Post-hybridization washing was performed in 1 ml of the same buffer, minus salmon sperm DNA, for 3 x 10 minutes, at the same temperature.

(c) Time-course studies indicate that, under the conditions selected, hybridization is essentially complete within one hour. This was found to be the case for a 483 bp probe labelled by random priming, as well as for a kinase end-labelled 30-mer oligonucleotide.

(d) Optimum hybridization efficiencies are obtained only if the diazotization reaction resulting in the immobilization of the support-bound fragment was allowed to proceed for a full 48 hours.

(e) The concentration of immobilized DNA does not significantly affect hybridization efficiency, within the range 62-202 pmole of DNA per

gram of resin. However, if this concentration is too low, the mass of resin required for each assay results in an unacceptably high non-specific background signal; on the other hand, no advantage is gained by having extremely high immobilized DNA concentrations, because the mass of resin required per assay is too small to be measured accurately. An ideal concentration appears to be around 80-100 pmole/g of resin.

(f) Immobilized fragment size (within the range 3,601 to 216 bp) has no effect on the efficiency of hybridization. However, a 20-mer oligonucleotide immobilized by diazotization onto Sephacryl S-1000 was found to be unavailable for hybridization.

(g) Increasing the immobilized DNA (A) to probe DNA (B) ratio up to 100:1 increases the hybridization efficiency; any further increase in this ratio appears to have no advantageous effect.

Having investigated the hybridization properties of DNA immobilized by diazotization onto Sephacryl S-1000, DNA-Sephacryl was used to develop a model two-step (sandwich) hybridization assay.

4.6 Two-step Hybridizations.

All results obtained for two-step (or sandwich) hybridizations were obtained using DNA covalently immobilized by diazotization onto Sephacryl S-1000 beads. The buffer used for hybridizations involving restriction fragments as probes consisted of 40 mM PIPES, pH 6.5, 0.6 M NaCl, 1 mM EDTA, 0.1% w/v SDS, 250 ug/ml denatured, sonicated salmon sperm DNA; post-hybridization washing was performed for 3 x 10 minutes, at 65^{o}C, in 1 ml of hybridization buffer (minus salmon sperm DNA). The results of immobilization and one-step studies clearly demonstrated that immobilized fragment size has little effect on immobilization and hybridization efficiencies; consequently, the restriction fragments

chosen to serve as immobilized probes in the two-step assays designed to distinguish between HPV types 6b, 11, 16 and 18 were selected largely on the basis of genomic location and ease of preparation. Fragments derived from the E1/E2 region and the long control region (LCR) were avoided where possible, because deletion in these regions has been observed on integration of the viral genome into cellular chromosomes (Choo et al., 1987; G.C.N. Parry, personal communication). In order to develop the sandwich assay, a model system was set up, using purified plasmid DNA as the target molecule. In the case of HPV types 11, 16 and 18, the intact linearized genome was purified as discussed in section 4.2. Since the entire genome was not available in the case of type 6b, a 5,368 bp sub-genomic fragment was used instead (see figure 14).

4.6.1 Two-step Assays Using A Labelled Restriction Fragment As Probe.

Preliminary experiments were performed as described in the legend to figure 37, using amounts of target DNA in the range 1-5 fmole. Consider the case of 1 fmole of target DNA; since 50 fmole of probe was used in each assay, if every single target molecule were to act as a bridge between immobilized and labelled probes, the theoretical maximum amount of probe which could be attached to the solid support would be 1 fmole (or approximately 10^5 dpm). The actual number of counts attached to the Sephacryl after hybridization was approximately 2.7×10^4, and the background level was 0.7×10^4 dpm; thus, the signal resulting specifically due to hybridization with the target sequence was 2×10^4 dpm. The overall hybridization efficiency may be calculated using the equation:

$$\% \text{ Hybridization Efficiency} = \frac{\text{Experimental dpm} - \text{Background dpm}}{\text{Theoretical maximum dpm}} \times 100$$

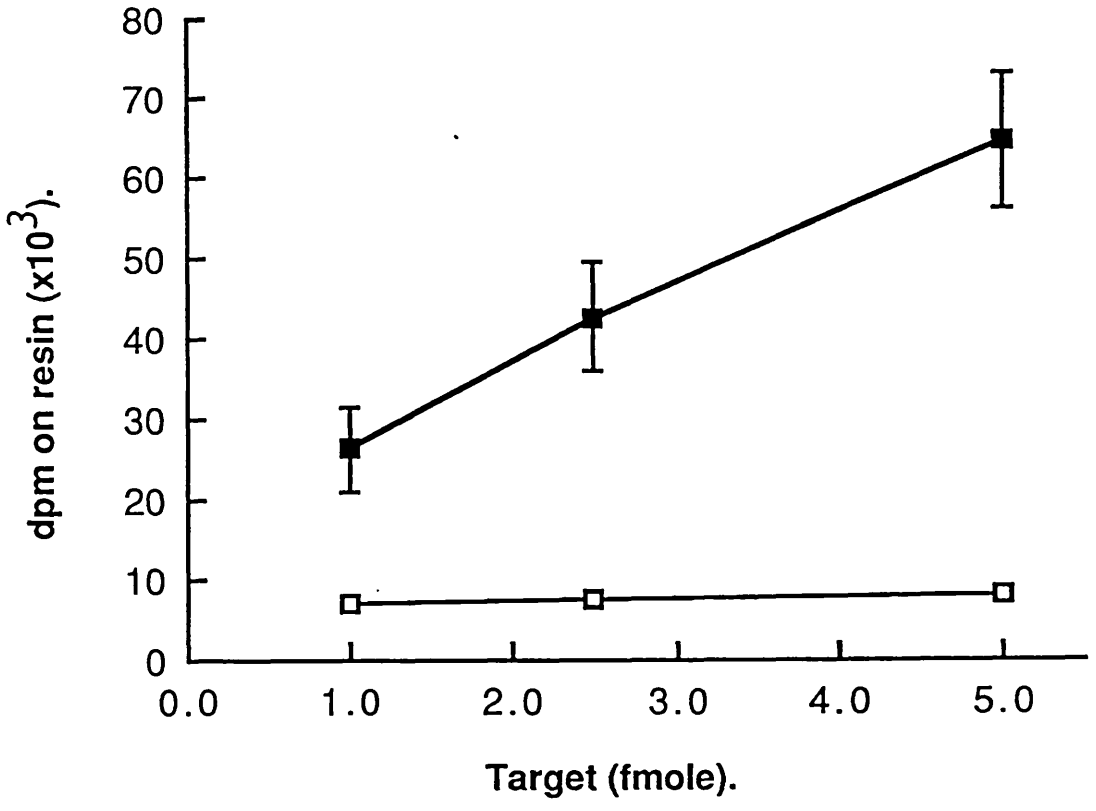


FIGURE 37

Two-step sandwich hybridization for the detection of HPV 18 DNA.

Immobilized DNA used was HPV 18 555 bp Pst1 fragment (A). 200 fmole of A immobilized on Sephacryl S-1000 was hybridized at 65°C, in 50 µl of buffer, with 50 fmole of probe (HPV 18 441 bp Pst1 fragment, labelled by random priming with ^{32}P to an activity of 1.0 x 10^5 dpm/fmole, together with the indicated amount of either: linear HPV 18 (7857 bp) [■], or linear HPV 16 (7904 bp) [□].
Each point represents the average of three determinations.

In the case of the example described above, this figure turns out to be 20%. The corresponding values for 2.5 and 5 fmole of target DNA are 14% and 11% respectively. In reality, the hybridization reaction between immobilized DNA (A), labelled probe (B) and target sequences (C) is complex; both B and C are free in solution, and for each single-stranded region of DNA, there is a complementary molecule. Although both strands of A are also present, these are not free to re-anneal, because they are immobilized on the solid support. If A, B and C are allowed to hybridize, any of the following may occur: (i) B-B re-annealing; (ii) C-C re-annealing; (iii) A-C hybrid formation; (iv) C-B hybrid formation; (v) A-C-B hybrid formation. A positive signal will be produced only by the A-C-B hybrid; if A and B are in a large excess over C, a single-stranded C molecule would theoretically be far more likely to form a duplex with A and/or B than it would be to re-anneal with a complementary C strand. If, in addition, A were in a large excess over B, the formation of the A-C complex (with subsequent formation of the A-C-B hybrid) should be favoured. It is important to point out that these are purely qualitative speculations; prediction of the hybridization kinetics between A, B and C is further complicated by the following points: (i) the experimentally observed efficiency with which the A-C-B complex forms may be greater than the actual efficiency, due to the formation of a probe network at the point of hybridization with the target strand (Wahl et al., 1979); (ii) once two complementary DNA strands hybridize, the duplex formed is not necessarily stable. For example, target re-annealing may result in the displacement of probe molecule(s) from A-C, C-B or A-C-B hybrids already formed (Ellwood et al., 1986; Vary, 1987).

The maximum experimentally observed hybridization efficiency in the example discussed above was obtained at the highest labelled

probe:target ratio (50:1). A decrease in this ratio resulted in a decrease in efficiency. An example of a study designed to determine the sensitivity of the two-step assay is shown in figure 38. Input target DNA amounts of 200, 150, 100, 50 and 25 amole resulted in the following respective hybridization efficiencies (figures in brackets refer to the labelled probe:target ratio): 26% (25:1), 28% (33:1), 30% (50:1), 25% (100:1), 22% (200:1).

The results of two-step sandwich assays designed to discriminate between HPV types 6b, 11, 16 and 18 are shown in table 9. In each case, two control assays were performed, as described in the legend to table 9. Using a labelled probe:target ratio of 50:1, hybridization efficiencies of 28%, 29%, 34% and 32% were observed for HPV types 6b, 11, 16 and 18 respectively. It is important to point out that these values do not take into account the results of the control experiments; it would be more realistic to calculate hybridization efficiency after subtraction of the non-specific binding control value. The amended efficiencies for virus types 6b, 11, 16 and 18 are 26%, 27%, 32% and 29% respectively. In each case, assays involving hybridization of target DNA with immobilized and labelled probes derived from a different virus type resulted in a signal of the same order of magnitude as that obtained for the non-specific binding control (C2). Despite the high degree of homology between types 6b and 11 (82% at the nucleic acid level), the hybridization efficiencies of assays involving HPV 6b derived probes with HPV 11 target (or HPV 11 derived probes with HPV 6b target) were not significantly above non-specific background levels. This suggests that the conditions used for two-step assays are sufficiently stringent to discriminate between closely related virus types, without being so stringent that the correct hybrids are prevented from

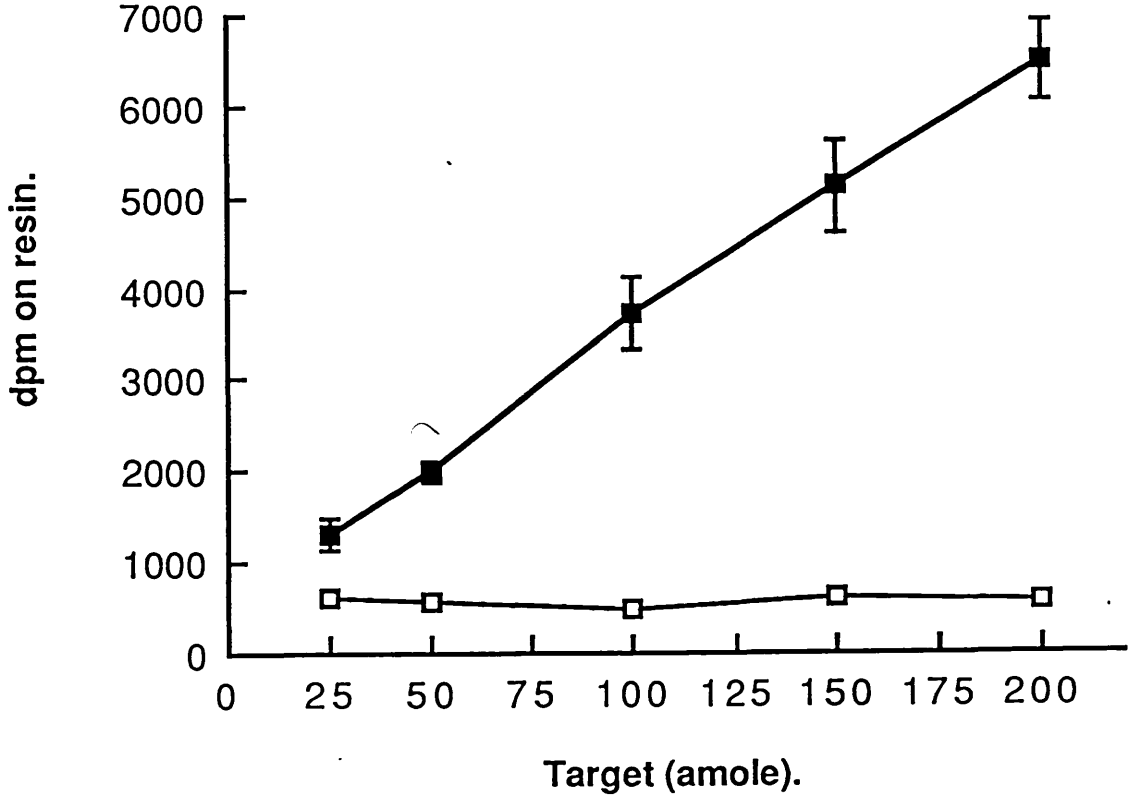


FIGURE 38

Sensitivity of two-step hybridization using radiolabelled DNA probes.

Immobilized DNA used was HPV 18 555 bp Pst1 fragment (A). 100 fmole of A immobilized on Sephacryl S-1000 was hybridized at 65°C, in 50 µl of buffer, with 5 fmole of probe (HPV 18 441 bp Pst1 fragment, labelled by random priming with ^{32}P to an activity of 1.1 x 10^5 dpm/fmole), together with the indicated amount of either: linear HPV 18 (7857 bp) [■], or linear HPV 16 (7904 bp) [□].
Each point represents the average of five determinations.

Target DNA	Origin of Probes		Initial dpm In Reaction ($x10^3$)	Final dpm In Reaction ($x10^3$)	% Hyb.
HPV 6b	6b	E	1,786	10.058	28
		C1	1,755	2.086	
		C2	1,724	0.904	
	11	E	1,549	0.879	
	16	E	1,696	1.047	
	18	E	1,542	0.943	
HPV 11	11	E	1,621	9.521	29
		C1	1,568	0.898	
		C2	1,692	0.743	
	6b	E	1,821	1.108	
	16	E	1,684	0.987	
	18	E	1,539	0.912	
HPV 16	16	E	1,702	11.661	34
		C1	1,675	0.775	
		C2	1,686	0.892	
	6b	E	1,792	1.143	
	11	E	1,607	0.994	
	18	E	1,577	0.826	
HPV 18	18	E	1,569	10.090	32
		C1	1,592	1.198	
		C2	1,607	1.047	
	6b	E	1,801	0.936	
	11	E	1,628	1.142	
	16	E	1,717	0.877	

TABLE 9

Two-step sandwich assays, using oligolabelled restriction fragment probes, for discriminating between HPV types 6b, 11, 16 and 18.

Two-step sandwich assays were performed using 100 fmole of immobilized DNA, 5 fmole of labelled probe, and 100 amole of target DNA. The immobilized DNA fragments (all generated by the enzyme PstI) used for the four HPV types were as follows: HPV 6b, 1,081 bp; HPV 11, 1,042 bp; HPV 16, 1,776 bp; HPV 18, 555 bp. The labelled probes used for the four HPV types (all PstI restriction fragments, labelled with ^{32}P by random priming to an activity of approximately 3×10^5 dpm/fmole) were as follows: HPV 6b, 686 bp; HPV 11, 435 bp; HPV 16, 483 bp; HPV 18, 441 bp. Experimental assays (E) were performed by hybridizing 100 amole of target DNA derived from the virus type indicated in the first column with immobilized and labelled probes derived from the source indicated in the second column. The controls included were as follows: **C1**, 100 amole of PstI digested target DNA from the indicated source was hybridized with probes derived from the same virus type; **C2**, 100 amole of intact target DNA from the indicated source was hybridized with a labelled probe derived from the same virus type, in the presence of immobilized calf thymus DNA.

Each result is the average of three determinations. More details regarding the source of immobilized and labelled probes are given in figures 14 to 17. The results are discussed fully in the text.

forming. The unexpectedly high signal obtained for PstI digested HPV 6b target DNA hybridized with HPV 6b derived probes (approximately twice the non-specific background signal) may be due to incomplete restriction enzyme digestion of the target DNA.

Having demonstrated that two-step sandwich assays can readily distinguish between even closely related HPV types, the effect on hybridization efficiency of using different sized HPV 16 fragments as immobilized and labelled probes was investigated; the results obtained are presented in table 10. Assays involving the use of HPV 16 2,817 bp and 1,776 bp fragments as immobilized (A) and labelled (B) probes were not attempted, because of previous results demonstrating hybridization between these two restriction fragments (see section 4.3). The maximum hybridization efficiency (34%) was attained using 2,817 bp and 483 bp fragments (or 1,776 bp and 1,063 bp fragments) as A and B respectively. The corresponding efficiencies resulting from immobilizing the smaller fragment, and using the larger fragment of each pair as a probe were 22% (the lowest efficiency observed) and 29%. There appears to be no obvious pattern relating the size of probes A and B with hybridization efficiency.

4.6.2 Two-step Assays Using A Labelled Oligonucleotide As Probe.

Pre-hybridization and hybridization were performed at 65^{o}C, in a buffer consisting of 6 x SSC, 0.1% w/v SDS, 250 ug/ml denatured, sonicated salmon sperm DNA. Post-hybridization washing was performed for 3 x 10 minutes at the same temperature, in hybridization buffer (minus salmon sperm DNA). In order to test the sensitivity of the sandwich assay using oligonucleotide probes, target DNA amounts in the range 0.625-20 fmole were hybridized with 100 fmole of immobilized DNA and 50 fmole of kinase end-labelled oligonucleotide probe. The results

Immobilized Fragment (bp)	Labelled Probe (bp)	Initial dpm ($\times 10^3$)	Final dpm ($\times 10^3$)	% Hyb.
2,817	1,776	NA	NA	NA
	1,063	3,761	17.32	23
	483	1,786	12.17	34
	216	972	5.17	27
	CT	1,981	0.87	--
1,776	2,817	NA	NA	NA
	1,063	3,929	26.52	34
	483	1,984	12.42	31
	216	1,021	5.87	29
	CT	2,186	0.91	--
1,063	2,817	10,819	63.74	29
	1,776	8,617	50.19	29
	483	2,034	11.49	28
	216	987	6.58	33
	CT	2,097	0.78	--
483	2,817	11,437	51.06	22
	1,776	8,798	45.97	26
	1,063	3,864	25.07	32
	216	1,089	6.54	30
	CT	1,898	0.87	

TABLE 10

Efficiency of sandwich hybridization using different HPV 16 PstI fragments as immobilized and labelled probes.

Two-step sandwich assays were performed using 100 fmole of immobilized DNA, 5 fmole of labelled probe, and 100 amole of target HPV 16 DNA. The probes were labelled with ^{32}P by random priming to the following activities (dpm/fmole): 2,817 bp, 2.2×10^6; 1,776 bp, 1.9×10^6; 1,063 bp, 7.8×10^5; 483 bp, 4.0×10^5; 216 bp, 1.7×10^5. Assays involving 2,817 bp and 1,776 bp fragments as immobilized and labelled probes were not performed (NA), because of previously encountered cross-hybridization between these fragments. The control hybridization (CT) involved replacing the HPV 16 specific probe with a calf thymus DNA probe (specific activity, 1×10^9 dpm/µg). Each result is the average of three determinations.

Details on the source of the HPV 16 PstI restriction fragments are given in figure 16. The results are discussed fully in the text.

are shown in figure 39. The hybridization efficiencies at input target DNA amounts of 0.625, 1.25, 2.5, 5, 10, 15 and 20 fmole (after subtraction of the non-specific binding figure) are 208%, 116%, 112%, 66%, 54%, 48% and 39% respectively. Clearly, the first three of these values are theoretically impossible, because the quantity of probe bound to the support after hybridization was, on a molar basis, greater than the amount of target DNA in the hybridization reaction. Possible explanations for this are: (i) inaccurate dilution of the target DNA resulted in a higher concentration than required; (ii) the estimation of the activity of the probe (6.9×10^2 dpm/fmole) is inaccurately low, so that the actual amount of probe added is considerably less than 50 fmole. In subsequent experiments under identical conditions, the hybridization efficiencies observed for target amounts greater than 5 fmole were similar to those observed in figure 39, suggesting that a dilution error was the cause of the anomalous hybridization efficiencies. The lower limit of sensitivity of the two-step assay, using oligonucleotide probes, is in the range 5-10 fmole of target DNA. This is 100- to 200-fold less sensitive than a similar assay using random-primed restriction fragments as probes. Bearing in mind that the activity of ^{32}P kinase end-labelled oligonucleotide (6.9×10^2 dpm/fmole) is approximately 150-fold lower than the activity of a 441 bp restriction fragment labelled with ^{32}P by random priming (1.1×10^5 dpm/fmole; see figure 38), this result is not unreasonable.

The results of two-step sandwich assays designed to discriminate between HPV types 6b, 11, 16 and 18, using labelled oligonucleotide probes, are shown in table 11. In each case, two control assays were performed, as described in the legend to table 11. Using 10 fmole of target DNA, and a labelled probe:target ratio of 10:1, hybridization efficiencies of 42%, 50%, 42% and 53% were observed for HPV types 6b,

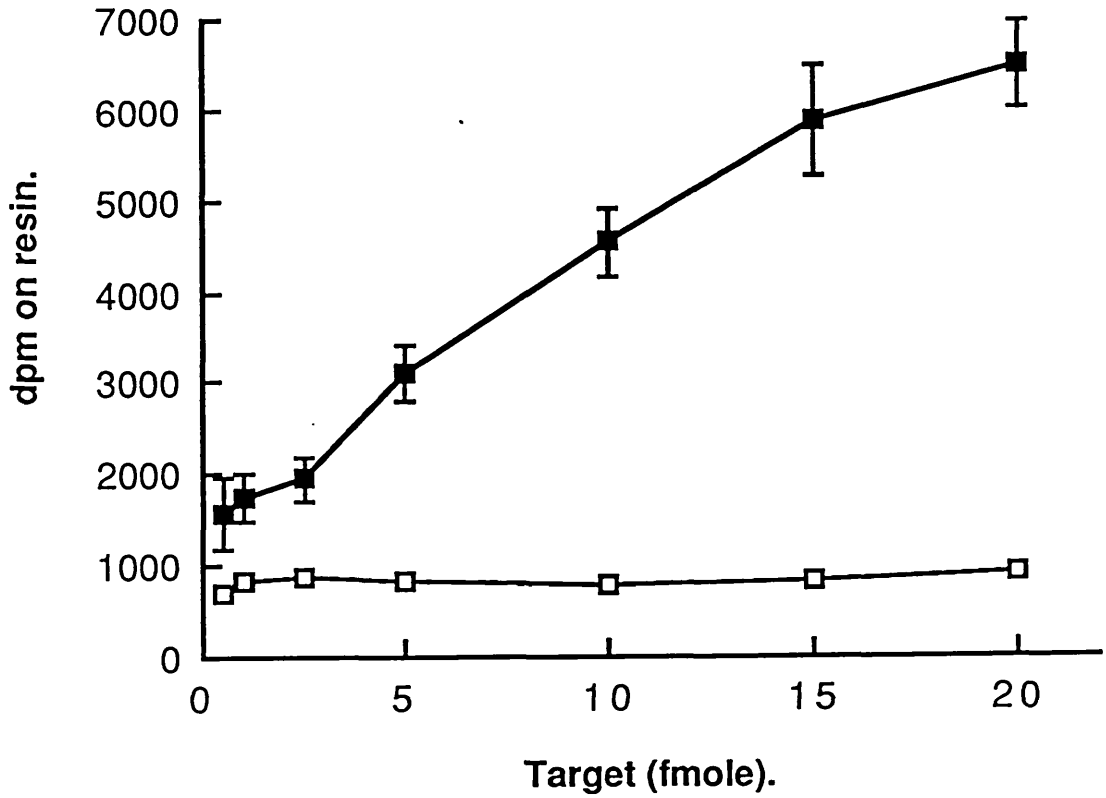


FIGURE 39

Sensitivity of two-step hybridization using radiolabelled oligonucleotide probes.

Immobilized DNA used was HPV 16 483 bp Pst1 fragment (A). 100 fmole of A immobilized on Sephacryl S-1000 was hybridized at 65°C, in 50 µl of buffer, with 50 fmole of probe (30-mer oligonucleotide PR16-1, kinase end-labelled with ^{32}P to an activity of 6.9 x 10^2 dpm/fmole), together with the indicated amount of either: linear HPV 16 (7904 bp) [■], or linear HPV 18 (7857 bp) [□].
Each point represents the average of five determinations.

Target DNA	Origin of Probes		Initial dpm In Reaction	Final dpm In Reaction	% Hyb.
HPV 6b	6b	E	23,210	2,510	42
		C1	21,980	490	
		C2	24,160	610	
	11	E	27,290	1,010	
	16	E	24,970	670	
	18	E	28,990	490	
HPV 11	11	E	28,220	3,500	50
		C1	27,870	590	
		C2	26,940	780	
	6b	E	22,470	1,250	
	16	E	26,250	880	
	18	E	29,340	540	
HPV 16	16	E	24,870	2,950	42
		C1	25,210	780	
		C2	26,010	940	
	6b	E	23,180	650	
	11	E	27,940	770	
	18	E	30,010	680	
HPV 18	18	E	29,890	3,910	53
		C1	27,990	960	
		C2	28,170	580	
	6b	E	21,220	690	
	11	E	29,320	780	
	16	E	26,870	910	

TABLE 11

Two-step sandwich assays, using radiolabelled oligonucleotide probes, for discriminating between HPV types 6b, 11, 16 and 18.

Two-step sandwich assays were performed using 100 fmole of immobilized DNA, 50 fmole of labelled probe, and 10 fmole of target DNA. The immobilized DNA fragments (all generated by the enzyme PstI) used for the four HPV types were as follows: HPV 6b, 1,081 bp; HPV 11, 435 bp; HPV 16, 483 bp; HPV 18, 441 bp. The oligonucleotides used as probes for the four HPV types (all kinase end-labelled with ^{32}P to an activity of approximately 5×10^2 dpm/fmole) were as follows: HPV 6b, PR6b-1; HPV 11, PR11-1; HPV 16, PR16-1; HPV 18, PR18-1. Experimental assays (E) were performed by hybridizing 10 fmole of target DNA derived from the virus type indicated in the first column with immobilized and labelled probes derived from the source indicated in the second column. The controls included were as follows: **C1**, 10 fmole of PstI digested target DNA from the indicated source was hybridized with probes derived from the same virus type; **C2**, 10 fmole of intact target DNA from the indicated source was hybridized with a labelled probe derived from the same virus type, in the presence of immobilized calf thymus DNA.

Each result is the average of three determinations; the final percentage hybridization value is calculated after subtraction of the average of the controls (C1 and C2). More details regarding the source of immobilized probes are given in figures 14 to 17. Details on the sequence and origin of the oligonucleotide probes are given in figures 42 and 43. The results are discussed fully in the text.

11, 16 and 18 respectively. Each result is adjusted to take into account non-specific binding of the probe to the solid support. The results clearly show that it is possible to discriminate between the virus types using oligonucleotide probes; this is not unexpected, since even if the hybridization between the oligonucleotide and the target DNA were not adequately type-specific, hybridization between the immobilized and target DNAs should result in the desired specificity; in other words, hybrids formed between an oligonucleotide and target DNA will not be captured by the solid support unless the target DNA is from the same HPV source as the immobilized capture probe. The actual hybridization efficiencies (in the range 42%-53%) are high in comparison with efficiencies observed with probes labelled by random priming (typically in the range 25%-35%). In the former case, the labelled probe was in 10-fold excess over the target; an increase in this excess was not found to increase the hybridization efficiency, but rather increased the non-specific binding background signal (data not shown). In the latter case, optimum efficiencies were attained using the probe in a 50-fold excess over target (see section 4.6.1). The lower hybridization efficiencies observed for probes labelled by random priming may be due to re-annealing of probe strands in solution; the effective withdrawal of a proportion of the probe from the hybridization equilibrium may result in a labelled probe:target ratio considerably less than 50:1. Oligonucleotides are free to anneal only to target strands, and since the labelled probe:target ratio is 10:1, the probe concentration should remain relatively constant throughout the assay.

4.6.3 Time-course Of Two-step Hybridization.

The results of experiments designed to determine the time-course of two-step assays using random primed restriction fragments and

oligonucleotides as probes are shown in figures 40 and 41 respectively. In the former case, an immobilized probe:labelled probe:target ratio of 1,000:50:1 resulted a hybridization efficiency after 24 hours of around 25%. The efficiency after just 16 hours was 24%, indicating that the hybridization was essentially complete after this time. If the same amount of target DNA were immobilized onto a membrane, and subsequently hybridized with 5 fmole of single-stranded labelled probe, the theoretical $t_{1/2}$ of the reaction (according to the equation shown in section 4.5.3) would be around 15 minutes, and hybridization would be 99% complete after 105 minutes. This indicates that the kinetics of the sandwich hybridization reaction are considerably slower than those of 'ideal' mixed phase reactions in which the target molecules are immobilized; this corresponds well with data presented by Ranki et al. in 1983.

The results obtained for the time-course of the two-step hybridization using oligonucleotide probes indicate that the reaction is essentially complete after 8 hours, and the hybridization efficiency after this time is around 68%. If the same amount of target DNA were immobilized on a membrane, and hybridized with 50 fmole of end-labelled 30-mer oligonucleotide probe, the theoretical $t_{1/2}$ of the reaction would be 6 minutes, and the reaction would be 99% complete after approximately 42 minutes. As in the case of the longer probe, the kinetics of the two-step sandwich assay with an oligonucleotide probe are considerably slower than those of 'ideal' mixed phase reactions in which the target is immobilized.

4.6.4 Two-step Assays With Human Samples.

As discussed in sections 4.6.2 and 4.6.3, the lower limits of sensitivity of sandwich assays using labelled restriction fragments and

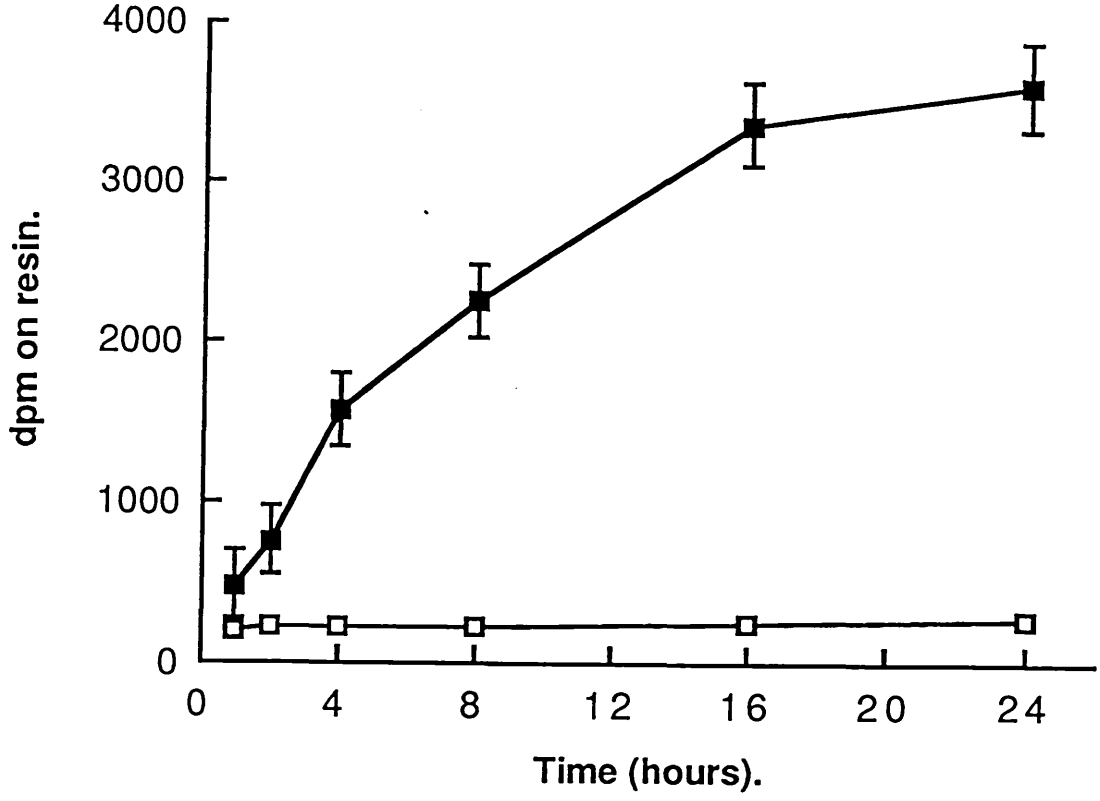


FIGURE 40

Time-course of two-step hybridization of restriction fragments to diazotized DNA-Sephacryl S-1000.

Immobilized DNA used was HPV 18 555 bp Pst1 fragment (A). 100 fmole of A immobilized on Sephacryl S-1000 was hybridized at 65°C, in 50 µl of buffer, with 5 fmole of probe (HPV 18 441 bp Pst1 fragment, labelled by random priming with ^{32}P to an activity of 1.3 x 10^5 dpm/fmole), together with either: 100 amole of linear HPV 18 (7857 bp) [■], or 100 amole of linear HPV 16 (7904 bp) [□]. Hybridizations were allowed to procede for the times indicated. The number of counts recorded is after subtraction of a control background figure (obtained from an identical assay performed with the omission of target). Each point represents the average of three determinations.

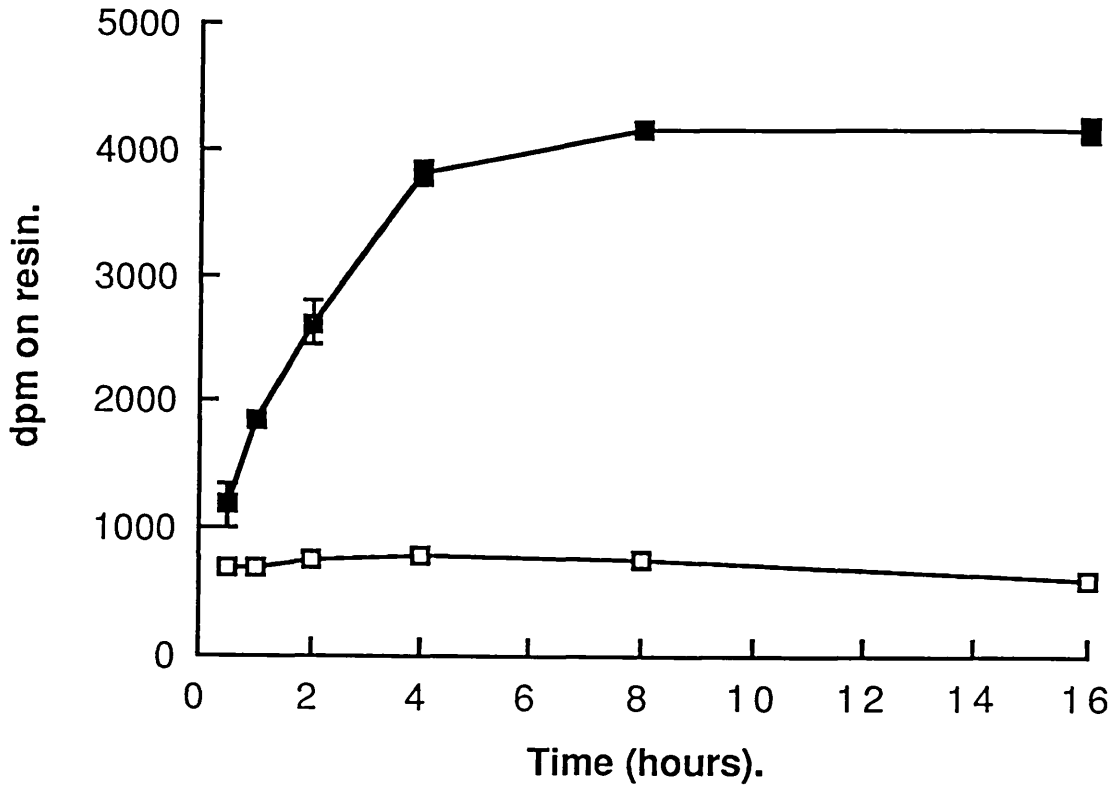


FIGURE 41

Time-course of two-step hybridization using an oligonucleotide probe.

Immobilized DNA used was HPV 16 483 bp PstI fragment (A). 100 fmole of A immobilized on Sephacryl S-1000 was hybridized at 65°C, in 50 µl of buffer, with 50 fmole of probe (30-mer oligonucleotide PR16-1, kinase end-labelled with ^{32}P to an activity of 4.8 x 10^2 dpm/fmole), together with either: 10 fmole of linear HPV 16 (7904 bp) [■], or 10 fmole of linear HPV 18 (7857 bp) [□]. Hybridization was allowed to proceed for the time indicated.
Each point represents the average of three determinations.

oligonucleotides as probes are around 25-50 amole and 5-10 fmole respectively.

As discussed in chapter 2, there is evidence to suggest that integration of HPV nucleic acid into the host cell chromosome may initiate the neoplastic process (Gissmann & Schwarz, 1986). Studies have demonstrated that the number of copies of the viral genome per cell may be much lower when the DNA is integrated, rather than episomal (Choo et al., 1987). Hence, any assay designed to detect HPV infection must be capable of identifying positive samples, regardless of the integrational status and abundance of the viral genome. Conventional blotting techniques- Southern or slot-blotting, for example -are capable of detecting as little as 0.5-1 amole of target DNA. The sensitivity of the two-step sandwich assay with an oligonucleotide probe (around 5-10 fmole) makes the technique unsuitable for use with clinical samples in this form. Using a labelled restriction fragment as the probe, the sensitivity can be improved so that around 25 amole of target DNA may be detected reliably. Nevertheless, it was considered that attempts should be made to increase the sensitivity of the assay before important samples were studied. Several options for achieving this were considered: (i) an increase in the size of the probe should result in a higher activity (dpm/fmole); attempts at using longer restriction fragments as probes resulted in signal:background ratios similar to those obtained with shorter probes. In effect, the signal for a given amount of target DNA increased, but the concomitant increase in background resulted in a lower sensitivity limit of around 25 amole of target DNA. This effect was also observed when a mixture of several restriction fragments, rather than a single fragment, were used as the probe (data not shown); (ii) the sensitivity of the two-step assay is limited by the background signal; attempts to increase the

signal/noise (S/N) ratio (for example, by increasing the pre-hybridization time, or increasing the concentration of salmon sperm DNA in the hybridization buffer) were unsuccessful (data not shown); (iii) single-stranded DNA probes produced from the M13 phage vector were not studied because of reports that M13 probes result in high non-specific background levels when used in hybridizations involving Sephacryl beads as the solid support (Langdale & Malcolm, 1985); (iv) single-stranded RNA probes produced from pGEM vectors containing promoters for both T7 and SP6 RNA polymerases were not used, because the aim of this study was to produce an assay which could be used on relatively crude samples; the presence of RNAses in such samples could lead to difficulties in interpreting negative results, in the case of an RNA probe. The most obvious means of dramatically increasing the sensitivity of the test was to incorporate a polymerase chain reaction (PCR)-mediated amplification of the number of target sequences, prior to sample assay by sandwich hybridization; the advantages of PCR, together with details on the development of a system for amplification of specific regions of the genomes of HPV types 6b, 11, 16 and 18, are given in the following section.

4.6.5 Summary Of Two-step Hybridization Data.

The results obtained from studies on two-step hybridization can be summarized as follows:

(a) Using plasmid-derived HPV DNA in a model system, the sensitivity of the two-step assay (using restriction fragments labelled by random priming as the probe) was around 25-50 amole. The sensitivity using an oligonucleotide probe was around 5-10 fmole.

(b) HPV types 6b, 11, 16 and 18 were readily distinguished, using both types of probe.

(c) In the case of restriction fragment probes, pre-hybridization was performed for 2 hours, at 65°C, in a buffer consisting of 40 mM PIPES, pH 6.5, 0.6 M NaCl, 1 mM EDTA, 0.1% w/v SDS, 250 ug/ml denatured, sonicated salmon sperm DNA. Hybridization was performed in the same conditions, for a minimum of 16 hours, with a 50-fold molar excess of probe over target. Post-hybridization washing was performed at the same temperature, for 3 x 10 minutes, in hybridization buffer (minus salmon sperm DNA).

(d) In the case of oligonucleotide probes, pre-hybridization was performed for 2 hours, at 65°C, in 6 x SSC, 0.1% w/v SDS, 250 ug/ml denatured, sonicated salmon sperm DNA. Hybridization was performed in the same conditions, for a minimum of 4 hours, with a 10-fold molar excess of probe over target. Post-hybridization washing was performed at the same temperature, for 3 x 10 minutes, in hybridiz--tion buffer (minus salmon sperm DNA).

(e) Hybridization time-courses showed that, using restriction fragment probes, the reaction is essentially complete after 16 hours; the equivalent figure in the case of oligonucleotide probes is 4 hours.

4.7 Polymerase Chain Reaction (PCR) For Amplification Of Specific Sub-genomic HPV Regions.

In an attempt to increase the sensitivity of the two-step sandwich hybridization assay, it was decided to use the polymerase chain reaction (PCR) to amplify the number of target sequences, prior to assay. The first reported use of PCR was for the amplification of a 110 bp region of the human beta-globin gene (Saiki et al., 1985 a); the technique has since been applied in many other biochemical studies (reviewed in section 1.7).

There are several advantages inherent in the use of the PCR

technique to increase the sensitivity of the sandwich assay: (i) the model two-step assay was developed using linear plasmid-derived HPV fragments as the target DNA; HPV genomes in infected cells exist either extrachromasomally, as covalently closed, circular episomes, or integrated within a host cell chromosome. The efficiency with which the HPV DNA would partake in hybridization is unknown; it is possible that the denatured strands of the episomal form would rapidly re-anneal in solution, rather than hybridize to the immobilized and labelled probes. HPV sequences integrated within cellular genes may also hybridize inefficiently; it has been reported that the hybridization efficiency of large intact bacterial genomes is only 50% of that of smaller viral genomes (Palva, 1983). An integrated HPV genome would be flanked by chromosomal sequences, and the size of the molecule may result in extremely low hybridization efficiencies. Although both of these problems could be surmounted if the sample nucleic acid were subjected to mechanical shearing prior to assay, the extent of fragmentation can be difficult to control. PCR amplification results in an accumulation of a linear, double-stranded DNA fragment of pre-determined size and composition; such a fragment would be more likely to hybridize efficiently with immobilized and labelled probes than episomal or integrated DNA; (ii) since the full nucleotide sequence of HPV types 6b, 11, 16 and 18 is known, it would be possible to choose primers so that the size of the amplified fragment varies depending on which HPV type is present. This would provide an excellent means of checking sandwich assay results during the development of the procedure; (iii) the primers could be chosen so that the amplified sequence is complementary to the restriction fragments already chosen as immobilized and labelled probes in the sandwich assay. This would result in the amplified sequence containing a recognition site for

PstI; (iv) PCR analysis would allow a single sample to be screened for many different infectious organisms. The typical yield of DNA from a single cervical scrape is around 5-10 ug; this is sufficient for a single Southern blot, or sandwich assay.

The main disadvantage of using PCR amplification is that it would be extremely difficult to accurately quantitate the amount of HPV DNA initially present in a sample. However, since it has been shown that there is no association between viral copy number and progression to malignancy (Wickenden et al., 1987 b), it is more important to detect the presence of HPV infection than it is to accurately quantitate the levels of HPV DNA.

4.7.1 Choice Of Oligonucleotide Primer Sequences.

The oligonucleotide sequences chosen to serve as PCR primers, together with their origins on the HPV genome, are shown in figures 42 and 43. All primers were 20 nucleotides in length, with the exception of primer PCR16-A (23 nucleotides). The purpose of the extra three residues in the case of PCR16-A was to increase the stability of the hybrid formed between this primer and a complementary sequence (the oligonucleotide has a low G/C content [22%]). The size of the amplification products for HPV types 6b, 11, 16 and 18 are 239 bp, 199 bp, 159 bp and 119 bp respectively. An alternative pair of primers, suitable for amplification of a 206 bp region of HPV 6b or HPV 11, were also synthesized. Each amplified sequence contains a PstI site, as shown in figure 43.

4.7.2 Optimization Of Amplification Conditions.

In order to optimize the efficiency of the PCR reaction, a number of reaction parameters were varied, including enzyme source,

POLYMERASE CHAIN REACTION (PCR) PRIMERS.

PCO3	5'-ACA CAA CTG TGT TCA CTA GC-3'
PCO4	5'-CAA CTT CAT CCA CGT TCA CC-3'
PCR18-A	5'-TGA GGT ACC ATT GGA TAT TT-3'
PCR18-B	5'-TAG CAA AAA GCT GCT CAC GC-3'
PCR16-A	5'-TAT TAA TAA TAC TGT TAC TAC TG-3'
PCR16-B	5'-GTA TCC ATA GGA ATT TCT TC-3'
PCR6b/11-A	5'-TTT GGT GCT ATG AAT TTT GC-3'
PCR11-B	5'-CAG GTT CCC CCA CAG TAC CG-3'
PCR6b-A	5'-CCG ACG TGG CCT TGT GCG GT-3'
PCR6b-B	5'-CTG ATA TAT TTG TAA CAG GG-3'
PCR6b/11-B	5'-TCA GGC ACA GGT TCC CCC AC-3'

HYBRIDIZATION PROBES.

PR6b-1	5'-ATT TTT ATG ATA TTT CAC CTA TTG CAC AGG-3'
PR6b-2	5'-CAT GTA AAT ATC CAG ATT ATT TAC AAA TGG-3'
PR11-1	5'-TCT GCA AAT ATC CTG ATT ATT TGC AAA TGG-3'
PR16-1	5'-CTG ACC CAT CTG TAT TGC AGC CTC CAA CAC-3'
PR18-1	5'-TTT GTA AAT ATC CTG ATT ATT TAC AAA TGT-3'
PRGLOBIN-1	5'-CTC AAA CAG ACA CCA TTG TGC ACC TGA CTC-3'

FIGURE 42

SEQUENCES OF THE OLIGODEOXYRIBONUCLEOTIDES USED IN THIS STUDY.

PCR primer pairs were designed to amplify a region of the following: PCO3+PCO4, human ß-globin gene; PCR18-A+PCR18-B = HPV 18; PCR16-A+PCR16-B = HPV 16; PCR6b/11-A+PCR11-B = HPV 11; PCR6b-A+PCR6b-B = HPV6b; PCR6b/11-A+PCR6b/11-B = HPV 6b or HPV 11. Hybridization probes were designed for the detection of the following genomes: PR6b-1, PR6b-2 = HPV 6b; PR11-1 = HPV 11; PR16-1 = HPV 16; PR18-1 = HPV 18; PRGLOBIN-1 = human ß-globin gene.
More details on the origin of each oligonucleotide are given in figure 43. The temperature at which 50% of hybridized oligonucleotides dissociate (Tm) was calculated according to the equation:

$$Tm\ (^{o}C) = 4(G+C) + 2(A+T).$$

This was intended to give an approximate indication of the Tm, and is probably not accurate in the case of the oligonucleotide probes. N/A = not applicable.

POLYMERASE CHAIN REACTION (PCR) PRIMERS.

Name	Tm	Location	Target	ORF	Amplified Region	PstI Site	Size Of Product
PCO3	58	-40 to -21	BETA-GLOBIN GENE	N/A	-40 to +70	N/A	110 bp
PCO4	60	51 to 70					
PCR18-A	54	6266-6285	HPV 18	L1	6266-6384	6325	119 bp
PCR18-B	60	6365-6384					
PCR16-A	56	4675-4697	HPV 16	L2	4675-4833	4755	159 bp
PCR16-B	54	4814-4833					
PCR6b/11-A	54	6374-6393	HPV 11	L1	6374-6572	6474	199 bp
PCR11-B	66	6553-6572					
PCR6b-A	68	5289-5308	HPV 6b	L2	5289-5527	5408	239 bp
PCR6b-B	54	5508-5527					
PCR6b/11-A	54	6389-6408	HPV 6b	L1	6389-6594	6489	206 bp
PCR6b/11-B	66	6575-6594					
PCR6b/11-A	54	6374-6393	HPV 11	L1	6374-6579	6474	206 bp
PCR6b/11-B	66	6560-6579					

HYBRIDIZATION PROBES.

Name	Tm	Location	Target	ORF	Adjacent Fragment.
PR6b-1	78	5378-5407	HPV 6b	L2	1081 bp PstI
PR6b-2	76	6459-6488	HPV 6b	L1	686 bp PstI-BamHI
PR11-1	80	6444-6473	HPV 11	L1	435 bp PstI
PR16-1	92	4725-4754	HPV 16	L2	483 bp PstI
PR18-1	72	6295-6324	HPV 18	L1	441 bp PstI
PRGLOBIN-1	90	-17 to 13	GLOBIN	N/A	201 bp DdeI

FIGURE 43

ORIGINS OF THE OLIGODEOXYRIBONUCLEOTIDES USED IN THIS STUDY.

reaction buffer, pH and magnesium ion concentration.

Two commercial preparations of Thermus aquaticus DNA polymerase were tested; one was supplied by Perkin Elmer Cetus (PEC), and the other was purchased from Anglian Biotec Limited (AB). The results of an assay designed to test these two enzymes with two different buffers are shown in figure 44. Buffer I (10 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 200 uM each of dATP, dCTP, dGTP and dTTP, 1 uM of each oligonucleotide primer, 100 ug/ml gelatin, 1 unit/25 ul DNA polymerase) was recommended by the manufacturer for use with the PEC enzyme, and Buffer II (67 mM Tris-HCl, pH 8.8, 16.6 mM ammonium sulphate, 6.7 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 6.7 mM EDTA, 33 uM each of dATP, dCTP, dGTP and dTTP, 170 ug/ml BSA, 1 uM of each oligonucleotide primer, 1 unit/25 ul DNA polymerase) was recommended for use with the AB enzyme. The results clearly show that the amplification efficiency with the AB enzyme is considerably lower than that with the PEC enzyme; in fact, there is no evidence on the gel that any amplification has resulted from the AB enzyme in either buffer. The PEC enzyme is active in both buffers, but the amplification efficiency is greatest in buffer I. Consequently, the PEC enzyme was used with buffer I for all subsequent studies. Variation of the magnesium ion concentration in the range 0.25-25 mM had no effect on amplification efficiency, judging by the intensity of the bands on an agarose gel (data not shown). The temperature cycle found to be most effective was as follows: sample DNA was denatured by heating to 95°C for 7 minutes, and the oligonucleotide primers were allowed to anneal to their target sequences by cooling to 55°C, followed by incubation at this temperature for a further 2 minutes. Polymerase-mediated elongation of the annealed primers was achieved by heating the sample to 70°C, and maintaining this temperature for a further 4 minutes. For subsequent cycles, denaturation, primer

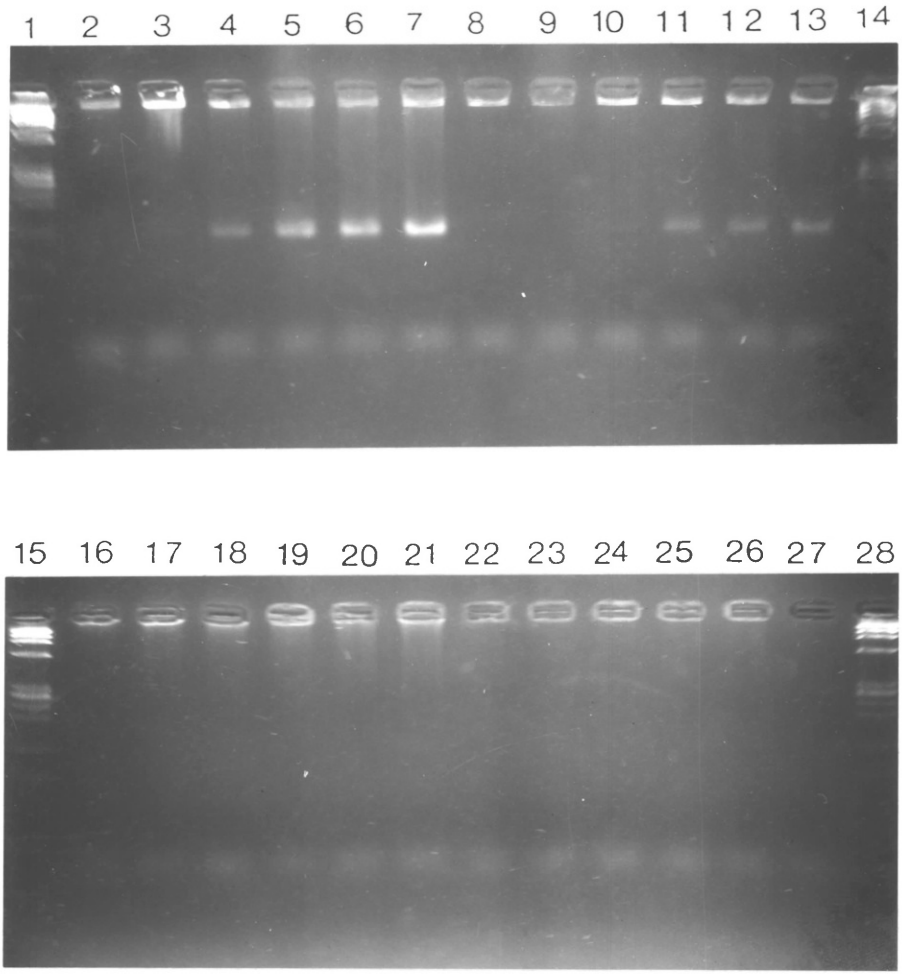


FIGURE 44

Comparison of PCR amplification of a 119 bp HPV 18 region with two commercial preparations of Thermus aquaticus DNA polymerase.

1 ng of linear HPV 18 DNA was subjected to 30 cycles of PCR amplification with primers PCR18-A and PCR18-B, as described in section 3.5.17. Two commercial preparations of Thermus aquaticus polymerase were tested, supplied by Perkin Elmer Cetus (PEC) and Anglian Biotec Limited (AB). Two different buffers were also tested: **Buffer I**, 10 mM Tris-HCl pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP and dTTP, 1 µM of each oligonucleotide primer, 100 µg/ml gelatin, 1 unit/25 µl DNA polymerase; **Buffer II**, 67 mM Tris-HCl pH 8.8, 16.6 mM ammonium sulphate, 6.7 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 6.7 mM EDTA, 33 µM each of dATP, dCTP, dGTP and dTTP, 170 µg/ml BSA, 1 µM of each oligonucleotide primer, 1 unit/25 µl DNA polymerase. Both PEC and AB polymerases were tested with both buffers, I and II. 5 µl aliquots were removed from each of the four samples (100 µl total), after 5, 10, 15, 20, 25 and 30 rounds of amplification, and subjected to electrophoresis through a 3% NuSieve GTG agarose/1% normal agarose gel (section 3.4.4.1). The gel was loaded as follows: Lanes 2-7, PEC/Buffer I (5, 10, 15, 20, 25, 30 cycles respectively). Lanes 8-13, PEC/Buffer II (5, 10, 15, 20, 25, 30 cycles respectively). Lanes 16-21, AB/Buffer I (5, 10, 15, 20, 25, 30 cycles respectively). Lanes 22-27, AB/Buffer II (5, 10, 15, 20, 25, 30 cycles respectively). Lanes 1, 14, 15 and 28 each contain 0.5 µg of øX174-HaeIII markers (fragment sizes 1,353, 1,078, 872, 605, 310, 281, 271, 234, 194, 118 and 72 bp).

The results are discussed fully in the text.

annealing and elongation were performed at the temperatures indicated above, for 1, 0.5 and 4 minutes respectively.

4.7.3 Effect Of Target DNA Conformation (Linear Or Circular) On Amplification Efficiency.

As discussed above, papillomavirus genomes persist in infected cells in two forms; they may be free-replicating covalently closed, circular, extrachromosomal episomes, or they may be integrated within a host cell chromosome. Before the PCR technique was used to amplify the number of target sequences prior to sandwich assay, the efficiency of amplification using covalently closed, circular DNA as template was investigated. If the amplification efficiency were to be low (or zero) with such a template (possibly due to rapid re-annealing of the two strands because of the close association caused by supercoiling), there would be a risk that clinical samples harbouring HPV DNA in episomal form would be incorrectly diagnosed. The results of the study are shown in figure 45. 1 ng of linear HPV 18 DNA and 1 ng of covalently closed, circular pBR322-HPV 18 plasmid were separately subjected to 30 rounds of amplification, with primers PCR18-A and PCR18-B. The amplification efficiency was examined qualitatively by agarose gel electrophoresis, as described in the legend to figure 45. The results demonstrate that the amplification product is the correct size (119 bp), and contains a PstI site in the correct position (producing two fragments of equal size). In addition, there is little difference between the extent of amplification achieved with linear and covalently closed, circular templates.

The amplification efficiency using integrated DNA as target was not examined; since it is possible to efficiently amplify specific regions of genes contained within a chromosome (for example, the beta-

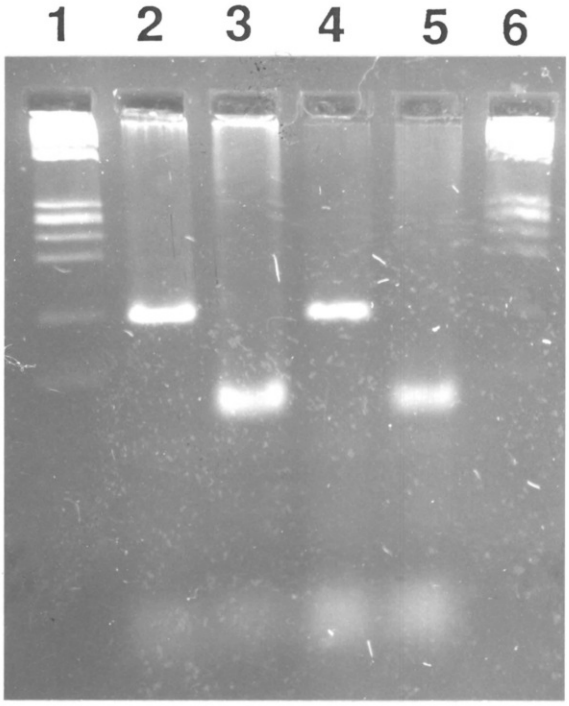


FIGURE 45

Comparison of the extent of PCR amplification using linear and covalently closed circular template.

1 ng of linear HPV 18 DNA (L) and 1 ng of covalently closed, circular pBR322-HPV 18 plasmid (C) were separately subjected to 30 rounds of PCR amplification, with primers PCR18-A and PCR18-B, exactly as described in section 3.5.17. After amplification, one tenth of each reaction mix was removed, and digested with the restriction enzyme PstI. A 3% NuSieve GTG agarose/1% normal agarose gel was prepared, and electrophoresed with the following samples loaded (section 3.4.4.1): Lanes 1 and 6, 0.5 µg of øX174-HaeIII markers (fragment sizes 1,353, 1,078, 872, 605, 310, 281, 271, 234, 194, 118 and 72 bp). Lanes 2 and 4, PCR amplified L (undigested) and C (undigested) respectively. Lanes 3 and 5 , PCR amplified L (PstI digested) and C (PstI digested) respectively. Lanes 2-5 contain one tenth of the original sample volume.

The results demonstrate that: (i) The amplification product is the correct size (119 bp), and contains a PstI site in the correct position (producing two fragments of equal size). (ii) There is little difference between the extent of amplification achieved with linear and covalently closed, circular templates.

globin gene), the same should be possible for integrated viral sequences.

4.7.4 'Sensitivity' Of Polymerase Chain Reaction.

In theory, it should be possible to PCR amplify a region of just one double-stranded DNA molecule (Saiki et al., 1988 b). In order to test the lower limit below which efficient amplification does not occur, 2 ng of HPV 18 DNA was serially diluted 22 times, so that the first sample contained 1 ng of DNA, and the final sample contained approximately 0.238 fg (around 30 molecules) of DNA. Each sample was subjected to 40 cycles of PCR amplification, and the amplification efficiency was qualitatively studied by agarose gel electrophoresis; the results are shown in figure 46. The results demonstrate that the expected amplification product (119 bp) is clearly present in lanes 1-12 and 14-16; the sample represented in lane 16 initially contained approximately 7,600 molecules of target DNA. Faint bands 119 bp in size are also present in lanes 19, 20 and 21 (samples initially containing 960, 480 and 240 molecules of target DNA respectively). The reason for the apparently sudden dramatic decrease in amplification efficiency starting in lane 17 is unknown. Interestingly, as the intensity of the 119 bp band decreases with increasing dilution, a band of smaller size (equivalent to a double-stranded DNA fragment of approximately 60 bp) increases in intensity. The origin of this band is unknown; it is very unlikely that it is formed from single-stranded 119 bp DNA molecules, because exponential increase in the amount of the target sequence occurs only if both strands are amplified concomitantly. If both strands were present in the amplification product in equal amounts, annealing should result in the presence of double-stranded fragments of around 119 bp. Interestingly, digestion with the restriction enzyme PstI had no effect on the size of the anomalous

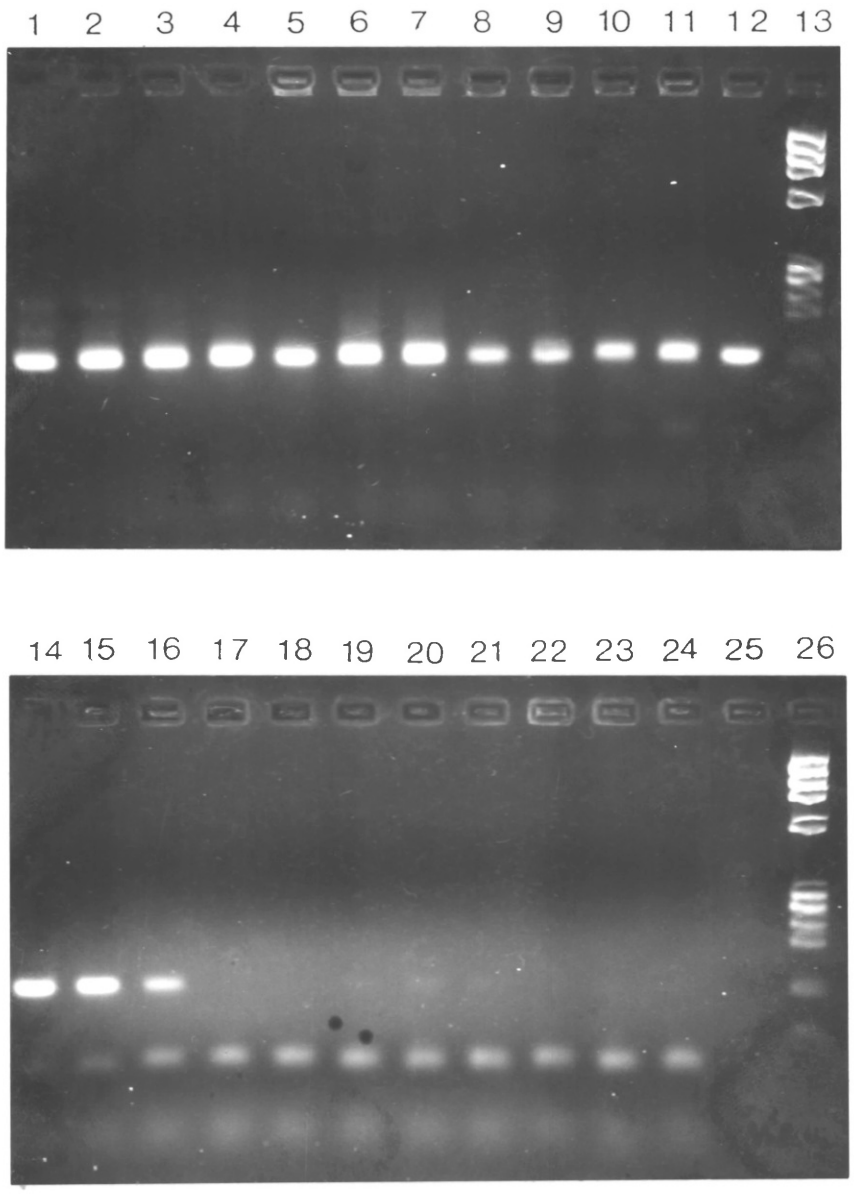


FIGURE 46

PCR amplification of serially diluted samples of HPV 18 DNA, with a primer pair designed to amplify a 119 bp region of HPV 18.

2 ng of linear HPV 18 DNA was serially diluted 22 times, so that the flrst sample contained 1 ng of DNA, the second 0.5 ng, and so on; the final sample thus contained approximately 0.238 fg (around 30 molecules) of HPV 18 DNA. The contents of all 23 tubes were then subjected to 40 cycles of PCR amplification (section 3.5.17), using primers PCR18-A and PCR18-B, designed to amplify a 119 bp region of HPV 18. One tenth of the contents of each tube was then subjected to electrophoresis through a 3% NuSieve GTG agarose /1% normal agarose gel, as described in section 3.4.4.1. The gel was loaded as follows: Lanes 1-12 and 14-24, amplified dilutions of HPV 18 (lane 1=lowest dilution, through to lane 24=highest dilution). Lanes 13 and 26, 1 µg of øX174-HaeIII markers (fragment sizes 1,353, 1,078, 872, 605, 310, 281, 271, 234, 194, 118 and 72 bp). Lane 25 is empty.

The results are discussed fully in the text.

band, indicating that it contains no recognition sites for this enzyme. Unfortunately, attempts to probe the gel shown in figure 46 with an oligonucleotide specific for the amplified HPV 18 119 bp sequence were unsuccessful, so the origin of the anomalous band remains a mystery.

4.7.5 PCR Amplification Of HPV DNA Sequences In Human Samples.

Twenty clinical specimens in the form of cervical scrapes were selected by a clinician from those obtained from a large cohort of women enrolled for a study of the risk associated with HPV infection of the uterine cervix. Each sample was collected, as described in section 3.5.5; the cells remaining after preparation of a routine Papanicolau smear were placed in 10 ml of filter sterilized PBS, pH 7.2, 0.5% SDS, and then sent to the Department of Cytology, St. Mary's Hospital Medical School, London. Results of the smear tests (determined by the Department of Cytology, at the Christie Hospital, Manchester) were sent to St. Mary's, London. Each sample was assigned the appropriate cytological code(s): 1, specimen unsatisfactory; 2, normal cells only; 3, atypical cells; 4, Trichomonas vaginalis present; 5, monilia present; 6, herpes/wart virus present; 7, actinomyces present. The twenty samples to be tested in the PCR/sandwich assay were selected by a clinician on the basis that the majority had cellular abnormalities characteristic of wart virus (HPV) infection. The cytological code(s) for each sample were not revealed until PCR/sandwich assays were completed.

DNA was prepared from each clinical sample, as described in section 3.5.6, with a typical yield of approximately 3ug. 0.5 ug of extracted DNA was subjected to PCR amplification, as described in the legend to figure 47; in each case, a mixture of primer pairs (PCR18-A/

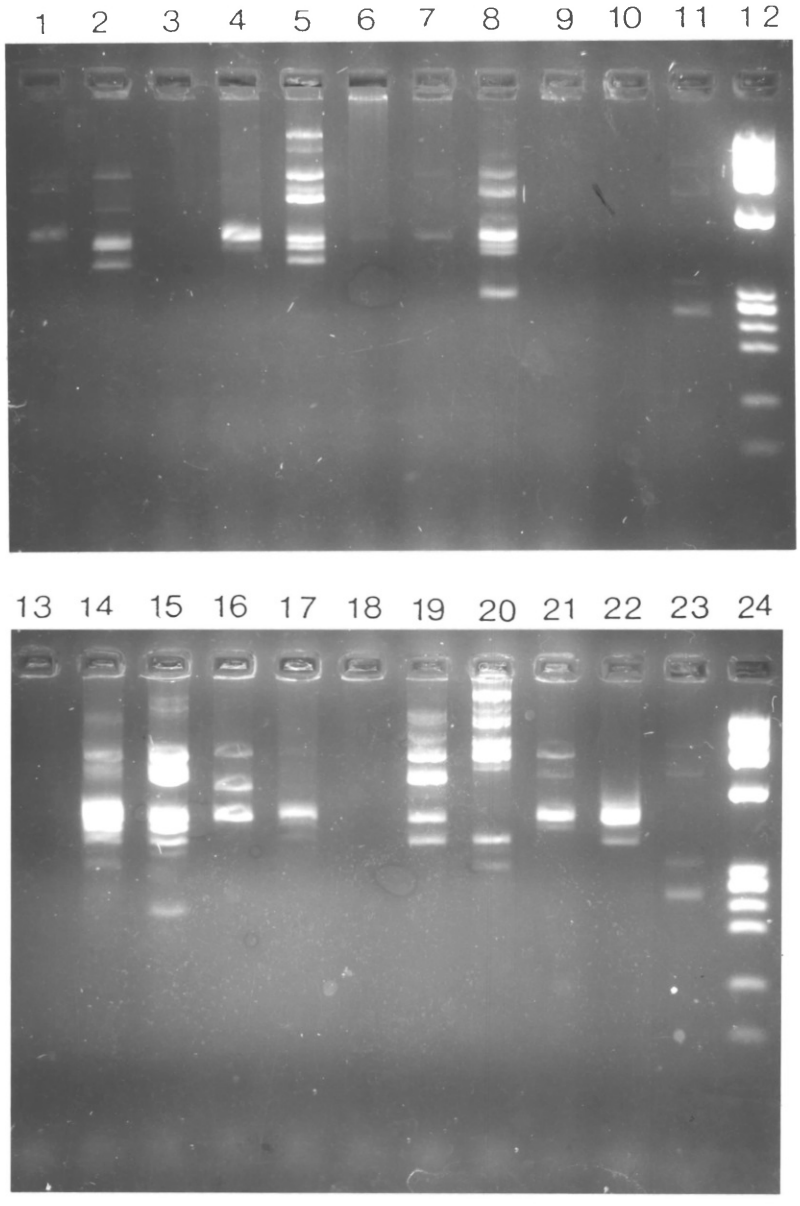


FIGURE 47

Analysis of human samples by PCR amplification with a mixture of primers designed to amplify regions of HPV types 6b, 11, 16 and 18.

0.5 µg of DNA extracted from 20 cervical scrape samples was subjected to PCR amplification (section 3.5.17) with primer pairs PCR18-A/PCR18-B, PCR16-A/PCR16-B and PCR6b/11-A/PCR6b/11-B. All three primer pairs were included in each case. One tenth of the volume of each amplified sample was subjected to electrophoresis through a 3% NuSieve GTG agarose/1% normal agarose gel (section 3.4.4.1). The gel was loaded as follows: Lanes 1-10, samples A to J. Lanes 13-22, samples K to T. Lanes 11 and 23, amplified linear DNA of HPV types 6b, 11, 16 and 18 (initially 0.25 ng of each DNA type). Lanes 12 and 24 contain 1 µg of øX174-HaeIII markers (fragment sizes 1,353, 1,078, 872, 605, 310, 281, 271, 234, 194, 118 and 72 bp).

The results are discussed fully in the text.

PCR18-B, PCR16-A/PCR16-B and PCR6b/11-A/PCR6b/11-B) were used, so that the presence of HPV types 6b, 11, 16 or 18 should result in amplification. In addition, the size of the amplification product should be indicative of the virus type(s) present. After amplification, samples were analysed by agarose gel electrophoresis, and the results are shown in figure 47. There are clearly amplification products present in the majority of the lanes, but the size of the bands does not correlate with the predicted sizes (206 bp in the case of HPVs 6b and 11, 159 bp for HPV 16 and 119 bp for HPV 18). In addition, the number of bands present in many of the lanes indicates that specific amplification of the desired target region has not occured. Each of the oligonucleotides chosen as a PCR primer was screened against the sequences present in the EMBL database, using a program known as XMATCH, and found to be unique to the HPV type it was designed to amplify. Unfortunately, only exact matches are detected by this program, so complementary sequences with one or more mismatch(es) were not identified; it is possible that regions bearing a high degree of homology with primers designed to amplify a different virus type are present on a given HPV genome. For example, HPV type 16 may have a region of high homology with primer PCR18-A, in addition to the binding sites for primers PCR16-A and PCR16-B. If all three primers were present during amplification, the expected 159 bp product would be produced, as well as an unexpected product of size determined by the distance between the binding sites for primers PCR18-A and PCR16-B. Clearly, the greater the number of binding sites, the more complex the pattern of amplification products will be.

In an attempt to increase the specificity of PCR amplification, 0.5 ug of DNA from each sample was amplified separately with each of the three primer pairs. The results of amplification with primers

PCR6b/11-A and PCR6b/11-B are shown in figure 48. The results demonstrate that a band of the correct size is present in the lanes corresponding to samples A, B, D, E, F, G, H, I, L, M, N, O, Q, R, S and T. An additional band of higher molecular weight is obvious in the case of sample E.

Amplification of the 20 samples with primers PCR16-A and PCR16-B, designed to amplify a 159 bp region of HPV 16, resulted in a negative result in each case (data not shown). Amplification with primers PCR18-A and PCR18-B, designed to amplify a 119 bp region of HPV 18, was negative with all samples, except sample M. In both cases, a control amplification performed with 1 ng of plasmid DNA was successful (data not shown).

A control amplification, using a pair of primers designed to amplify a 110 bp region of the human beta-globin gene, was subsequently performed on each of the twenty samples, as described in the legend to figure 49. The primers used were identical to those described by Saiki et al. in 1985. The purpose of the control amplification was: (i) to ensure that total loss of human DNA from a sample had not occurred during purification; (ii) to ensure that contaminants within the sample did not have an inhibitory effect on the amplification reaction; (iii) to check that the sample DNA was not totally degraded. Any sample which gives a negative result with the globin primers must be considered unsatisfactory, because even if HPV DNA were initially present, this DNA could have been lost or degraded during purification, or contaminants present in the sample could be preventing amplification. The results of control amplification of the twenty clinical samples, shown in figure 49, clearly show the expected 110 bp band in the case of all samples, with the exception of sample P. Sample P was also negative for HPV types 6b, 11, 16 and 18, and must be classed as 'sample unsatis-

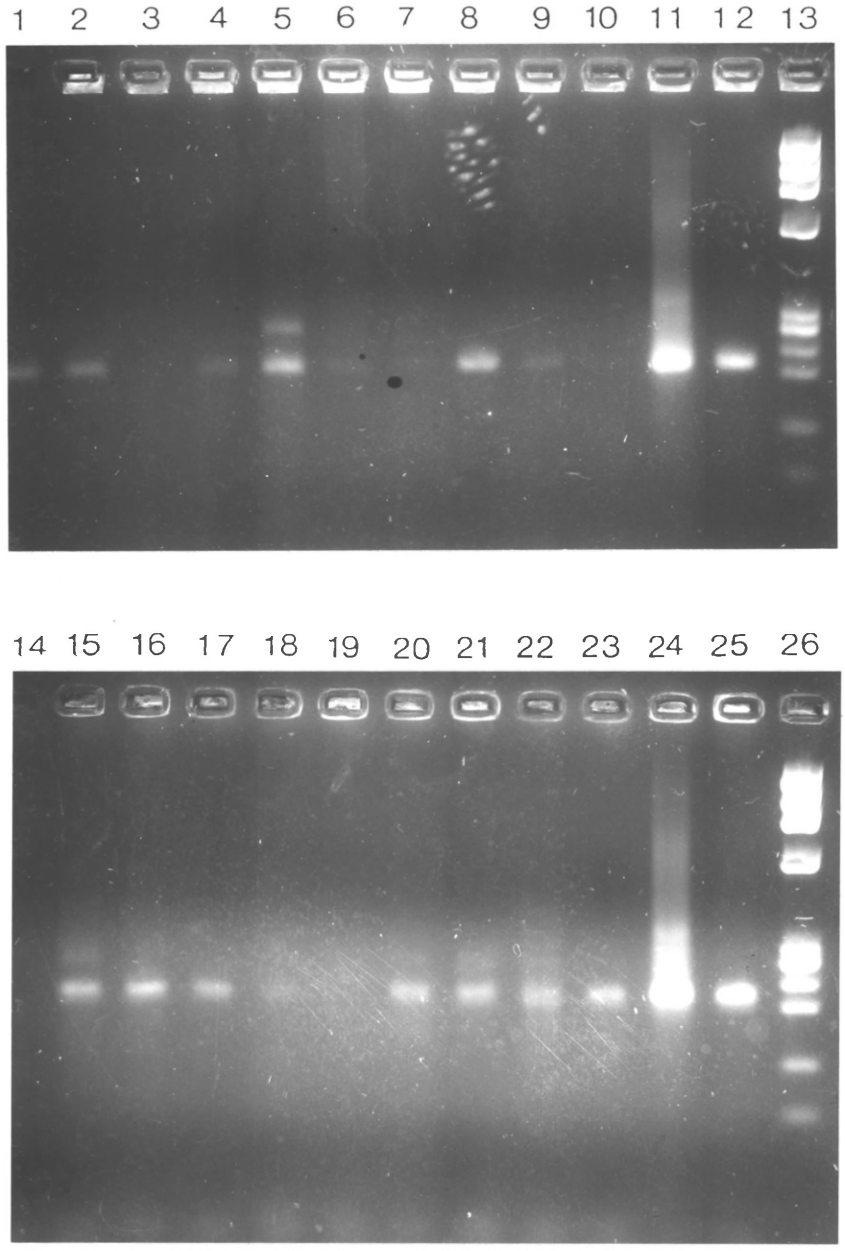


FIGURE 48

Analysis of human samples by PCR amplification with a primer pair designed to amplify a 206 bp region of HPV types 6b and 11.

0.5 µg of DNA extracted from 20 cervical scrape samples was subjected to 40 cycles of PCR amplification with primers PCR6b/11-A and PCR6b/11-B, exactly as described in section 3.5.17. One tenth of the volume of each amplified sample was subjected to electrophoresis through a 3% NuSieve GTG agarose/1% normal agarose gel (section 3.4.4.1). The gel was loaded as follows: Lanes 1-10, samples A to J. Lanes 14-23, samples K to T. Lanes 11 and 24, amplified linear HPV 6b DNA (initially 1 ng). Lanes 13 and 26 contain 1 µg of øX174-HaeIII markers (fragment sizes 1,353, 1,078, 872, 605, 310, 281, 271, 234, 194, 118 and 72 bp).

The results are discussed fully in the text.

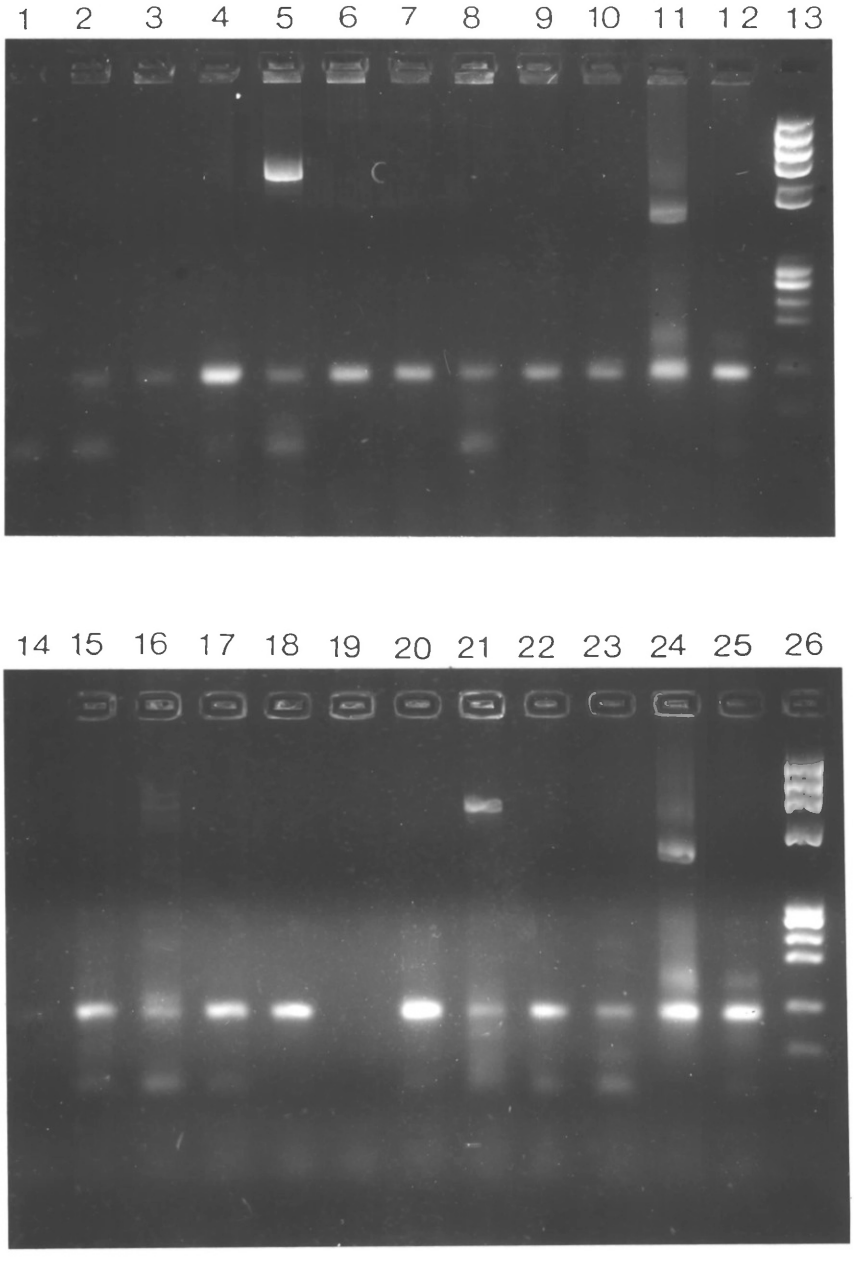


FIGURE 49

Analysis of human samples by PCR amplification with a primer pair designed to amplify a 110 bp region of the ß-globin gene.

0.5 µg of DNA extracted from 20 cervical scrape samples was subjected to 40 cycles of PCR amplification with primers PCO3 and PCO4, exactly as described in section 3.5.17. One tenth of the volume of each amplified sample was subjected to electrophoresis through a 3% NuSieve GTG agarose/1% normal agarose gel (section 3.4.4.1). The gel was loaded as follows: Lanes 1-10, samples A to J. Lanes 14-23, samples K to T. Lanes 11 and 24, amplified ß-globin gene 1.9 kb fragment (initially 1 ng). Lanes 12 and 25, amplified total human DNA (initially 1 µg). Lanes 13 and 26 contain 1 µg of øX174-HaeIII markers (fragment sizes 1,353, 1,078, 872, 605, 310, 281, 271, 234, 194, 118 and 72 bp).

The results are discussed fully in the text.

factory' for the reasons described above. The origin of the higher molecular weight amplification products in the case of samples E and R, and the lower molecular weight products in many of the samples, is unknown.

4.7.6 Summary Of PCR Data.

The results obtained from PCR studies can be summarized as follows:

(a) Maximum amplification efficiencies were attained using thermostable DNA polymerase supplied by Perkin Elmer Cetus, in a buffer consisting of 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 200 uM each of dATP, dCTP, dGTP and dTTP, 1 uM of each oligonucleotide primer, 100 ug/ml gelatin, 1 unit/25 ul DNA polymerase.

(b) PCR primer pairs were chosen to amplify different sized fragments of HPV types 6b, 11, 16 and 18. A universal primer pair was selected for use with types 6b and 11.

(c) Episomal (covalently closed, circular) DNA was amplified to the same efficiency as linear DNA.

(d) As few as 5,000-10,000 DNA molecules were detected by PCR amplification.

(e) Amplification of a sample with a mixture of primer pairs designed to amplify HPV types 6b, 11, 16 and 18 resulted in non-specific amplification products, of variable size. Amplification with individual primer pairs resulted in the production of amplification products of the predicted size.

(f) A control amplification, using a pair of primers designed to amplify a 110 bp region of the human beta-globin gene, was included to ensure that the reaction conditions were suitable for amplification.

4.8 Combined PCR-Sandwich Hybridization Assay For The Detection Of HPV Infection In Cervical Scrapes.

Twenty clinical samples (identical to those used in section 4.7.5) were subjected to 40 cycles of PCR amplification with primer pairs designed to amplify regions of HPV types 6b, 11, 16 and 18, exactly as described in section 4.7.5. A control reaction involving amplification of a 110 bp region of the human beta-globin gene was included in each case. After amplification, samples were diluted to a volume of 1 ml, and 5 ul aliquots were subjected to sandwich hybridization, exactly as described in the legend to table 12. The logic behind the dilution step was as follows: (i) 40 cycles of amplification theoretically results in a 1.1×10^{12} fold increase in the amount of the target sequence; clearly, the actual increase is considerably lower than this, because the amplification efficiency decreases as the concentration of target increases (Saiki et al., 1988 b). This effect is probably due to complementary target strands annealing to each other, rather than to the oligonucleotide primers. Regardless of the cause of this effect, it is reasonable to assume that (in the case of an infected sample), after 40 cycles, the reaction will have reached a plateau, and any further increase in target concentration will be very small in comparison with the concentration already present; (ii) studies with plasmid DNA indicated that, using the conditions described in section 3.5.17, the maximum amount of amplification product obtained from a single reaction (initially containing approximately 10,000 target molecules) was around 0.5-1 ug. In the case of a 200 bp amplification product, this represents 3.7-7.4 pmole of DNA. On dilution up to 1 ml, and extraction of a 5 ul aliquot, the total DNA removed would be between 18.5 and 37 fmole. This should be the ideal amount of target for assay by sandwich hybridization, using HPV type-specific kinase

	PCR-Sandwich Assay Result (Final dpm).			
	GLOBIN	HPV 6b/11	HPV 16	HPV 18
CONTROLS.				
HPV 6b	985	9,375	859	1,038
HPV 11	749	11,014	1,186	639
HPV 16	869	783	9,573	523
HPV 18	1,012	1,108	782	8,783
GLOBIN	10,764	594	927	863
SAMPLE.				
A	5,073	6,790	898	1,086
B	8,127	8,763	1,026	998
C	9,816	896	1,063	1,026
D	12,085	4,173	912	1,235
E	7,824	10,685	784	1,387
F	9,027	3,278	697	686
G	8,975	2,987	912	904
H	4,675	11,695	1,006	787
I	5,826	5,213	1,230	1,091
J	6,095	912	609	984
K	7,096	607	827	1,063
L	9,203	8,216	898	879
M	8,078	9,803	998	9,870
N	10,063	7,405	1,012	1,224
O	11,895	2,860	1,187	878
P	802	1,012	909	904
Q	9,873	8,376	877	637
R	5,827	9,027	612	789
S	8,698	10,011	788	524
T	11,826	5,403	1,096	876

TABLE 12

Combined PCR-sandwich assay for the detection of HPV types 6b, 11, 16 and 18.

The results of combined PCR-sandwich assays for the detection of HPV infection in clinical samples are tabulated above. 0.5 µg of DNA extracted from 20 cervical scrape samples was subjected to 40 cycles of PCR amplification with four different sets of primers: PCR6b/11-A + PCR6b/11-B; PCR16-A + PCR16-B; PCR18-A + PCR18-B; PCO3 + PCO4. These primers were designed to amplify specific regions of DNA from HPV 6b or HPV 11, HPV 16, HPV 18 and the human ß-globin gene respectively (see section 3.5.17). For the control experiments, 0.1 ng of the following DNAs were used in place of the clinical sample: HPV 6b, 5,368 bp EcoRI-BamHI fragment; HPV 11, complete genome (7,931 bp); HPV 16, complete genome (7,904 bp); HPV 18, complete genome (7,857 bp); ß-globin, 1,900 bp ß-globin gene fragment (Langdale & Malcolm, 1985). After amplification, samples were diluted to a volume of 1 ml, and 5 µl aliquots were subjected to sandwich hybridization. The following immobilized probes (100 fmole/assay) were used: HPV 6b, 686 bp PstI-BamHI fragment; HPV 11, 435 bp PstI fragment; HPV 16, 483 bp PstI fragment; HPV 18, 441 bp PstI fragment; ß-globin, 201 bp DdeI fragment. The probes used were the following 30-mer oligonucleotides, kinase end-labelled to an activity of approximately 5 x 10^2 dpm/fmole (50 fmole/assay): HPV 6b, PR6b-2; HPV 11, PR11-1; HPV 16, PR16-1; HPV 18, PR18-1; ß-globin, PRGLOBIN-1.

The results are discussed fully in the text.

end-labelled oligonucleotides as probes. After amplification of 20 clinical samples with the four primer pairs listed in the legend to table 12, the results were quantitated by sandwich hybridization. The results, presented in table 12, indicate the following: (i) the dilution used prior to assay was clearly suitable; the range of values within which samples were positive was 2,860-12,085 dpm; (ii) the total number of radioactive counts included in each reaction was approximately 25,000 dpm; the proportion of the probe which, in the case of positive samples, was involved in the formation of the A-C-B complex was between 11% and 48%; (iii) samples were considered positive if the signal they produced exceeded the mean of the negative control values, plus three times the standard deviation (range 523-1,186 [mean $\pm$ SD, 861 $\pm$ 183]); using this criterion, all of the clinical samples, with the exception of sample P, were positive according to the globin control assay, and were therefore suitable for assay (range 4,675-12,085 [mean $\pm$ SD, 8,425 $\pm$ 2,196]); (iv) sample P was negative for all four assays, and must therefore be considered unsuitable for study; (v) 16/20 samples were positive for HPV 6b and/or HPV 11 (range 2,987-11,695 [mean $\pm$ SD, 7,168 $\pm$ 2,781]); 4/20 were negative for these virus types (range 607-1,012 [mean $\pm$ SD, 857 $\pm$ 151]); (vi) 20/20 samples were negative for HPV type 16 (range 609-1,230 [mean $\pm$ SD, 917 $\pm$ 164]); (vii) 19/20 samples were negative for HPV type 18 (range 524-1,387 [mean $\pm$ SD, 945 $\pm$ 208]); (viii) sample M was positive for HPV type 6b or 11, and also for type 18. These results are presented as a scatterplot in figure 50.

Having studied each of the twenty clinical samples by the PCR-sandwich hybridization assay, the results of the cytology for each sample were obtained; these results (presented in table 13) indicate the following: (i) of 10 samples showing cytological evidence of

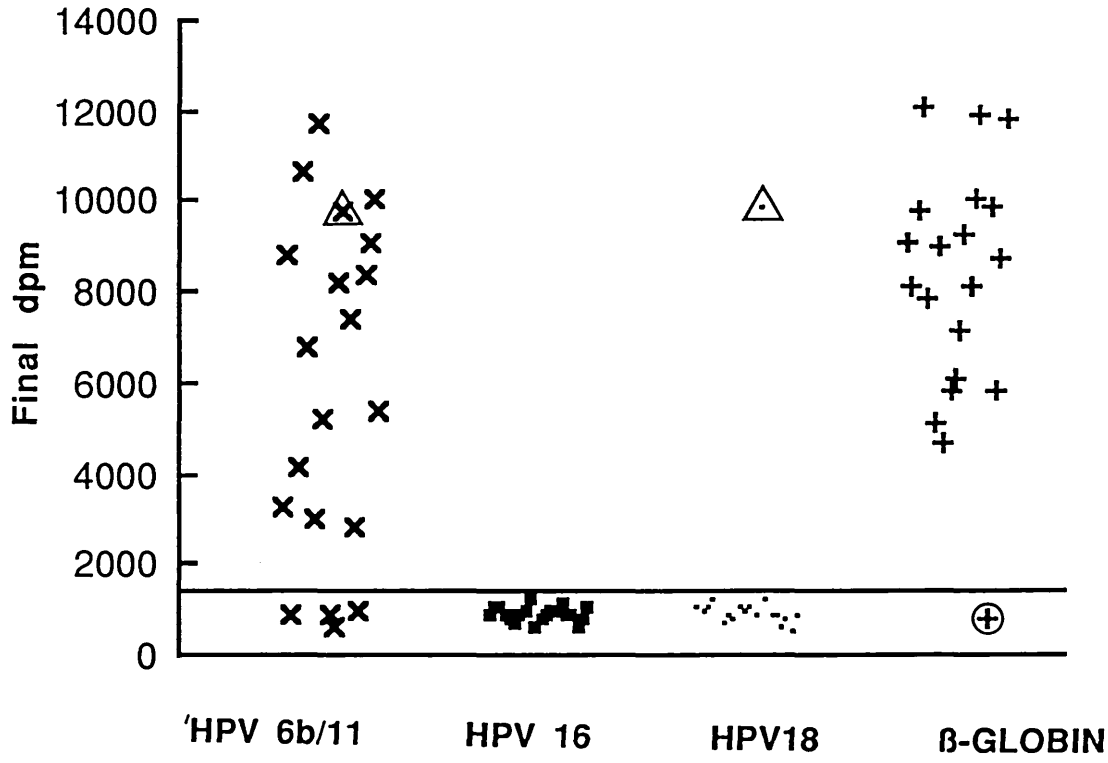


FIGURE 50

Scatter plot of results of combined PCR-sandwich assay for the detection of HPV types 6b, 11, 16 and 18.

PCR-sandwich assays were performed on 20 clinical samples, as described in the legend to table 12. The results are plotted as a scatter in the diagram above. The horizontal line represents the mean of the negative control values, + 3 standard deviations (S.D.) (861 + [3 x 183] = 1,410). Samples represented by points below the horizontal line are considered negative. A single specimen was negative for the globin amplification (◯), and was thus considered unsuitable for assay. A single sample was positive for globin, and also positive for HPV types 6b or 11, and 18 (△). Fifteen additional samples were positive for HPV types 6b or 11 only, and none were positive for HPV type 16.

The results are discussed fully in the text.

herpes/wart virus infection, 3 were negative in the PCR-sandwich assay. Of the remaining 7 samples, 6 were positive for HPV type 6b and/or 11, and 1 was positive for HPV 6b and/or 11, as well as for HPV 18 (sample M); (ii) 2/20 samples were considered cytologically normal, but were found to be positive for HPV 6b and/or 11 by nucleic acid hybridization; (iii) samples L and O were considered unsatisfactory for cytological screening (although both specimens show evidence of cellular atypia); both samples were positive for HPV 6b and/or 11, according to the sandwich assay; (iv) of 5 samples with cytological evidence of cellular atypia (of undefined origin), 3 were positive for HPV 6b and/or 11, one was negative for the four virus types tested, and one sample (P) was unsatisfactory for assay.

The results described above raise several interesting points; however, it is important to remember that the number of samples incorporated into this trial study was small, and it would be unrealistic to attach great statistical significance to the results. The aim of this investigation was to produce a novel assay format suitable for the detection of HPV infection in clinical samples, rather than to contribute to our understanding of the progression of the cellular changes resulting from cervical HPV infection. 30% (3/10) of the samples which showed cytological evidence of herpes virus or HPV infection were negative according to the sandwich assay; infection with both of these viruses can result in irregularities in the cell nucleus, and it can be difficult to firmly implicate one or other virus on this basis alone. Thus, it is possible that this figure (30%) represents samples which are infected with herpes virus, but not with HPV. 10% (2/20) of the samples studied were cytologically normal, but were HPV-positive according to the sandwich assay. Recent evidence suggests that the level of HPV infection in the cytologically normal adult population (as

Sample Code	Cytological Code(s).	PCR-Sandwich Hybridization Result. GLOBIN	HPV 6b/11	HPV 16	HPV 18
CONTROLS.					
HPV 6b	N/A	-	+	-	-
HPV 11	N/A	-	+	-	-
HPV 16	N/A	-	-	+	-
HPV 18	N/A	-	-	-	+
GLOBIN	N/A	+	-	-	-
SAMPLES.					
A	3,4	+	+	-	-
B	3,6	+	+	-	-
C	3,6	+	-	-	-
D	3	+	+	-	-
E	3,6	+	+	-	-
F	3,6	+	+	-	-
G	3,6	+	-	-	-
H	3,6	+	+	-	-
I	3	+	+	-	-
J	3,6	+	-	-	-
K	3	+	-	-	-
L	1,3	+	+	-	-
M	3,6	+	+	-	+
N	3	+	+	-	-
O	1,3,4	+	+	-	-
P	3	-	-	-	-
Q	3,6	+	+	-	-
R	2	+	+	-	-
S	3,6	+	+	-	-
T	2	+	+	-	-

TABLE 13

Summary of results of combined PCR-sandwich assay for the detection of HPV types 6b, 11, 16 and 18.

The results of combined PCR-sandwich assays for the detection of HPV infection in clinical samples are tabulated above. The results of assays designed for the detection of HPV types 6b or 11, 16 and 18, together with the results of a control assay for the detection of the human ß-globin gene, are shown; '-' indictes that a sample was negative in the assay, and '+' indicates a positive sample. The cytological codes used correspond to the following: **1**, specimen unsatisfactory; **2**, normal cells only; **3**, atypical cells present; **4**, Trichomonas vaginalis present; **6**, herpes/wart virus (HPV) present. Clinical specimen **'P'** is negative in the assays designed to detect HPV, and is also negative for the ß-globin control; for the reasons discussed in section 4.7.5, the sample must be designated 'unsuitable for assay'.

The results tabulated above are discussed fully in the text.

determined by PCR) may be greater than 80% (G.C.N. Parry, personal communication). Great care was taken to ensure that sample contamination was not the reason for this surprising result, and further studies aimed at identifying HPV sub-types with different 'oncogenic potential' are currently in progress. It is interesting to note that 10% (2/20) of the samples studied were unsuitable for cytological screening, but were studied successfully by the PCR-sandwich assay; this is an example of a situation in which nucleic acid-based tests are superior to cytology.

How certain can we be that the specimens used for the two screening procedures were identical? A single cervical scrape was used for the preparation of both samples (see section 3.5.5), but it is possible that the majority of the cells obtained were used to prepare the smear, rather than stored for nucleic acid analysis (or vice versa). Cervical HPV infection can be focal, so it is possible that an individual could be incorrectly diagnosed because the infected region of the cervix was accidently avoided on taking the scrape. Since both of these potential sources of error are avoidable, it is essential to stress the importance of good technique to the individual collecting the sample.

In summary, it seems unlikely that cytology or nucleic acid hybridization alone will be use for screening the population for cervical HPV infection; far more likely, a combination of the two techniques will be used to improve the efficacy of the cervical screening program.

4.9 Application Of Non-radioactive Detection Systems To PCR-Sandwich Assay.

In an attempt to adapt the sandwich hybridization assay for use with non-radioactive probes, two commercially available detection

systems were investigated: (i) a non-radioactive labelling and detection kit supplied by Boehringer Mannheim (BCL). In this system, the probe is labelled by random priming, with the incorporation of digoxigenin-labelled deoxyuridine triphosphate. The dUTP is linked via a spacer arm to the steroid hapten digoxigenin (Dig-dUTP). After hybridization with the target DNA, the hybrids are detected by enzyme-linked immunoassay using an antibody conjugate, and subsequent enzyme-catalyzed colour reaction with a suitable substrate; (ii) BluGENE, a non-radioactive nucleic acid detection system developed by Gibco-BRL, involves the use of a biotinylated probe, labelled by nick translation; after hybridization and washing, the probe-target hybrid is detected by incubation with a streptavidin-alkaline phosphatase (AP) conjugate, and subsequent enzyme-catalyzed colour reaction with a suitable substrate.

Two-step assays were performed using 100 fmole of immobilized HPV 16 483 bp PstI fragment, 5 fmole of labelled probe (1,776 bp HPV 16 PstI fragment), and 100 amole of target DNA (HPV 16 genome [7,904 bp], isolated from a plasmid). Initial studies performed with the BCL kit were totally unsuccessful, because the reaction product (formed by the addition of nitroblue tetrazolium [NBT] salt, and 5-bromo-4-chloro-3-indolyl phosphate [BCIP]) was insoluble, and adhered to the Sephacryl beads (data not shown). Washing with ethanol dissolved the precipitate, but on measuring the $O.D._{650\ nm}$ of the supernatant, no correlation was observed between the amount of target DNA, and the signal obtained (data not shown). In fact, equivalent signals were attained in control assays in which the immobilized probe was omitted, indicating that the <Dig>-AP conjugate bound non-specifically to the Sephacryl bead. Although attempts were made to decrease this background (for example, pre-incubation of the resin with BSA), no satisfactory results were obtained, and the BCL kit was discarded in favour of the BRL detection

Immobilized Probe (100 fmole)	Target DNA (100 amole)	Labelled Probe (5 fmole)	Initial dpm	Final dpm	O.D.$_{650\ nm}$
483 bp	HPV 16	1,776 bp	92,105	816	0.406
483 bp	NONE	1,776 bp	102,207	278	0.247
NONE	HPV 16	1,776 bp	98,817	192	0.252

TABLE 14

Two-step hybridization using a probe labelled with ^{32}P and biotin.

A two-step sandwich assay using a probe labelled with ^{32}P and biotin was performed exactly as described in the text. Each result is the average of two determinations. The immobilized DNA used (A) was a 483 bp HPV 16 PstI restriction fragment, derived from the genome of HPV 16. 100 fmole of A was hybridized at 65^{o}c with 5 fmole of dual-labelled probe, and 100 amole of HPV 16 target DNA. The detection of the non-radioactive probe was performed as described in section 3.5.8.

system.

The results of a two-step sandwich assay using a probe labelled by nick translation with biotin and ^{32}P (to an activity of approximately 2 x 10^4 dpm/fmole) are shown in table 14. The following points are clear from the table: (i) the dual-labelled probe hybridizes specifically; after subtraction of the average of the control values, the hybridization efficiency turns out to be approximately 31%; (ii) the non-specific binding of the probe to the Sephacryl beads (on average, 0.23%) is comparable with the equivalent values obtained for probes labelled just with ^{32}P; (iii) despite the fact that the hybridization is clearly specific, the results obtained for the non-radioactive assay are relatively poor. The level of non-specific binding is comparatively high, and is probably the result of direct binding between the streptavidin-AP conjugate and the solid support; subsequent studies performed with the omission of the biotinylated probe confirmed this hypothesis (data not shown). Although attempts were made to decrease the level of non-specific binding (for example, pre-incubation of the resin with BSA), extensive studies to optimize the amount of probe, conjugate and substrate used were not performed, because of limited time.

Although, in this form, non-radioactive probes are not suitable for use in the sandwich assay, the results are not totally discouraging, and it is possible that further study with alternative conjugates or substrates (for example, the pre-formed biotin-streptavidin complex available from Amersham International) would improve the results considerably.

4.10 Amplified Signal Affinity Capture (ASAC).

Studies performed with plasmid DNA indicated that one-step

assays (involving the hybridization of a labelled probe directly to an immobilized DNA fragment) were extremely rapid; in the case of a kinase end-labelled oligonucleotide probe, the reaction was found to be essentially complete after just 20 minutes. The amplified signal affinity capture (ASAC) technique was developed in order to take advantage of the efficiency and favourable kinetics of the one-step assay.

The principle of the procedure (shown diagrammatically on page 166) is simple; DNA extracted from a clinical sample known to be positive for HPV 6b and/or HPV 11 (see table 13, sample H) was subjected to PCR amplification with primers PCR6b/11-A and PCR6b/11-B; the latter of this pair was pre-biotinylated at the 5' end, as described in section 3.5.18. This results in one half of the amplified strands (210 nucleotides in length) being biotinylated at the 5' end. Qualitative analysis of the amplification efficiency by agarose gel electrophoresis demonstrated that the use of a biotinylated primer did not significantly affect the reaction (data not shown). After the final cycle of PCR, 5 ul of the reaction volume (equivalent to between 0.15 and 0.35 pmole of amplification product) was removed, and hybridized in solution with 25 fmole each of oligonucleotides PR6b-2 and PR11-1, kinase end-labelled to an activity of approximately 5 x 10^2 dpm/fmole. After hybridization, all biotinylated molecules were captured by incubation with a suspension of streptavidin-agarose; after washing (as described in section 3.5.18), the results were quantitated by Cerenkov counting. The results of an ASAC assay performed on DNA extracted from sample H are shown in table 15. The following points should be noted: (i) in each assay, the affinity-captured amplification product is in approximately 3-fold to 7-fold excess over the labelled probe; however, since two different probes were used in each assay, one of which was complementary to HPV 6b, and the other to HPV 11, the theoretical maximum

amount of probe which could become attached to the solid support is 50% (assuming that the sample is infected with just one HPV type); (ii) the control assays involving HPV 6b or HPV 11 plasmid-derived DNA as the amplified target have clearly been successful, whereas assays involving non-complementary target-probe combinations result in a much lower proportion of the probe being captured; (iv) the result of the ASAC assay for the detection of HPV 6b and/or 11 in sample H is clearly positive; this corresponds well with the result of the PCR-sandwich assay shown in table 12.

Unfortunately, further studies involving the use of the ASAC technique were precluded, because of the expense involved in purchasing streptavidin-agarose. It would have been interesting to attempt to quantify the amount of amplification product, by comparison of results with a standard curve prepared using known amounts of biotinylated DNA. In addition, it is possible that the efficiency of affinity capture could have been improved by incorporation of more than one biotin group into each amplification product.

[Note: After completion of this section, a study describing a similar technique was published; the reference is as follows: Syvanen A-C, Bengtstrom M, Tenhunen J, Soderlund H: Quantification of polymerase chain reaction product by affinity-based hybrid collection. Nucleic Acids Res 16:11327-11338, 1988].

TARGET	Initial dpm	Final dpm	% Probe Bound
PLASMID			
HPV 6b	21,868	7,842	36
HPV 11	23,297	6,158	26
HPV 16	24,874	1,794	7
HPV 18	20,085	2,257	11
SAMPLE			
H	22,731	7,140	31

TABLE 15

Detection of HPV infection by amplified signal affinity capture.

Amplified signal affinity capture (ASAC) was performed exactly as described in section 3.5.18. Each result represents a single determination. The results are discussed fully in section 4.10.

5. DISCUSSION.

5.1 Sandwich Hybridization As A Method Of Detecting HPV Infection.

A nucleic acid sandwich hybridization assay has been developed, initially using cloned human papillomavirus (HPV) sequences as a model, and subsequently for the detection and typing of HPV infection in cervical scrapes. Genital infection with one of this genus of viruses is clinically significant, because of the relatively recent accumulation of evidence linking specific HPV types with the aetiology of cervical cancer. The sandwich technique was developed using DNA fragments covalently immobilized onto Sephacryl S-1000 beads, for use as the 'catching' reagent; a ^{32}P labelled, HPV type-specific DNA probe (either a labelled restriction fragment, or a synthetic oligonucleotide) was used in conjunction with the immobilized probe. A number of parameters were varied, in order to optimize the covalent immobilization of nucleic acids, and study their hybridization properties.

Having chosen PstI as the most suitable restriction enzyme for producing sub-genomic HPV-derived DNA fragments for use in the sandwich assay, the appropriate fragments were sub-cloned into pUC8, and their hybridization properties were investigated by Southern and slot-blotting; where possible, the fragments chosen as immobilized (A) and labelled (B) probes were adjacent on the viral genome, in order to minimize the likelihood of obtaining a false negative signal as a result of degradation of the 'target' DNA. After confirming that the selected fragment pairs were HPV type-specific, and did not cross-hybridize, two chemistries suitable for covalently immobilizing nucleic acids were investigated. The first method tested, carbodiimide-mediated coupling via the 5' phosphate group, was totally unsuitable because of consistently low (and highly unreliable) immobilization

efficiencies. The second method investigated, involving diazonium ion-mediated coupling at random nucleotide base positions, was far more suitable and reliable, typically resulting in an immobilization efficiency within the range 53%-85%. Over the range of input DNA concentrations 140-560 pmole/g of solid support, the maximum immobilization efficiency was observed at the lowest input concentration. Over the range of fragment sizes 3,601-216 bp, size had no significant effect on immobilization efficiency. Of four particulate solid supports tested, DPTE-activated Sephacryl S-1000 resulted in the highest binding efficiency, with the lowest levels of non-covalent attachment. Sephacryl S-500 was also suitable (although average binding efficiencies were lower); Dynospheres M450 and Sepharose CL4B were totally unsuitable, because the extent of coupling was extremely low. Although time-course studies indicated that the immobilization reaction was complete after approximately one hour, most stable coupling resulted if the reaction was allowed to proceed for 48 hours. The stability of DNA immobilized by diazotization onto Sephacryl S-1000 was such that approximately 5% of the DNA was lost each month, for at least 12 months after immobilization. Of the two methods used to determine the quantity of immobilized DNA, the ^{32}P tracer assay was found to give higher results than the nuclease assay; this probably reflects the fact that the former is a measure of the total DNA coupled, whereas the latter is a representation of the amount of bound nucleic acid which is accessible to the nuclease molecule.

Having identified a suitable chemistry for the covalent immobilization of HPV-derived restriction fragments to Sephacryl beads, the hybridization properties of the coupled nucleic acid strands were investigated, using a one-step assay. Of three hybridization buffers tested, the best results were obtained using 40 mM PIPES, pH 6.5, 0.6 M

NaCl, 1 mM EDTA, 0.1% w/v SDS, 250 ug/ml denatured, sonicated salmon sperm DNA, at 65^{o}C. Post-hybridization washing was performed in 1 ml of the same buffer, minus salmon sperm DNA, for 3 x 10 minutes, at the same temperature. Time-course studies indicated that, under the conditions selected, the one-step assay was essentially complete within one hour. Optimum hybridization efficiencies (typically within the range 55%-70%) were only obtained if the immobilization of the support-bound fragment was allowed to proceed for a full 48 hours. The concentration of immobilized DNA (within the range 62-202 pmole/g of resin) did not significantly affect the hybridization efficiency. However, it was found that, if this concentration was too low, the mass of resin required for each assay resulted in unacceptably high non-specific background levels; on the other hand, no advantage was gained by having immobilized DNA concentrations at the high end of this range, because the mass of resin required per assay was too small to be measured accurately. The ideal concentration of immobilized DNA, with respect to ease of manipulation, and efficiency of subsequent hybridization (with an acceptable background signal) was approximately 80-100 pmole/g of resin. Immobilized fragment size (within the range 3,601 to 216 bp) had no effect on the efficiency of hybridization. However, a 20-mer oligonucleotide immobilized by diazotization was found to be unavailable for hybridization. Increasing the immobilized probe:labelled probe ratio up to 100:1 resulted in an increase in the hybridization efficiency; any further increase in this ratio had no advantageous effect.

Having ascertained that DNA immobilized by diazotization onto Sephacryl S-1000 beads was available for hybridization, a model sandwich (two-step) assay for the detection of cloned HPV target DNA was developed. The lower sensitivity limit of the assay, using restriction fragments labelled by random priming as probes, was around 25 amole.

No increase in hybridization efficiency was observed on dividing the sandwich assay into two separate reactions (allowing the hybrid between target and immobilized probe to form, with the subsequent addition of labelled probe, or allowing the hybrid between target and labelled probe to form, with the subsequent addition of immobilized probe). Pre-hybridization was performed for 2 hours, at 65^{o}C, in a buffer consisting of 40 mM PIPES, pH 6.5, 0.6 M NaCl, 1 mM EDTA, 0.1% w/v SDS, 250 ug/ml denatured, sonicated salmon sperm DNA; pre-hybridization for longer than 2 hours, or increasing the concentration of salmon sperm DNA, had no effect on the non-specific background signal. Hybridization was performed in the same conditions, for a minimum of 16 hours, with a 50-fold molar excess of probe over target. Post-hybridization washing was performed at the same temperature, for 3 x 10 minutes, in hybridization buffer (minus salmon sperm DNA); more extensive (and/or stringent) washing was found to have no effect on the background signal, but did result in a decrease in the expected hybridization signal, with a concomitant reduction in the signal to noise (S/N) ratio. The sensitivity of the two-step assay using a kinase end-labelled oligonucleotide probe was around 5-10 fmole. Pre-hybridization was performed for 2 hours, at 65^{o}C, in 6 x SSC, 0.1% w/v SDS, 250 ug/ml denatured, sonicated salmon sperm DNA. Hybridization was performed in the same conditions, for a minimum of 4 hours, with a 10-fold molar excess of probe over target. Post-hybridization washing was performed at the same temperature, for 3 x 10 minutes, in hybridization buffer (minus salmon sperm DNA). HPV types 6b, 11, 16 and 18 were readily distinguished using both types of labelled probe. Hybridization time-courses showed that, using restriction fragments as probes, the reaction was essentially complete after 16 hours; the equivalent figure in the case of oligonucleotide probes was 4 hours. Although 25 amole of plasmid target

DNA was detected using a probe labelled by random-priming, attempts were made to increase the sensitivity of the assay before important samples were tested by sandwich hybridization; since the samples used in this investigation were collected as part of a large prospective study, it was essential that accurate results were obtained. The yield of DNA recovered from a cervical smear (typically around 5 ug) is sufficient to perform a single blot or sandwich assay; in the case of blotting, this is not a problem, because it is possible to remove hybridized probe (a procedure known as strip-washing), allowing a filter to be investigated by hybridization with a different probe. Thus, a single filter may be analyzed with four different probes, derived from HPV types 6b, 11, 16 and 18. In theory, it should be possible to subject the DNA extracted from a sample to successive sandwich hybridizations with four different immobilized probes isolated from the HPV types listed above. Consider, for example, a sample which is positive for HPV type 11, and not for any other virus type; the DNA extracted from the sample could first be hybridized with immobilized and labelled probes derived from HPV type 6b. Since there is no HPV 6b target DNA, no hybrids would form, and the HPV 11 DNA should remain free in solution. The supernatant could then be removed prior to washing the Sephacryl beads, and subjected to a second round of hybridization, with immobilized and labelled probes derived from virus type 11; this time, the sandwich hybrid should form, leading to a positive result. Although such sequential hybridization is possible in theory, the simultaneous addition of four different labelled probes would probably result in an unacceptably high non-specific background signal (and corresonding low sensitivity).

The most obvious means of increasing the sensitivity of the sandwich assay was to include a polymerase chain reaction (PCR)-

mediated amplification step prior to hybridization. This had the additional advantage that a single clinical sample would provide ample DNA for many separate amplifications, allowing it to be tested for HPV types 6b, 11, 16 and 18. Maximum amplification efficiencies were attained using a thermostable DNA polymerase supplied by Perkin Elmer Cetus, in a buffer consisting of 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 200 uM each of dATP, dCTP, dGTP and dTTP, 1 uM of each oligonucleotide primer, 100 ug/ml gelatin, and 1 unit/25 ul DNA polymerase. PCR primer pairs were chosen to amplify different sized genomic segments of HPV types 6b, 11, 16 and 18; each amplified region contained a recognition site for the enzyme PstI. A single primer pair was selected for the amplification of a 206 bp region of types 6b and 11. HPV DNA persists within infected cells either as covalently closed, extrachromosomal, circular elements (known as episomes), or integrated within a host cell chromosome. Covalently closed, circular (plasmid) DNA was amplified to the same efficiency as linear DNA, suggesting that the PCR technique is effective, regardless of the physical structure of the target DNA. Dilution studies with plasmid DNA indicated that as few as 5,000-10,000 molecules of target were amplified with high efficiency. Studies involving amplification of a single sample with all three primer pairs (with target regions on HPV types 6b or 11, 16 and 18) resulted in non-specific amplification products, of variable size. However, amplification with individual primer pairs generated products of the predicted size. A control amplification was included for each sample, using a pair of primers designed to amplify a 110 bp region of the human beta-globin gene. The purpose of this was to ensure that the conditions were suitable for the PCR reaction. Having developed the sandwich assay, and the PCR amplification technique, the two procedures were combined, and used for the analysis of clinical samples. The

results, obtained in well under 24 hours, clearly demonstrated that the PCR-sandwich assay was capable of detecting HPV infection, as well as identifying the type of the virus involved.

There are a number of experiments included in this study which, in retrospect, could have been better designed. For example, a great deal of effort purifying restriction fragments could have been circumvented if the immobilized and labelled probes for a given HPV type were sub-cloned into different vectors; it was essential that at least one of each fragment pair was thoroughly purified from contaminating pUC8 vector sequences, or a false positive signal could have resulted from hybridization between these sequences in the immobilized and labelled probes. If two different (non-complementary) vectors had been used, it is possible that purification of probes prior to immobilization and labelling would not have been necessary. In addition, in assays where the labelled probe was a synthetic oligonucleotide, it was probably not essential to isolate the HPV restriction fragment from the vector prior to immobilization, since it is highly unlikely that a binding site for the HPV-specific oligonucleotide was present on pUC8.

A second study which could have been better designed was the use of a pair of beta-globin gene specific primers to ensure that the conditions were suitable for the PCR reaction. In order to be an ideal control, the globin primers should have been included in the same tube as the sample and HPV-specific primers, rather than used in a totally separate assay. The main reason for performing two separate reactions was that studies involving amplification of plasmid DNA with a mixture of three pairs of primers specific for HPV types 6b or 11, 16 and 18 resulted in the generation of a complex mixture of non-specific amplification products; these products were probably the result of primers binding to regions of partial homology on an HPV genome which they were

not designed to amplify. This should not be a problem in the case of the globin primers, because it is highly unlikely that a region of sufficient homology was present on the genome of any of the HPVs tested for.

The great advantage of the PCR technique is that it results in an amplification in the number of target sequences present in a sample. The main disadvantage of the method is that a false positive signal could easily be obtained as a result of contamination. Consider a clinical sample (initially free from HPV infection) which accidently becomes contaminated with a small amount of pBR322-HPV 18 plasmid DNA; if half of this sample were to be tested by slot-blotting, the result should be negative, because the number of contaminating plasmid molecules would be below the lower limit of sensitivity of the assay. Many more contaminating molecules would be required before the sample was incorrectly identified as positive. Next, consider the same sample tested by PCR amplification, followed by sandwich hybridization; PCR would result in an exponential increase in the number target sequences, and the result of the sandwich assay would be positive. It is clear from this example that, if PCR is to be used in routine diagnosis, great care must be taken to ensure that sample contamination is avoided. If plasmids were the only likely source of contamination, it would be possible to include a control amplification involving a primer pair designed to amplify a fragment spanning one of the junctions between vector and insert sequences. An amplification product would not result from this primer pair if the sample were genuinely infected with HPV DNA, because there would be no binding site for the primer complementary to the vector sequence. Unfortunately, plasmids are not the only potential source of contamination; a sample would be far more likely to be contaminated with the amplification products of a previous PCR

reaction (possibly spread in an aerosol produced from a micropipette), and these sequences would obviously be excellent templates for subsequent amplification. Contamination from this source would clearly be impossible to detect, because the PCR products would be indistinguishable from those resulting due to a 'genuine' infection.

Recent evidence suggests that the size of the region to be amplified has a significant effect on the efficiency of the PCR reaction (G.C.N. Parry, personal communication). If this is the case, the primer pairs chosen for HPV types 6b, 11, 16 and 18 should ideally have been separated on the target genomes by the same number of nucleotides, in an attempt to attain comparable amplification efficiencies for the four virus types. In fact, the shortest sequence amplified (in the case of HPV type 18) was 119 bp, and the longest sequence amplified (in the case of types 6b and 11) was 206 bp; significant reductions in amplification efficiency have only been observed for sequences greater than 500 nucleotides in length (G.C.N. Parry, personal communication).

A notable omission which should have been investigated thoroughly was the effect of target size on the efficiency of the two-step hybridization. However, considering that target DNA molecules of a size of around 100-200 bp (the products of PCR-amplification) appeared to hybridize as efficiently as intact viral genomes around 8 kb in size, it seems unlikely that, within this range, target size has a highly significant effect on the efficiency of hybrid formation.

The sensitivity of the sandwich assay was limited by the non-specific background signal; as discussed in chapter 1, the sensitivity of any technique may be improved by increasing the signal to noise (S/N) ratio. This may be achieved either by increasing the signal resulting from the presence of the target sequence, or by decreasing the level of background. Although some attempt was made to reduce

background (for example, the pre-hybridization time, and concentration of salmon sperm DNA, BSA and Denhardt's solution were varied), in retrospect, potentially more effective measures could have been taken. The non-specific signal was the result of probe molecules becoming attached to the surface of the Sephacryl bead; it is possible that this interaction was ionic in nature, and may have been reduced by pre-incubation of the DNA-Sephacryl with a buffer containing an anionic small molecule.

In summary, the sandwich hybridization assay for the detection of HPV infection developed during the course of this investigation has significant advantages over more conventional detection methods; it is reliable and simple, and results can be obtained in under 24 hours. This represents a considerable improvement in the ease of sample manipulation, and time taken to achieve a result. It is possible that the PCR-sandwich technique could be adapted for use with a crude cell lysate (thus avoiding the time-consuming purification of nucleic acid from large numbers of samples); further experiments are required in order to clarify this point. The use of a bead rather than a membrane as the solid support also has advantages; beads are easily pelleted by centrifugation, making washing and changing buffers facile. The fact that each sample is assayed in a separate tube means that results can be quantitated simply and efficiently, using Cerenkov counting. It should be easy to adapt the procedure for the detection of other infectious agents, or specific genes, providing suitable probes are available. If the modification of the technique for use with differentially-melting oligonucleotides were successful, point mutations could also be identified (see section 5.2). Despite the fact that the sandwich assay was found to be approximately ten times less sensitive than

Southern or slot-blotting, this disadvantage is outweighed by the inclusion of a PCR amplification step, prior to assay. Unlike techniques which require the immobilization of the sample DNA (and in which the amount of sample assayed is limited by the binding capacity of the membrane), the amount of target used in sandwich hybridization can be increased until a suitable signal is attained. Unfortunately, the non-radioactive signal detection methods tested were largely unsuccessful; it is possible that probes labelled directly with a suitable enzyme (HRP or AP) would be more suitable, because the main problem associated with biotinylated probes was the non-specific binding between the solid support and the streptavidin-enzyme conjugates used to detect the probe. Possibly the most attractive advantage of the PCR-sandwich method is that the technology exists to automate the procedure. For example, an intelligent heating block could be used to perform PCR amplification on the sample; the amplified DNA could be denatured in the heating block by increasing the temperature, and the probes required for sandwich assay (one immobilized on Sephacryl, and the other labelled in a suitable manner) could be added to each sample, using an XYZ-type robotic workstation. This instrument was initially designed to transfer reagents to and from 96-well microtitre plates, but could easily be adapted for use with a uniform array of Eppendorf tubes in a heating block (Landegren et al., 1988). After hybridization at 65^{o}C, the beads could be pelleted, and washing could be performed using the workstation. The signal could then be measured in a suitable manner (depending on the nature of the labelled probe). The main problem with this procedure is the step involving pelleting of the beads. There are two possible routes for circumventing this problem: (i) if the beads used as the hybridization support were magnetic, an electro-magnet incorporated in the heating block could be used in place

of a centrifuge; (ii) pelleting may not be essential. An immunoassay system using Sephacryl as the solid support is being developed by Celltech (and is known commercially as SucroSep™); instead of centrifugal pelleting, the beads are washed by allowing them to settle by gravity through a layer of sucrose solution. Initial studies have shown that this method of washing away unbound reagents is extremely effective, and each wash is complete in two minutes. It is possible that this procedure could be adapted for use with DNA hybridization assays which use Sephacryl as the solid support.

A considerable amount of work remains to be done before the precise role played by papillomaviruses in the aetiology of cervical cancer is fully understood; in order to determine whether the association between HPV infection and cancer is casual or causal, large numbers of clinical samples will need to be examined. The sandwich assay developed during the course of this investigation should contribute to the success of such studies. It seems unlikely at present that a DNA-based assay will be used in place of cytology and histology for the detection of cervical HPV infection; far more likely, the techniques will be used to complement each other, resulting in a considerable improvement in the efficacy of the screening program. For example, if a sample were found to be HPV positive by nucleic acid hybridization, but was negative according to histology and cytology, the patient involved could be screened on a regular basis in the expectation of detecting cellular abnormalities as soon as possible after they occur.

5.2 Future Work Arising From This Study.

In order to be ideal for routine diagnostic use, the signal detection step in the PCR-sandwich assay should be based on a non-radioactive probe. Studies involving the use of such probes were un-

successful, largely because of non-specific binding of the <Dig>-enzyme and streptavidin-enzyme conjugates directly to the solid support. It is possible that this binding could be prevented by pre-incubation of the resin with preformed biotin-streptavidin complexes. Alternatively, probes labelled directly with a suitable enzyme (for example, HRP or AP) may have been less susceptible to non-specific binding. It would have been interesting to attempt to apply non-radioactive detection systems to the ASAC technique, but further studies were precluded because of the considerable expense involved in purchasing streptavidin -agarose.

There are circumstances in which it is necessary to detect a single base mutation which does not result in the creation or destruction of a restriction enzyme recognition site; an example is the mutation associated with $alpha_1$-antitrypsin deficiency (Abbott et al., 1988). Recent evidence suggests that point mutations (together with small insertions or deletions) in the long control region (LCR) of HPV type 16 may have a significant effect on the 'oncogenic potential' of a particular virus sub-type (G.C.N Parry, personal communication). It is possible that these variations (initially identified directly by sequencing) will have to be detected on a large-scale basis using the differential melting properties of synthetic oligonucleotides (see section 1.7.2). There is no obvious reason why the sandwich hybridization assay should not be adapted for use with differentially melting oligonucleotide probes; oligos are simple and relatively cheap to manufacture, but have not been used extensively in studies involving large numbers of samples, because the resulting sensitivities are lower than those achieved using probes produced by nick translation, or random priming. Clearly, if the differential melting properties of oligos were to be used to discriminate between sequences differing in a

single nucleotide, the hybridization and melting characteristics of matched and mis-matched oligos in a sandwich assay would have to be extensively investigated. Recent studies involving the use of the base analogue 2-aminoadenine have demonstrated that hybrids formed between target sequences and oligos containing this analogue are considerably more stable than the equivalent hybrids formed between the same target and a non-modified oligo. This is because substitution of adenine for 2-aminoadenine results in changes in the minor groove of the DNA helix, and creates an additional (stabilizing) hydrogen bond in the Watson-Crick base pair with thymine (Chollet & Kawashima, 1988). Consider the case of a point mutation resulting in the substitution of an adenine residue with a thymine residue; the hybrid formed between a modified oligo (incorporating a 2-aminoadenine residue at the position of the mutation) and the mutated target sequence would be far more stable than the hybrid formed between the same oligo and the non-mutated sequence. Thus, the use of 2-aminoadenine-modified oligos should simplify the adaptation of differential melting assays for use in sandwich hybridization; recent studies have confirmed this hypothesis (U.B. Voss, personal communication). Experiments involving the immobilization of a 20-mer oligo by diazotization resulted in an immobilization efficiency of 40% (according to the tracer assay); the efficiency of hybridization of the immobilized oligo with a labelled restriction fragment free in solution was very low (3%), indicating that immobilization randomly via the bases prevents the oligo from forming stable duplexes with complementary DNA. It is likely that immobilization via one or other end would have allowed the oligo to hybridize freely. Studies involving the end-coupling of oligos to magnetic beads (Lund et al., 1988), Sephacryl S-500 (Gingeras et al., 1987) and AH Sepharose 4B (Voss & Malcolm, 1988 a) have demonstrated that the hybridization properties of

oligos immobilized via the 5' end allow them to be used in one-step assays (in which the labelled probe is free in solution), but such systems have, to date, never been extensively tested in a two-step sandwich assay.

One of the barriers preventing the widespread use of non-radioactive probes in diagnostic assay is that the sensitivity of a test incorporating such probes is, in general, around ten-fold lower than a similar assay performed with a radioactive probe. However, PCR-mediated target amplification prior to assay allows non-radioactive probes to be used (Saiki et al., 1988 a). The hybridization properties of oligonucleotides labelled with fluorescent dyes (fluoescein, Texas Red and rhodamine) have been investigated (Urdea et al., 1988); the use of such probes in a sandwich assay (using a capture probe immobilized on streptavidin-agarose beads) resulted in a sensitivity capable of detecting around 100 fmole of target DNA. Although the sensitivity of this test appears to be comparitively low, PCR-mediated target amplification prior to assay should allow oligos labelled with fluorescent dyes to be used successfully as probes. A sandwich assay for the detection of HPV types 6b, 11, 16 and 18 could be developed using four oligos, each labelled with a different fluorescent dye; in fact, the primers could be labelled with the dyes used in the recently developed automated non-radioactive DNA sequencing technique (Smith et al., 1985). Each dye has a different wavelength of maximum emission, and it is possible to distinguish between the four compounds on this basis. After sandwich hybridization (using all four labelled oligos) and washing, the oligonucleotide probe captured as a result of the presence of HPV target DNA could be released into solution by heating; the supernatant could then be examined by illumination at each of the wavelengths corresponding to the excitation maximum of the four dyes.

The infecting HPV type could be identified by examination of the wavelength of the emission maximum of the oligonucleotide probe within the supernatant.

Another important factor which is helping to prevent the widespread use of nucleic acid-based assays in clinical diagnosis is the fact that, in the majority of cases, nucleic acid must be extracted from a sample, prior to assay. The extraction procedures generally involve incubating the sample with a protease, followed by phenol/chloroform extraction and ethanol precipitation; although instruments are available for the automation of this process, it is highly time-consuming, especially if large numbers of samples are involved. As discussed in section 1.3, one of the major advantages of sandwich hybridization over conventional blotting formats is that the technique has been found to be insensitive to the presence of a crude sample (Ranki et al., 1983 a,b). Crude samples were not tested with the sandwich assay developed in this study, mainly because DNA extraction prior to PCR amplification was considered necessary (the reasons for this are discussed on page 117). Had more time been available, the results of amplification of a set of samples before and after DNA purification would have been investigated; had identical results been obtained, a combined PCR-sandwich assay for use with a crude cell lysate could have been developed.

5.3 Alternative Uses For Immobilized DNA Probes.

In addition to their application in diagnostic tests, immobilized DNA probes can be used for the isolation, identification and purification of specific DNA sequences (Gilham, 1964), as well as for the isolation of DNA binding proteins (Litman, 1968). In fact, nucleic acids were first covalently immobilized onto supports like powdered

cellulose in order to facilitate the purification of such binding proteins, rather than to be used in hybridization-based assays.

As discussed in section 1.6.2, single-stranded recombinant M13 phage DNA bound to DBM-cellulose has been used for the production of DNA probes; this is achieved by adding reverse transcriptase (Ashley & MacDonald, 1984), or DNA polymerase (Hansen et al., 1987), in the presence of either oligo(dT), or M13 primer. After synthesis, unincorp-

orated labelled nucleotides are washed away, and the labelled single-strand is eluted with formamide. The great advantage of this technique is that the immobilized template can be used repeatedly, and the purification of the single-stranded probe from the immobilized template is facile.

To date, the majority of the reports involving the use of DNA immobilized on resins have used cellulose as the solid support; results arising from this investigation (in addition to data published by Bunemann et al. in 1983) suggest that Sephacryl may be a superior alternative.

5.4 The Future Of DNA Diagnostics.

The great potential of DNA diagnostic methods (in terms of both improving the standard of health care and boosting the bank balance) have been recognized for several years; nucleic acid probes have distinct advantages over more traditional diagnostic methods. For example, probes with the desired strain, sub-group or group specificity can be selected, and the stringency of the hybridization reaction can be adjusted to optimize the efficiency of the assay. Pathogenic and apathogenic strains of the same organism can be distinguished, and genes conveying antibiotic resistance can be detected directly, without the need for microbial cultivation in the presence of the antibiotic. Viral nucleic acids can be detected, regardless of the integrational status and transcriptional or translational activity of the invading genome. In the case of some viruses (for example, rotavirus, hepatitis A and B viruses and Epstein-Barr virus), cultivation in established cell-lines is often not possible, so nucleic acid hybridization is the only satisfactory direct diagnostic method. Similarly, some important bacterial pathogens (for example, Treponema pallidum) cannot be cultiv-

ated on artificial media, and are best detected using DNA probes (Viscidi & Yolken, 1987).

One of the most striking facts which comes to light on studying the literature published in the last five years is the rapid advance which has been made in the development of nucleic acid-based diagnostic assays. Many of the new techniques (for example, polymerase chain reaction [Saiki et al., 1985 a], affinity-based hybrid collection [Syvanen & Korpela, 1986], time-resolved fluorometry [Syvanen et al., 1986 b] and multiple sandwich assay using oligonucleotide complexes [Urdea et al., 1987 a]) are discussed in detail in chapter 1. There is no doubt that, of the techniques listed above, PCR has had the biggest impact on molecular biology. Although, at first glance, PCR appears to solve many of the problems which have prevented nucleic acid-based tests from becoming widely used in routine diagnostics, a considerable amount of work remains to be done before the technique is reliable and specific enough to be universally used. Over the last three years, important improvements which have been made to the PCR assay format; these include the development of a reliable, robust DNA polymerase by the Cetus Corporation in the U.S.A. (Saiki et al., 1988 b), and the concomitant introduction of commercially available apparatus for the automation of the procedure. However, it is not yet obvious which primer G/C:A/T content results in the most efficient amplification; many different primer pairs have been used by different research groups for the amplification of various regions of the same HPV genome. Ideally, in order that the results obtained by different workers could be compared directly with each other, a universal primer pair should be agreed upon for each HPV type. The length of the amplified region should also be standardized, because this can have a significant effect on amplification efficiency (G.C.N. Parry, personal communication). As

discussed in section 5.1, great care will need to be taken to prevent the contamination of clinical samples with DNA which would be a suitable template for the PCR reaction, in order to avoid the possibility of obtaining false positive results.

In summary, although a considerable amount of ground has been covered in the race to bring DNA diagnostics within the realm of routine use, much work remains to be done. It seems unlikely that a single technique will be used to the exclusion of all others for the routine detection of specific nucleic acid sequences. Far more likely, a range of assays will be used, in conjunction with immunological tests, depending on the nature of the problem which needs to be tackled. Already, the enormous potential of nucleic acid hybridization has been recognized; the ability to detect variations in gene structure will facilitate the identification of the inherited mutations leading to disorders such as sickle-cell anaemia, thalassaemia, Huntington's chorea, muscular dystrophy and cystic fibrosis, to name a few of the diseases currently under study (Gillespie, 1987, and references therein). Progeny from two carrier parents can be evaluated in utero to permit the detection of recessive autosomal inherited disorders such as cystic fibrosis, haemophilia B and Tay-Sachs disease; recessive X-linked diseases such as Duchenne muscular dystrophy, Lesch-Nyhan syndrome, and X-linked mental retardation may also de identified. It is possible that, in the near future, individuals will be genetically screened at (or before) birth to evaluate the risk they face from succumbing to environmental stresses; for example, the probability that a given individual will suffer from neuromuscular disorders, artery disease, hypertension, diabetes or schizophrenia shows a large inherited component (Lowe, 1986; Gillespie, 1987).

Other commercial applications of DNA-based diagnostic tests

include the detection of infectious disease in animals and plants, and the identification of contaminants in foodstuffs and water supplies. A conservative estimate predicts that the size of the gene diagnosis gross market in the 1990s will be between 3 and 30 billion pounds annually (Gillespie, 1987). The development of nucleic acid-based tests will undoubtedly be an effective way of improving the standard of health care of the general population.

6. REFERENCES.

Abbott CM, McMahon CJ, Whitehouse DB, Povey S: Prenatal diagnosis of alpha-1-antitrypsin deficiency using polymerase chain reaction. Lancet i:763-764, 1988.

Agrawal S, Christodoulou C, Gait MJ: Efficient methods for attaching non-radioactive labels to the 5' ends of synthetic oligodeoxyribonucleotides. Nucleic Acids Res 14:6227-6245, 1986.

Ahola H, Stenlund J, Moreno-Lopez J, Pettersson U: Sequences of bovine papillomavirus type 1 DNA-functional and evolutionary implications. Nucleic Acids Res 11:2639-2650, 1983.

Al-Hakim AH, Hull R: Studies towards the development of chemically synthesized non-radioactive biotinylated nucleic acid hybridization probes. Nucleic Acids Res 14:9965-9976, 1986.

Al-Hakim AH, Hull R: Chemically synthesized non-radioactive biotinylated long-chain nucleic acid hybridization probes. Biochem J 251:935-938, 1988.

Alberts B, Herrick G: DNA-cellulose chromatography. Methods Enzymol 21:198-217, 1971.

Alwine JC, Kemp DJ, Stark GR: Method for detection of specific RNA's in agarose gels by transfer to diazobenzyloxymethyl paper and hybridization with DNA probes. Proc Natl Acad Sci USA 74:5350-5354, 1977.

Amasino RM: Acceleration of nucleic acid hybridization rate by polyethylene glycol. Anal Biochem 152:304-307, 1986.

Amersham International plc, Amersham, England. Nucleic acid labelling, Customer Information Booklet.

Androphy EJ, Hubbert NL, Schiller JT, Lowy DR: Identification of the HPV-16 E6 protein from transformed mouse cells and human cervical carcinoma cell lines. EMBO J 6:989-992, 1987.

Arndt-Jovin DJ, Jovin TM, Bahr W, Frischauf A-M, Marquardt M:

Covalent attachment of DNA to agarose. Improved synthesis and use in affinity chromatography. Eur J Biochem 54:411-418, 1975.

Ashley PL, MacDonald RJ: Synthesis of single-stranded hybridization probes from reusable DNA templates bound to solid support. Anal Biochem 140:95-103, 1984.

Astell CR, Smith M: Synthesis and properties of oligonucleotide-Cellulose columns. Biochemistry 11:4114-4120, 1972.

Avraham H, Golenser J, Gazitt Y, Spira DT, Sulitzeanu D: A highly sensitive solid-phase radioimmunoassay for the assay of Plasmodium falciparum antigens and antibodies. J Immunol Methods 53:61-68, 1982.

Banks L, Spence P, Androphy E, Hubbert N, Matlashewski G, Murray A, Crawford L: Identification of human papillomavirus type 18 E6 polypeptide in cells derived from human cervical carcinomas. J Gen Virol 68:1351-1359, 1987.

Beaudenon S, Kremsdorf D, Croissant O, Jablonska S, Wain-Hobson S, Orth G: A novel type of human papillomavirus associated with genital neoplasias. Nature 321:246-248, 1986.

Beaudenon S, Kremsdorf D, Obalek S, Jablonska S, Pehau-Arnaudet G, Croissant O, Orth G: Plurality of genital human papillomaviruses: characterization of two new types with distinct biological properties. Virology 161:374-384, 1987.

Beck BN, Ho SN: Increased specificity of PCR-amplified products by size-fractionation of restriction enzyme-digested template genomic DNA. Nucleic Acids Res 16:9051, 1988.

Beck S: Colorimetric-detected DNA sequencing. Anal Biochem 164:514-520, 1987.

Beck S, Pohl FM: DNA sequencing with direct blotting electrophoresis. EMBO J 3:2905-2909, 1984.

Bedell MA, Jones KH, Laimins LA: The E6-E7 region of human papillomavirus type 18 is sufficient for transformation of NIH 3T3 and rat-1 cells. J Virol 61:3635-3640, 1987.

Berger SL, Krug MS: A simple method for quantifying phosphorous-32 in submicroliter samples. Biotechniques 3:38-46, 1985.

Biagioni S, Sisto R, Ferraro A, Caiafa P, Turano C: A new method for the preparation of DNA-cellulose. Anal Biochem 89:616-619, 1978.

Birnboim HC, Doly J: A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7:1513-1523, 1979.

Bittner M, Kupferer P, Morris CF: Electrophoretic transfer of proteins and nucleic acids from slab gels to diazobenzyloxymethyl cellulose or nitrocellulose sheets. Anal Biochem 102:459-471, 1980.

Bolivar F, Rodriguez RL, Greene PJ, Betlach MC, Heyneker HL, Boyer HW, Crosa JH, Falkow S: Construction and characterisation of new cloning vehicles II. A multipurpose cloning system. Gene 2:95-113, 1977.

Bolton ET, McCarthy BJ: A general method for the isolation of RNA complementary to DNA. Proc Natl Acad Sci USA 48:1390-1397, 1962.

Bos JL, Fearson ER, Hamilton SR, Verlaan-de Vries M, van Boom JH, van der Eb AJ, Vogelstein B: Prevalence of ras gene mutations in human colorectal cancers. Nature 327:293-297, 1987.

Boshart M, Gissmann L, Ikenberg H, Kleinheinz A, Scheurlen W, zur Hausen H: A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. EMBO J 3:1151-1157, 1984.

Bowles NE, Richardson PJ, Olsen EGJ, Archard LC: Detection of coxsackie-B virus-specific RNA sequences in myocardial biopsy

samples from patients with myocarditis and dilated cardiomyopathy. Lancet i:1120-1123, 1986.

Boyer HW, Roulland-Dussoix D: A complementation analysis of the restriction and modification of DNA in Escherichia coli. J Mol Biol 41:459-472, 1969.

Brandsma JL, Steinberg BM, Abramson AL, Winkler B: Presence of human papillomavirus type 16 related sequences in verrucous carcinoma of the larynx. Cancer Res 46:2185-2188, 1986.

Bresser J, Doering J, Gillespie D: Quick-blot: selective mRNA or DNA immobilization from whole cells. DNA 2:243-254, 1983.

British Medical Association Report: Cervical cancer and screening in Great Britain. Prepared by the Board of Science and Education, 1986.

Broker TR: Structure and genetic expression of papillomaviruses. Obstet Gynecol Clin North Am 14:329-348, 1987.

Bruce-Chwatt LJ: DNA probes for malaria diagnosis (letter). Lancet i:795, 1984.

Bruun Petersen K, Kolvraa S, Bolund L, Bruun Petersen G, Koch J, Gregersen N: Detection of alpha-1-antitrypsin genotypes by analysis of amplified DNA sequences. Nucleic Acids Res 16:352, 1988.

Bunemann H: Immobilization of denatured DNA to macroporous supports: II. Steric and kinetic parameters of heterogeneous hybridization reactions. Nucleic Acids Res 10:7181-7196, 1982.

Bunemann H, Westhoff P: Hybrid selection of specific RNAs using DNA covalently coupled to macroporous supports. Methods Enzymol 100:400-407, 1983.

Bunemann H, Westhoff P, Herrmann RG: Immobilization of denatured DNA to macroporous supports: I. Efficiency of different coupling

procedures. Nucleic Acids Res 10:7163-7180, 1982.

Campo MS, Jarrett WFH: Papillomaviruses and diseases. In Molecular Basis Of Virus Disease, WC Russel and JW Almond eds. Cambridge University Press, Cambridge, 1987, pp 215-243.

Campo MS, Moar MH, Satirana ML, Kennedy IM, Jarrett WFH: The presence of bovine papillomavirus type 4 DNA is not required for the progression to, or the maintenance of, the malignant state in cancers of the alimentary canal in cattle. EMBO J 4:1819-1825, 1985.

Cannon G, Heinhorst S, Weissbach A: Quantitative molecular hybridization on nylon membranes. Anal Biochem 149:229-237, 1985.

Casey J, Davidson N: Rates of formation and thermal stabilities of RNA:DNA and DNA:DNA duplexes at high concentrations of formamide. Nucleic Acids Res 4:1539-1552, 1977.

Cavalieri LF, Carroll E: A DNA-acrylamide gel column for analyzing proteins that bind to DNA, I. DNA polymerase. Proc Natl Acad Sci USA 67:807-812, 1970.

Chehab FF, Doherty M, Cai S, Kan YW, Cooper S, Rubin EM: Detection of sickle cell anaemia and thalassaemias. Nature 329:293-294, 1987.

Chen EY, Howley PM, Levinson AD, Seeburg PH: The primary structure and genetic organization of the bovine papillomavirus type 1 genome. Nature 299:529-534, 1982.

Chien A, Edgar DB, Trela JM: Deoxyribonucleic acid polymerase from the extreme thermophile Thermus aquaticus. J Bacteriol 127:1550-1557, 1976.

Chollet A, Kawashima E: DNA containing the base analogue 2-aminoadenine: preparation, use as hybridization probes and cleavage by restriction endonucleases. Nucleic Acids Res 16:305-317, 1988.

Choo K-B, Pan C-C, Han S-H: Integration of human papillomavirus type 16 into cellular DNA of cervical carcinoma: Preferential deletion of the E2 gene and invariable retention of the long control region and the E6/E7 open reading frames. Virology 161:259-261, 1987.

Chou S, Merigan TC: Rapid detection and quantitation of human cytomegalovirus in urine through DNA hybridization. N Engl J Med 308:921-925, 1983.

Clertant P, Seif I: A common function for polyoma virus large-T and papillomavirus E1 proteins? Nature 311:276-279, 1984.

Coggin JR, zur Hausen H: Workshop on papillomaviruses and cancer. Cancer Res 39:545-546, 1979.

Cole ST, Danos O: Nucleotide sequence and comparative analysis of the human papillomavirus type 18 genome. J Mol Biol 193:599-608, 1987.

Cole ST, Streeck RE: Genome organization and nucleotide sequence of human papillomavirus type 33, which is associated with cervical cancer. J Virol 58:991-995, 1986.

Collins ML, Hunsaker WR: Improved hybridization assays employing tailed oligonucleotide probes: A direct comparison with 5'-end labeled oligonucleotide probes and nick-translated plasmid probes. Anal Biochem 151:211-224, 1985.

Commerford SL: Iodination of nucleic acids in vitro. Biochemistry 10:1993-2000, 1971.

Cook AF, Vuocolo E, Brakel CL: Synthesis and hybridization of a series of biotinylated oligonucleotides. Nucleic Acids Res 16:4077-4095, 1988.

Coppleson M: Colposcopic features of papillomaviral infection and premalignancy in the female lower genital tract. Obstet Gynecol

Clin North Am 14:471-494, 1987.

Crawford L: Papilloma viruses and cervical tumours. Nature 310:16, 1984.

Crawford L: Criteria for establishing that a virus is oncogenic. In Papillomaviruses: Ciba Foundation Symposium 120. Wiley, Chichester, 1986, pp 104-116.

Crum CP, Nagai N, Levine RU, Silverstein S: In situ hybridization analysis of HPV 16 DNA sequences in early cervical neoplasia. Am J Pathol 123:174-182, 1986.

Cunningham M: Spot blot-A hybridization assay for specific DNA sequences in multiple samples. Anal Biochem 128:415-421, 1983.

Cunningham MW, Mundy CR: Labelling nucleic acids for hybridization. Nature 326:723-724, 1987.

Dahlen P: Detection of biotinylated DNA probes by using Eu-labeled streptavidin and time-resolved fluorometry. Anal Biochem 164:78-83, 1987.

Danos O, Katinka M, Yaniv M: Human papillomavirus 1a complete DNA sequence: a novel type of genome organization among Papovaviridae. EMBO J 1:231-236, 1982.

Dartmann K, Schwarz E, Gissmann L, zur Hausen H: The nucleotide sequence and genome organization of human papilloma virus type 11. Virology 151:124-130, 1986.

de Villiers EM, Gissmann L, zur Hausen H: Molecular cloning of viral DNA from human genital warts. J Virol 40:932-935, 1981.

Denhardt DT: A membrane-filter technique for the detection of complementary DNA. Biochem Biophys Res Commun 23:641-646, 1966.

Derman E, Krauter K, Walling L, Weinberger C, Ray M, Darnell JE: Transcriptional control in the production of liver-specific mRNA's. Cell 23:731-739, 1981.

DiLella AG, Huang W-M, Woo SLC: Screening for phenylketonuria mutations by DNA amplification with the polymerase chain reaction. Lancet i:497-499, 1988.

DiMaio D: Nonsense mutation in open reading frame E2 of bovine papillomavirus DNA. J Virol 57:475-480, 1986.

Dunn AR, Hassell JA: A novel method to map transcripts: Evidence for homology between an adenovirus mRNA and discrete multiple regions of the viral genome. Cell 12:23-36, 1977.

Dunn AR, Mathews MB, Chow LT, Sambrook J, Keller W: A supplementary adenoviral leader sequence and its role in messenger translation. Cell 15:511-526, 1978.

Dunn AR, Sambrook J: Mapping viral mRNA's by sandwich hybridization. Methods Enzymol 65:468-478, 1980.

Durst M, Croce CM, Gissmann L, Schwarz E, Huebner K: Papillomavirus sequences integrate near cellular oncogenes in some cervical carcinomas. Proc Natl Acad Sci USA 84:1070-1074, 1987.

Ellwood MS, Collins M, Fritsch EF, Williams JI, Diamond SE, Brewen JG: Strand displacement applied to assays with nucleic acid probes. Clin Chem 32:1631-1636, 1986.

Embury SH, Scharf SJ, Saiki RK, Gholson MA, Golbus M, Arnheim N, Erlich HA: Rapid prenatal diagnosis of sickle cell anemia by a new method of DNA analysis. N Engl J Med 316:656-661, 1987.

Engelke DR, Hoener PA, Collins FS: Direct sequencing of enzymatically amplified human genomic DNA. Proc Natl Acad Sci USA 85:544-548, 1988.

Erlich HA, Gelfand DH, Saiki RK: Specific DNA amplification. Nature 331:461-462, 1988.

Farr CJ, Saiki RK, Erlich HA, McCormick F, Marshall CJ: Analysis of RAS gene mutations in acute myeloid leukemia by polymerase chain

reaction and oligonucleotide probes. Proc Natl Acad Sci USA 85:1629-1633, 1988.

Feinberg AP, Vogelstein B: A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6-13, 1983.

Feinberg AP, Vogelstein B: A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Addendum. Anal Biochem 137:266-267, 1984.

Flavell RA, Birfelder EJ, Sanders JPM, Borst P: DNA-DNA hybridization on nitrocellulose filters I. General considerations and non-ideal kinetics. Eur J Biochem 47:535-543, 1974.

Forster AC, McInnes JL, Skingle DC, Symons RH: Non-radioactive hybridization probes prepared by the chemical labelling of DNA and RNA with a novel reagent, photobiotin. Nucleic Acids Res 13:745-761, 1985.

Fuchs PG, Iftner T, Weninger J, Pfister H: Epidermodysplasia verruciformis-associated human papillomavirus 8: genomic sequence and comparative analysis. J Virol 58:626-634, 1986.

Ghosh SS, Musso GF: Covalent attachment of oligonucleotides to solid supports. Nucleic Acids Res 15:5353-5372, 1987.

Gilham PT: The synthesis of polynucleotide-celluloses and their use in the fractionation of polynucleotides. J Am Chem Soc 86:4982-4985, 1964.

Gilham PT: The synthesis of celluloses containing covalently bound nucleotides, polynucleotides, and nucleic acids. Biochemistry 7:2809-2813, 1968.

Gillam IC: Non-radioactive probes for specific DNA sequences. Trends Biotechnol 5:332-334, 1987.

Gillespie DH: The state of the art of clinical gene diagnosis. J Clin

Lab Analysis 1:42-51, 1987.

Gingeras TR, Kwoh DY, Davis GR: Hybridization properties of immobilized nucleic acids. Nucleic Acids Res 15:5373-5390, 1987.

Ginsberg M, Lundholm-Beauchamp U, Prince G: Adenovirus as a model of disease. In Molecular Basis of Virus Disease: 40th Symposium of the Society for General Microbiology, WC Russell, JW Almond eds. Cambridge University Press, Cambridge, 1987, pp 245-258.

Giri I, Danos O, Yaniv M: Genomic structure of the cottontail rabbit (Shope) papillomavirus. Proc Natl Acad Sci USA 82:1580-1584, 1985.

Gissmann L, Boshart M, Durst M, Ikenberg H, Wagner D, zur Hausen H: Presence of human papillomavirus in genital tumors. J Invest Dermatol 83:26s-28s, 1984.

Gissmann L, Schwarz E: Persistence and expression of human papillomavirus DNA in genital cancer. In Papillomaviruses: Ciba Foundation Symposium 120. Wiley, Chichester, 1986, pp 190-207.

Gissmann L, Wolnik L, Ikenberg H, Koldovsky U, Schnurch HG, zur Hausen H: Human papillomavirus types 6 and 11 DNA sequences in genital and laryngeal papillomas and in some cervical cancers. Proc Natl Acad Sci USA 80:560-563, 1983.

Glenn J: Cytomegalovirus infections following renal transplantation. Rev Infect Dis 3:1151-1178, 1981.

Goldkorn T, Prockop DJ: A simple and efficient method for covalent attachment of DNA to cellulose. Application for hybridization-restriction analysis and for in vitro synthesis of DNA probes. Nucleic Acids Res 14:9171-9191, 1986.

Green M, Loewenstein PM: Demonstration that a chemically synthesized BPV1 oncoprotein and its C-terminal domain function to induce cellular DNA synthesis. Cell 51:795-802, 1987.

Green M, Mackey JK, Wold WSM, Rigden P: Thirty-one human adenovirus serotypes (Ad1-Ad31) form five groups (A-E) based upon DNA genome homologies. Virology 93:481-492, 1979.

Green NM: Avidin. Adv Protein Chem 29:85-133, 1975.

Griffin BE, Karran L, King D, Chang SE: Immortalising gene(s) encoded by Epstein-Barr virus. In Viruses and Cancer: 37th Symposium of the Society for General Microbiology, PWJ Rigby, NM Wilkie eds. Cambridge University Press, Cambridge, 1985, pp 93-110.

Groff DE, Lancaster WD: Molecular cloning and nucleotide sequence of deer papillomavirus. J Virol 56:85-91, 1985.

Halonen P, Sarkkinen P, Arstila E, Hjertsson E, Torfason E: Four-layer radioimmunoassay for detection of adenovirus in stool. J Clin Microbiol 11:614-617, 1980.

Hansen C, Libert F, Vassart G, Christophe D: Preparation of single-stranded deoxyribonucleic acid probes using an immobilized template. Anal Biochem 162:130-136, 1987.

Hansen P: Sandwich hybridization method for nucleic acid detection. European Patent Publication No. 139489, 1985.

Haralambidis J, Chai M, Tregear GW: Preparation of base-modified nucleosides suitable for non-radioactive label attachment and their incorporation into synthetic oligodeoxyribonucleotides. Nucleic Acids Res 15:4857-4876, 1987.

Harper ME, Marselle LM, Gallo RC, Wong-Staal F: Detection of lymphocytes expressing human T-lymphotropic virus type III in lymph nodes and peripheral blood from infected individuals by in situ hybridization. Proc Natl Acad Sci USA 83:772-776, 1986.

Hauber R, Geiger R: A sensitive, bioluminescent-enhanced detection method for DNA dot-hybridization. Nucleic Acids Res 16:1213, 1988.

Heller M, Henderson A, Kieff E: Repeat array in Epstein-Barr virus DNA is related to cell DNA sequences interspersed on human chromosomes. Proc Natl Acad Sci USA 79:5916-5920, 1982.

Higuchi R, von Beroldingen CH, Sensabaugh GF, Erlich HA: DNA typing from single hairs. Nature 332:543-546, 1988.

Hodgson CP, Fisk RZ: Hybridization probe size control: optimized 'oligolabelling'. Nucleic Acids Res 15:6295, 1987.

Howley PM, Yang Y-C, Spalholz BA, Rabson MS: Papillomavirus transforming functions. In Papillomaviruses: Ciba Foundation Symposium 120. Wiley, Chichester, 1986, pp 39-52.

Hu N, Messing J: The making of strand-specific M13 probes. Gene 17:271-277, 1982.

Impraim CC, Saiki RK, Erlich HA, Teplitz RL: Analysis of DNA extracted from formalin-fixed, paraffin-embedded tissues by enzymatic amplification and hybridisation with sequence-specific oligonucleotides. Biochem Biophys Res Commun 142:710-716, 1987.

Ish-Horowicz D, Burke JF: Rapid and efficient cosmid cloning. Nucleic Acids Res 9:2989-2998, 1981.

Jarrett WFH, McNeil PE, Grimshaw TR, Selman IE, McIntyre WIM: High incidence area of cattle cancer with a possible interaction between an environmental carcinogen and a papillomavirus. Nature 274:215-217, 1978.

Jenson AB, Rosenthal JR, Olson C, Pass F, Lancaster WD, Shah K: Immunological relatedness of papillomaviruses from different species. J Natl Cancer Inst 64:495-500, 1980.

Johnson K: PhD Thesis, University of London, 1985.

Jones FS, Grimberg JI, Fischer SG, Ford JP: Detection of sickle-cell mutation by electrophoresis of partial RNA:DNA hybrids following solution hybridization. Gene 39:77-83, 1985.

Kafatos FC, Jones CW, Efstratiadis A: Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. Nucleic Acids Res 7:1541-1552, 1979.

Kim H-S, Smithies O: Recombinant fragment assay for gene targetting based on the polymerase chain reaction. Nucleic Acids Res 16:8887-8903, 1988.

Kitchener HC: Genital virus infection and cervical neoplasia. Br J Obstet Gynaecol 95:182-191, 1988.

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 317:985-990, 1987.

Kremsky JN, Wooters JL, Dougherty JP, Meyers RE, Collins M, Brown EL: Immobilization of DNA via oligonucleotides containing an aldehyde or carboxylic acid group at the 5' terminus. Nucleic Acids Res 15:2891-2909, 1987.

Kulski JK, Norval M: Nucleic acid probes in diagnosis of viral diseases of man: Brief review. Arch Virol 83:3-15, 1985.

Kunkel LM, Smith KD, Boyer SH, Borgaonkar DS, Wachtel SS, Miller OJ, Breg WR, Jones HW, Rary JM: Analysis of human Y-chromosome-specific reiterated DNA in chromosome variants. Proc Natl Acad Sci USA 74:1245-1249, 1977.

Kurman RJ, Shah KV, Lancaster WD, Jenson AB: Immunoperoxidase localization of papillomavirus antigens in cervical dysplasia and vulvar condylomas. Am J Obstet Gynecol 140:931-935, 1981.

Kwok S, Mack DH, Mullis KB, Poiesz B, Ehrlich G, Blair D, Friedman-Kien A, Sninsky JJ: Identification of human immunodeficiency virus sequence by using *in vitro* enzymatic amplification and oligomer cleavage detection. J Virol

61:1690-1694, 1987.

Lai-Goldman M, Lai E, Grody WW: Detection of human immunodeficiency virus (HIV) infection in formalin-fixed, paraffin-embedded tissues by DNA amplification. Nucleic Acids Res 16:8191, 1988.

Lancaster WD, Jenson AB: Human papillomavirus infection and anogenital neoplasia: Speculations for the future. Obstet Gynecol Clin North Am 14:601-609, 1987.

Landegent JE, Jansen In De Wal N, Baan RA, Hoeijmakers JHJ, Van Der Ploeg M: 2-Acetylaminofluorene-modified probes for the indirect hybridocytochemical detection of specific nucleic acid sequences. Exp Cell Res 153:61-72, 1984.

Landegren U, Kaiser R, Caskey CT, Hood L: DNA diagnostics-molecular techniques and automation. Science 242:229-237, 1988.

Langdale JA, Malcolm ADB: A rapid method of gene detection using DNA bound to Sephacryl. Gene 36:201-210, 1985.

Langer PR, Waldrop AA, Ward DC: Enzymatic synthesis of biotin-labeled polynucleotides: Novel nucleic acid affinity probes. Proc Natl Acad Sci USA 78:6633-6637, 1981.

Law M-F, Lancaster WD, Howley PM: Conserved polynucleotide sequences among the genomes of papillomaviruses. J Virol 32:199-207, 1979.

Lazo PA: Accessibility of DNA-epoxycellulose to sequence-specific DNA-binding proteins. Biochem J 217:331-334, 1984.

Lazo PA: Structure, DNaseI hypersensitivity and expression of integrated papilloma virus in the genome of HeLa cells. Eur J Biochem 165:393-401, 1987.

Leary JJ, Brigati DJ, Ward DC: Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: Bio-blots. Proc Natl Acad Sci USA 80:4045-4049, 1983.

Legerski RJ, Robberson DL: Analysis and optimization of recombinant DNA joining reactions. J Mol Biol 181:297-312, 1985.

Lehtomaki K, Julkunen I, Sandelin K, Salonen J, Virtanen M, Ranki M, Hovi T: Rapid diagnosis of respiratory adenovirus infections in young adult men. J Clin Microbiol 24:108-111, 1986.

Lench N, Stanier P, Williamson R: Simple non-invasive method to obtain DNA for gene analysis. Lancet I:1356-1358, 1988.

Lewis AM, Levin MJ, Wiese WH, Crumpacker CS, Henry PH: A nondefective (competent) adenovirus-SV40 hybrid isolated from the Ad2-SV40 hybrid population. Proc Natl Acad Sci USA 63:1128-1135, 1969.

Liang W, Johnson JP: Rapid plasmid insert amplification with polymerase chain reaction. Nucleic Acids Res 16:3579, 1988.

Litman RM: A deoxyribonucleic acid polymerase from Micrococcus luteus (Micrococcus lysodeikticus) isolated on deoxyribonucleic acid-cellulose. J Biol Chem 243:6222-6233, 1968.

Little PR, Jackson IJ: Application of plasmids containing promoters specific for phage-encoded RNA polymerases. DNA Cloning Volume III: A Practical Approach, DM Glover ed. IRL Press, Oxford, 1987, pp. 1-18.

Lo MD, Mehal WZ, Fleming KA: Rapid production of vector-free biotinylated probes using the polymerase chain reaction. Nucleic Acids Res 16:8719, 1988.

Loche M, Mach B: Identification of HIV-infected seronegative individuals by a direct diagnostic test based on hybridisation to amplified viral DNA. Lancet ii:418-421, 1988.

Lookingbill DP, Kreider JW, Howett MK, Olmstead PM, Conner GH: Human papillomavirus type 16 in Bowenoid papulosis, intraoral papillomas, and squamous cell carcinoma of the tongue. Arch Dermatol 123:363-368, 1987.

Lorincz AT, Lancaster WD, Temple GF: Cloning and characterization of the DNA of a new human papillomavirus from a woman with dysplasia of the uterine cervix. J Virol 58:225-229, 1986.

Lowe JB: Clinical applications of gene probes in human genetic disease, malignancy, and infectious disease. Clinica Chimica Acta 157:1-32, 1986.

Lowy DR, Dvoretzky I, Shober R, Law MF, Engel L, Howley PM: In vitro tumorigenic transformation by a defined sub-genomic fragment of bovine papilloma virus DNA. Nature 287:72-74, 1980.

Lund V, Schmid R, Rickwood D, Hornes E: Assessment of methods for covalent binding of nucleic acids to magnetic beads, Dynabeads, and the characteristics of the bound nucleic acids in hybridization reactions. Nucleic Acids Res 16:10861-10880, 1988.

Lusky M, Botchan MR: Genetic analysis of bovine papillomavirus type 1 trans-acting replication factors. J Virol 53:955-965, 1985.

Macdougall AJ, Brown JR, Plumbridge TW: Immobilization of DNA for affinity chromatography and drug-binding studies. Biochem J 191:855-858, 1980.

Maingon R: PhD Thesis, University of London, 1982.

Malcolm ADB, Fallon RA, Langdale JA, Figueiredo H, Nicholls PJ, Voss UB, Wickenden C, Woodhead JL: DNA probes in human disease. Biochem Soc Symp 53:131-143, 1987.

Mandel M, Higa A: Calcium-dependent bacteriophage DNA infection. J Mol Biol 53:159-162, 1970.

Maniatis T, Fritsch EF, Sambrook J: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York, 1982.

Mark A, Trowell H, Dyall-Smith ML, Dyall-Smith DJ: Extraction of DNA from formalin-fixed paraffin-embedded pathology specimens and its use in hybridization (histo-blot) assays. Application to the

detection of human papillomavirus DNA. Nucleic Acids Res 15:8565, 1987.

Matthews JA, Batki A, Hynds C, Kricka LJ: Enhanced chemiluminescent method for the detection of DNA dot-hybridization assays. Anal Biochem 151:205-209, 1985.

Matthews JA, Kricka LJ: Analytical strategies for the use of DNA probes. Anal Biochem 169:1-25, 1988.

Maxam AM, Gilbert W: A new method for sequencing DNA. Proc Natl Acad Sci USA 74:560-564, 1977.

McCance DJ: Human papillomaviruses and cancer. Biochim Biophys Acta 823:195-205, 1986.

McDougall JK, Beckmann AM, Kiviat NB: Methods for diagnosing papillomavirus infection. In Papillomaviruses: Ciba Foundation Symposium 120. Wiley, Chichester, 1986, pp 86-103.

Meinkoth J, Wahl G: Hybridization of nucleic acids immobilized on solid supports. Anal Biochem 138:267-284, 1984.

Meisels A, Fortin R: Condylomatous lesions of the cervix and vagina. I. Cytologic patterns. Acta Cytol 20:505-509, 1976.

Messing J, Crea R, Seeburg PH: A system for shotgun DNA sequencing. Nucleic Acids Res 9:309-321, 1981.

Morse AR, Wickenden C, Byrne M, Taylor-Robinson D, Smith J, Anderson MC,, Smith C, Malcolm ADB, Coleman DV: DNA hybridisation of cervical scrapes: comparison with cytological findings in Papanicolaou smears. J Clin Path 41:296-299, 1988.

Mosely SL, Echeverria P, Seriwatana J, Tirapat C, Chaicumpa W, Sakuldaipeara T, Falkow S: Identification of enterotoxigenic Escherichia coli by colony hybridization using three enterotoxin gene probes. J Infect Dis 145:863-869, 1982.

Moss LG, Moore JP, Chan L: A simple, efficient method for coupling

DNA to cellulose. J Biol Chem 256:2655-12658, 1981.

Mullis K, Faloona F, Scharf S, Saiki R, Horn G, Erlich H: Specific enzymatic amplification of DNA in vitro: The polymerase chain reaction. Cold Spring Harbor Symp Quant Biol 51:263-273, 1986.

Myers RM, Larin Z, Maniatis T: Detection of single base substitutions by ribonuclease cleavage at mismatches in RNA:DNA duplexes. Science 230:1242-1246, 1985.

Myers RM, Lumelsky N, Lerman LS, Maniatis T: Detection of single base substitutions in total genomic DNA. Nature 313:495-498, 1985.

Mykoniatis MG: Immobilization of native and denatured DNA on Sephadex G200. J Biochem Biophys Methods 10:321-328, 1985.

Nagasawa J, Suehiro T, Yamauchi A: Immunosorbent consisting of DNA immobilized on oxirane-activated Sepharose. J Appl Biochem 7:430-437, 1985 a.

Nagasawa J, Suehiro T, Yamauchi A: Immobilization of DNA via covalent linkage for use as immunosorbent. J App Biochem 7:296-302, 1985 b.

Nagata Y, Yokota H, Kosuda O, Yokoo K, Takemura K, Kikuchi T: Quantification of picogram levels of specific DNA immobilized in microtiter wells. FEBS Lett 183:379-382, 1985.

Nakabayashi Y, Chattopadhyay SK, Lowy DR: The transforming function of bovine papillomavirus DNA. Proc Natl Acad Sci USA 80:5832-5836, 1983.

Nicholls PJ, Langdale JA, Malcolm ADB: Sandwich hybridization using immobilized DNA. Biochem Soc Trans 15:140, 1987.

Nicholls PJ, Malcolm ADB: Nucleic acid analysis by sandwich hybridization. J Clin Lab Analysis: In press, early 1989.

Noyes BE, Stark GR: Nucleic acid hybridization using DNA covalently coupled to cellulose. Cell 5:301-320, 1975.

Nyberg K, Palva A, Palva I: Exceptionally high copy numbers of a staphylococcal plasmid in Bacillus subtilis revealed by a sandwich hybridization technique. FEMS Microbiol Lett 29:305-310, 1985.

Olive DM, Atta AI, Setti SK: Detection of toxigenic Escherichia coli using biotin-labelled DNA probes following enzymatic amplification of the heat labile toxin gene. Mol Cell Probes 2:47-57, 1988.

Orth G: Epidermodysplasia verruciformis: a model for understanding the oncogenicity of human papillomaviruses. In Papillomaviruses: Ciba Foundation Symposium 120. Wiley, Chichester, 1986, pp 157-174.

Oser A, Roth WK, Valet G: Sensitive non-radioactive dot-blot hybridization using DNA probes labelled with chelate group substituted psoralen and quantitative detection by europium ion fluorescence. Nucleic Acids Res 16:1181-1196, 1988.

Oser A, Valet G: Improved detection by time-resolved fluorometry of specific DNA immobilized in microtiter wells with europium/metal-chelator labelled DNA probes. Nucleic Acids Res 16:8178, 1988.

Ostrow RS, Faras AJ: The molecular biology of human papillomaviruses and the pathogenesis of genital papillomas and neoplasms. Cancer Metastasis Rev 6:383-395, 1987.

Ostrow RS, Manias DA, Fong WJ, Zachow KR, Faras AJ: A survey of human cancers for human papillomavirus DNA by filter hybridization. Cancer 59:429-434, 1987.

Ou C-Y, Kwok S, Mitchell SW, Mack DH, Sninsky JJ, Krebs JW, Feorino P, Warfield D, Schochetman G: DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells.

Science 239:295-297, 1988.

Owens DA, Diener TO: Sensitive and rapid diagnosis of potato spindle tuber viroid disease by nucleic acid hybridization. Science 213:670-672, 1981.

Palva AM: ompA gene in the detection of Escherichia coli and other Enterobacteriaceae by nucleic acid sandwich hybridization. J Clin Microbiol 18:92-100, 1983.

Palva A: Nucleic acid spot hybridization for detection of Chlamydia trachomatis. FEMS Microbiol Lett 28:85-91, 1985.

Palva A, Jousimies-Somer H, Saikku P, Vaananen P, Soderlund H, Ranki M: Detection of Chlamydia trachomatis by nucleic acid sandwich hybridization. FEMS Microbiol Lett 23:83-89, 1984.

Palva A, Nyberg K, Palva I: Quantification of alpha-amylase mRNA in Bacillus subtilis by nucleic acid sandwich hybridization. DNA 7:135-142, 1988.

Palva A, Ranki M: Microbial diagnosis by nucleic acid sandwich hybridization. Clin Lab Med 5:475-490, 1985.

Panet A, Khorana HG: The linkage of deoxyribopolynucleotide templates to cellulose and its use in their replication. J Biol Chem 249:5213-5221, 1974.

Pardue ML: In situ hybridisation. In Nucleic Acid Hybridization: A Practical Approach, BD Hames and SJ Higgins eds. IRL Press, Oxford, 1985, pp 179-202.

Parkkinen S: Nucleic acid sandwich hybrdidization in detection of HPV 16 DNA: technique and its clinical applications. J Virol Methods 19:69-77, 1988.

Parkkinen S, Mantyjarvi R, Syrjanen K, Ranki M: Detection of human papillomavirus DNA by the nucleic acid sandwich hybridization method from cervical scraping. J Med Virol 20:279-288, 1986.

Parkkinen S, Syrjanen S, Syrjanen K, Yliskoski M, Tenhunen J, Mantyjarvi R, Ranki M: Screening of premalignant cervical lesions for HPV 16 DNA by sandwich and in situ hybridization techniques. Gynecol Oncol 30:251-264, 1988.

Parsons GH, Rogers CH, Polsky-Cynkin R Wood AM, Miles LEM, Rogers AH: Use of a solid-state multihead gamma counter in a second-generation system for solid phase immunoassay. Clin Chem 29:1620-1627, 1983.

Patel KR, Smith KT, Campo MS: The nucleotide sequence and genome organization of bovine papillomavirus type 4. J Gen Virol 68:2117-2128, 1987.

Peden K, Mounts P, Hayward GS: Homology between mammalian cell DNA sequences and human herpesvirus genomes detected by a hybridization procedure with high-complexity probe. Cell 31:71-80, 1982.

Pettersson U, Ahola H, Stenlund A, Bergman P, Ustav M, Moreno-Lopez J: Organization and expression of the genome of bovine papillomavirus type 1. In Papillomaviruses: Ciba Foundation Symposium 120. Wiley, Chichester, 1986, pp 23-38.

Pfister H: Relationship of papillomaviruses to anogenital cancer. Obstet Gynecol Clin North Am 14:349-361, 1987.

Pfister H, Krubke J, Dietrich W, Iftner T, Fuchs PG: Classification of the papillomaviruses-mapping the genome. In Papillomaviruses: Ciba Foundation Symposium 120. Wiley, Chichester, 1986, pp 3-22.

Polsky-Cynkin R, Parsons GH, Allerdt L, Landes G, Davis G, Rashtchian A: Use of DNA immobilized on plastic and agarose supports to detect DNA by sandwich hybridization. Clin Chem 31:1438-1443, 1985.

Poonian MS, Schlabach AJ, Weissbach A: Covalent attachment of nucleic

acids to agarose for affinity chromatography. Biochemistry 10:424-427, 1971.

Potuzak H, Dean PDG: Affinity chromatography on columns containing nucleic acids. FEBS Lett 88:161-166, 1978.

Purello M, Balazs I: Direct hybridization of labeled DNA to DNA in agarose gels. Anal Biochem 128:393-397, 1983.

Rando RF, Groff DE, Chirikjian JG, Lancaster WD: Isolation and characterization of a novel human papillomavirus type 6 DNA from an invasive vulvar carcinoma. J Virol 57:353-356, 1986.

Ranki M, Palva A, Virtanen M, Laaksonen M, Soderlund H: Sandwich hybridization as a convenient method for the detection of nucleic acids in crude samples. Gene 21:77-85, 1983 a.

Ranki M, Virtanen M, Palva A, Laaksonen M, Pettersson R, Kaarianen L, Halonen P, Soderlund H: Nucleic acid sandwich hybridization in adenovirus diagnosis. Curr Top Microbiol Immunol 104:307-318, 1983 b.

Ratner L, Sarin PS, Wong-Staal F, Gallo RC: Human and primate T-lymphotropic retroviruses (HTLV and PTLV): subtypes, biological activity, and role in neoplasias. In Viruses and Cancer: 37th Symposium of the Society for General Microbiology, PWJ Rigby, NM Wilkie eds. Cambridge University Press, Cambridge, 1985, pp 261-289.

Reid R, Laverty CR, Coppleson M, Isarangkul W, Hills E: Noncondylomatous cervical wart virus infection. Obstet Gynecol 55:476-483, 1980.

Renz M: Polynucleotide-histone H1 complexes as probes for blot hybridization. EMBO J 2:817-822, 1983.

Renz M, Kurz C: A colorimetric method for DNA hybridization. Nucleic Acids Res 12:3435-3444, 1984.

Rickwood D: An improved method for the insolubilization of DNA for affinity chromatography. Biochim Biophys Acta 269:47-50, 1972.

Rigby PWJ, Dieckmann M, Rhodes C, Berg P: Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J Mol Biol 113:237-251, 1977.

Rigoni-Stern D: Fatti statistici relativi alle mallattie cancrosi che servirono de base alle poche cose dette dal dott. Giornale Service Progr Pathol Terap Ser 2:507-517, 1842.

Riou G, Le MG, Le Doussal V, Barrois M, George M, Haie C: C-myc proto-oncogene expression and prognosis in early carcinoma of the uterine cervix. Lancet i:761-763, 1987.

Rollo F, Amici A, Salvi R: A simple and low cost DNA amplifier. Nucleic Acids Res 16:3105-3106, 1988.

Rous P, Beard JW: The progression to carcinoma of virus-induced rabbit papillomas (Shope). J Exp Med 62:523-548, 1935.

Saiki RK, Arnheim N, Erlich HA: A novel method for the detection of polymorphic restriction sites by cleavage of oligonucleotide probes: Application to sickle-cell anemia. Biotechnology 3:1008-1012, 1985 b.

Saiki RK, Bugawan TL, Horn GT, Mullis KB, Erlich HA: Analysis of enzymatically amplified beta-globin and HLA-DQ alpha DNA with allele-specific oligonucleotide probes. Nature 324:163-166, 1986.

Saiki RK, Chang C-A, Levenson CH, Warren TC, Boehm CD, Kazazian HH, Erlich HA: Diagnosis of sickle cell anemia and beta-thalassemia with enzymatically amplified DNA and nonradioactive allele-specific oligonucleotide probes. N Eng J Med 319:537-541, 1988 a.

Saiki RK, Gelfand DH, Stoffel S, Scharf S, Higuchi R, Horn GT, Mullis KB, Erlich HA: Primer-directed amplification of DNA with a

thermostable DNA polymerase. Science 239:487-491, 1988 b.

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N: Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230:1350-1354, 1985 a.

Sanger F, Nicklen S, Coulson AR: DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463-5467, 1977.

Sarkar G, Sommer SS: RNA amplification with transcript sequencing (RAWTS). Nucleic Acids Res 16:5197, 1988.

Sarkinnen HK, Halonen PE, Arstila PP, Salmi AA: Detection of respiratory syncytial, parainfluenza type 2, and adenovirus antigens by radioimmunoassay and enzyme immunoassay on nasopharyngeal specimens from children with acute respiratory disease. J Clin Microbiol 13:258-265, 1981.

Sarver N, Rabson MS, Yang Y-C, Byrne JC, Howley PM: Localization and analysis of bovine papillomavirus type 1 transforming functions. J Virol 52:377-388, 1984.

Scharf SJ, Horn GT, Erlich HA: Direct cloning and sequence analysis of enzymatically amplified genomic sequences. Science 233:1076-1078, 1986.

Schiller JT, Vass WC, Lowy DR: Identification of a second transforming region in bovine papillomavirus DNA. Proc Natl Acad Sci USA 81:7880-7884, 1984.

Schiller JT, Vass WC, Vousden KH, Lowy DR: E5 open reading frame of bovine papillomavirus type 1 encodes a transforming gene. J Virol 57:1-6, 1986.

Schwarz E, Durst M, Demankowski C, Lattermann O, Zech R, Wolfsperger E, Suhai S, zur Hausen H: DNA sequence and genome organization of

genital human papillomavirus type 6b. EMBO J 2:2341-2348, 1983.

Schwarz E, Freese UK, Gissmann L, Mayer W, Roggenbuck B, Stremlau A, zur Hausen H: Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature 314:111-114, 1985.

Seed B: Diazotizable arylamine cellulose papers for the coupling and hybridization of nucleic acids. Nucleic Acids Res 10:1799-1811, 1982.

Seedorf K, Krammer G, Durst M, Suhai S, Rowekamp WG: Human papillomavirus type 16 DNA sequence. Virology 145:181-185, 1985 a.

Seedorf K, Krammer G, Rowekamp WG: Human papillomavirus type 16 DNA: Expression of open reading frames in E.coli. In Papillomaviruses: Molecular and Clinical aspects. Alan R. Liss, Inc., 1985 b, pp 391-396.

Shibata DK, Arnheim N, Martin WJ: Detection of human papillomavirus in paraffin-embedded tissue using the polymerase chain reaction. J Exp Med 167:225-230, 1988.

Shih TY, Martin MA: Chemical linkage of nucleic acids to neutral and phosphorylated cellulose powders and isolation of specific sequences by affinity chromatography. Biochemistry 13:3411-3418, 1974.

Shope RE: Infectious papillomatosis of rabbits. J Exp Med 58:607-624, 1933.

Siegel RE, Scott Young W: Detection of preprocholecystokinin and preproenkephalin A mRNAs in rat brain by hybridization histochemistry using complementary RNA probes. Neuropeptides 6:573-580, 1986.

Singer A, French P: Natural history and epidemiology of cervical carcinoma. In Cancer Of The Uterine Cervix. Academic Press,

London, 1984, pp 5-30.

Smith GE, Summers MD: The bidirectional transfer of DNA and RNA to nitrocellulose or diazobenzyloxymethyl-paper. Anal Biochem 109:123-129, 1980.

Smith KT, Campo S: "Hit and run" transformation of mouse C127 cells by bovine papillomavirus type 4: The viral DNA is required for the initiation but not for maintenance of the transformed phenotype. Virology 164:39-47, 1988.

Smith LM, Fung S, Hunkapiller MW, Hunkapiller TJ, Hood LE: The synthesis of oligonucleotides containing an aliphatic amino group at the 5' terminus: synthesis of fluorescent DNA primers for use in DNA sequence analysis. Nucleic Acids Res 13:2399-2412, 1985.

Smith LM, Sanders JZ, Kaiser RJ, Hughes P, Dodd C, Connell CR, Heiner C, Kent SBH, Hood LE: Fluorescence detection in automated DNA sequence analysis. Nature 321:674-679, 1986.

Soini E, Kojola H: Time-resolved fluorometer for lanthanide chelates-a new generation of nonisotopic immunoassays. Clin Chem 29:65-68, 1983.

Southern EM: Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503-517, 1975.

Spalholz BA, Yang Y-C, Howley PM: Transactivation of a bovine papilloma virus transcriptional regulatory element by the E2 gene product. Cell 42:183-191, 1985.

Spence RP, Murray A, Banks L, Kelland LR, Crawford L: Analysis of human papillomavirus sequences in cell lines recently derived from cervical cancers. Cancer Res 48:324-328, 1988.

Steward M: Immunological tests. In Immunology, I Roitt, J Brostoff, D Male eds. Gower Medical Publishing, London, 1985, Chapter 25.

Stoflet ES, Koeberl DD, Sarkar G, Sommer SS: Genomic amplification

with transcript sequencing. Science 239:491-494, 1988.

Stremlau A, Gissmann L, Ikenberg H, Stark M, Bannasch P, zur Hausen H: Human papillomavirus type 16 related DNA in an anaplastic carcinoma of the lung. Cancer 55:1737-1740, 1985.

Studencki AB, Wallace RB: Allele-specific hybridization using oligonucleotide probes of very high specific activity: Discrimination of the human beta-A and beta-S globin genes. DNA 3:7-15, 1984.

Sundberg L, Porath J: Preparation of adsorbents for biospecific affinity chromatography I. Attachment of group-containing ligands to insoluble polymers by means of bifunctional oxiranes. J Chromatogr 90:87-98, 1974.

Syvanen A-C: Nucleic acid hybridization: From research tool to routine diagnostic method. Med Biol 64:313-324, 1986.

Syvanen A-C, Alanen M, Soderlund H: A complex of single-strand binding protein and M13 DNA as hybridization probe. Nucleic Acids Res 13:2789-2802, 1985.

Syvanen A-C, Korpela K: Detection of ampicillin and tetracycline resistance genes in uropathogenic Escherichia coli strains by affinity-based nucleic acid hybrid collection. FEMS Microbiol Lett 36:225-229, 1986.

Syvanen A-C, Laaksonen M, Soderlund H: Fast quantification of nucleic acid hybrids by affinity-based hybrid collection. Nucleic Acids Res 14:5037-5048, 1986 a.

Syvanen A-C, Tchen P, Ranki M, Soderlund H: Time-resolved fluorometry: a sensitive method to quantify DNA-hybrids. Nucleic Acids Res 14:1017-1028, 1986 b.

Szabo P, Ward DC: What's new with hybridization in situ? Trends Biol Sci 4:425-427, 1982.

Tchen P, Fuchs RPP, Sage E, Leng M: Chemically modified nucleic acids as immunodetectable probes in hybridization experiments. Proc Natl Acad Sci USA 81:3466-3470, 1984.

Thompson GH, Roman A: Expression of human papillomavirus type 6 E1, E2, L1 and L2 open reading frames in Escherichia coli. Gene 56:289-295, 1987.

Tindall KR, Kunkel TA: Fidelity of DNA synthesis by the Thermus aquaticus DNA polymerase. Biochemistry 27, 6008-6013, 1988.

Tomita Y, Shirasawa H, Sekine H, Simizu B: Expression of the human papillomavirus type 6b L2 open reading frame in Escherichia coli: L2-beta-galactosidase fusion proteins and their antigenic properties. Virology 158:8-14, 1987.

Triglia T, Peterson MG, Kemp DJ: A procedure for in vitro amplification of DNA segments that lie outside the boundaries of known sequences. Nucleic Acids Res 16:8186, 1988.

Tsunokawa Y, Takebe N, Kasamatsu T, Terada M, Sugimura T: Transforming activity of human papillomavirus type 16 DNA sequences in a cervical cancer. Proc Natl Acad Sci USA 83:2200-2203, 1986.

Urdea MS, Running JA, Horn T, Clyne J, Ku L, Warner BD: A novel method for the rapid detection of specific nucleotide sequences in crude biological samples without blotting or radioactivity; application to the analysis of hepatitis B virus in human serum. Gene 61:253-264, 1987 a.

Urdea MS, Warner B, Horn T: Solution phase nucleic acid sandwich assay and polynucleotide probes useful therein. European Patent Publication No. 225807 A2, 1987 b.

Urdea MS, Warner BD, Running JA, Stempien M, Clyne J, Horn T: A comparison of non-radioisotopic hybridization assay methods using

fluorescent, chemiluminescent and enzyme labeled synthetic oligodeoxyribonucleotide probes. Nucleic Acids Res 16:4937-4956, 1988.

Vary CPH: A homogeneous nucleic acid hybridization assay based on strand displacement. Nucleic Acids Res 15:6883-6897, 1987.

Verdlov ED, Monastyrskaya GS, Guskova LI, Levitan TL, Sheichenko VI, Budowsky EI: Modification of cytidine residues with a bisulfite-O-methylhydroxylamine mixture. Biochim Biophys Acta 340:153-165, 1974.

Vieira J, Messing J: The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19:259-268, 1982.

Virtanen M, Laaksonen M, Soderlund H, Palva A, Halonen P, Ranki M: Novel test for rapid viral diagnosis: Detection of adenovirus in nasopharyngeal aspirates by means of nucleic acid sandwich hybridization. Lancet i:381-383, 1983.

Virtanen M, Syvanen A-C, Oram J, Soderlund H, Ranki M: Cytomegalovirus in urine: Detection of viral DNA by sandwich hybridization. J Clin Microbiol 20:1083-1088, 1984.

Viscidi RP, Yolken RG: Molecular diagnosis of infectious diseases by nucleic acid hybridization. Mol Cell Probes 1:3-14, 1987.

Volpi A, Whitley RJ, Ceballos R, Stagno S, Pereira L: Rapid diagnosis of pneumonia due to cytomegalovirus with specific monoclonal antibodies. J Infect Dis 147:1119-1120, 1983.

Voss UB, Malcolm ADB: The immobilization of oligonucleotides and their hybridization properties. Biochem Soc Trans 16:216-217, 1988 a.

Voss UB, Malcolm ADB: A simple and efficient method for covalently attaching oligonucleotides to a cellulose support matrix. Biochem

Soc Trans 16:367-368, 1988 b.

Wahl GM, Stern M, Stark G: Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc Natl Acad Sci USA 76:3683-3687, 1979.

Waner JL, Weller TH: Analysis of antigenic diversity among human cytomegaloviruses by kinetic neutralization tests with high-titered rabbit antisera. Infect Immun 21:151-157, 1978.

Weller TH: The cytomegaloviruses: ubiquitous agents with protean clinical manifestations. N Engl J Med 285:203-214; 267-274, 1971.

Wells M, Griffiths S, Lewis F, Bird CC: Demonstration of human papillomavirus types in paraffin processed tissue from human ano-genital lesions by in situ DNA hybridisation. J Pathol 152:77-82, 1987.

Welsh J, Cantor CR: Protein-DNA cross-linking. Trends Biochem Sci 9:505-508, 1984.

Wetmur JG: Acceleration of DNA renaturation rates. Biopolymers 14:2517-2524, 1975.

Wetmur JG, Davidson N: Kinetics of renaturation of DNA. J Mol Biol 31:349-370, 1968.

Wickenden C, Coleman DV, Evans B, Malcolm ADB: Cross hybridization of human papillomavirus DNA on filters. J Virol Methods 15:249-255, 1987 a.

Wickenden C, Malcolm ADB, Smith C, Byrne M, Anderson MC, Coleman DV: Prevalence of HPV DNA and viral copy numbers in cervical scrapes from women with normal and abnormal cervices. J Pathol 153:127-135, 1987 b.

Wickenden C, Malcolm ADB, Coleman DV: DNA hybridization of cervical tissues. CRC Crit Rev Clin Lab Sci 25:1-18, 1987 c.

Wickenden C, Steele A, Malcolm ADB, Coleman DV: Screening for wart virus infection in normal and abnormal cervices by DNA hybridization of cervical scrapes. Lancet i:65-67, 1985.

Wilkins RJ, Snell RG: Centrifugal transfer and sandwich hybridisation permit 12-hour Southern blot analyses. Nucleic Acids Res 15:7200, 1987.

Wolf H, Leser U, Haus M, Gu SY, Pathmanathan R: Sandwich nucleic acid hybridization: a method with a universally usable labeled probe for various specific tests. J Virol Methods 13:1-8, 1986.

Wolf SF, Haines L, Fisch J, Kremsky JN, Dougherty JP, Jacobs K: Rapid hybridization kinetics of DNA attached to submicron latex particles. Nucleic Acids Res 15:2911-2926, 1987.

Wong C, Dowling CE, Saiki RK, Higuchi RG, Erlich HA, Kazazian HH: Characterization of beta-thalassaemia mutations using direct genomic sequencing of amplified single copy DNA. Nature 330: 384-386, 1987.

Yang Y-C, Okayama H, Howley PM: Bovine papillomavirus contains multiple transforming genes. Proc Natl Acad Sci USA 82:1030-1034, 1985 b.

Yang Y-C, Spalholz BA, Rabson MS, Howley PM: Dissociation of transforming and trans-activation functions for bovine papillomavirus type 1. Nature 318:575-577, 1985 a.

Yasumoto S, Burkhardt AL, Doninger J, DiPaolo JA: Human papillomavirus type 16 DNA-induced malignant transformation of NIH 3T3 cells. J Virol 57:572-577, 1986.

Young LS, Bevan IS, Johnson MA, Blomfield PI, Bromidge T, Maitland NJ, Woodman CBJ: The polymerase chain reaction: a new epidemiological tool for investigating cervical human papillomavirus infection. Br Med J 298:14-18, 1989.

Zachow KR, Ostrow RS, Faras AJ: Nucleotide sequence and genome organization of human papillomavirus type 5. Virology 158:251-254, 1987.

Zolg JW, Andrade E, Scott ED: Detection of Plasmodium falciparum DNA using repetitive DNA clones as species specific probes. Mol Biochem Parasitol 22:145-151, 1987.

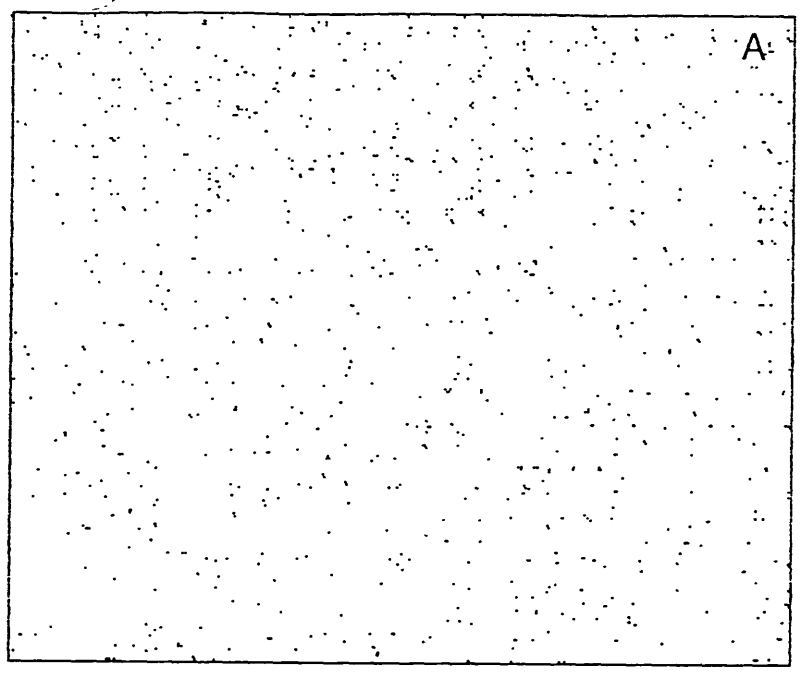
Zuckerman AJ: Biology and epidemiology of hepatitis B virus. In Viruses and Cancer: 37th Symposium of the Society for General Microbiology, PWJ Rigby, NM Wilkie eds. Cambridge University Press, Cambridge, 1985, pp 43-65.

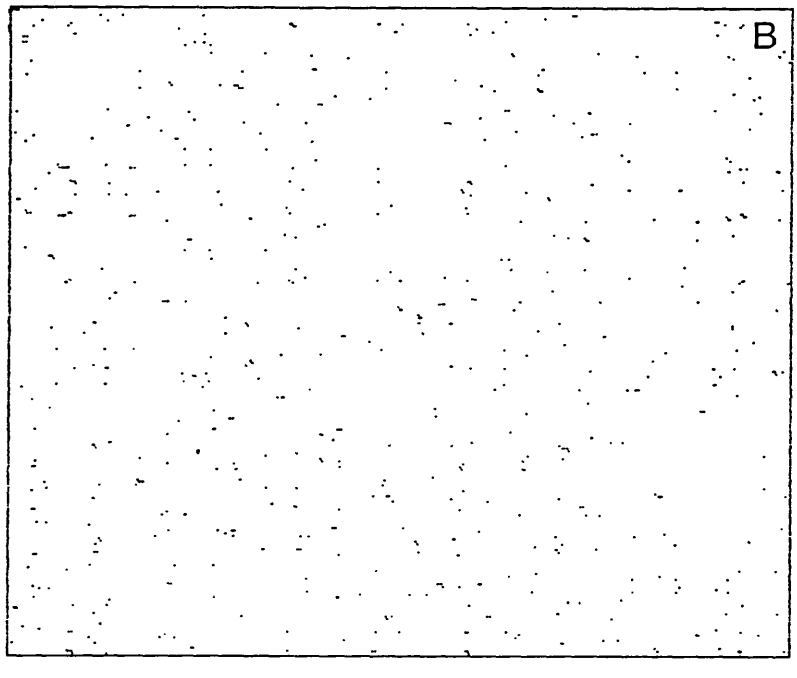
zur Hausen H: Intracellular surveillance of persisting viral infections. Lancet ii:489-491, 1986.

zur Hausen H: Papillomaviruses in human cancer. Cancer 59:1692-1696, 1987.

7. APPENDIX.

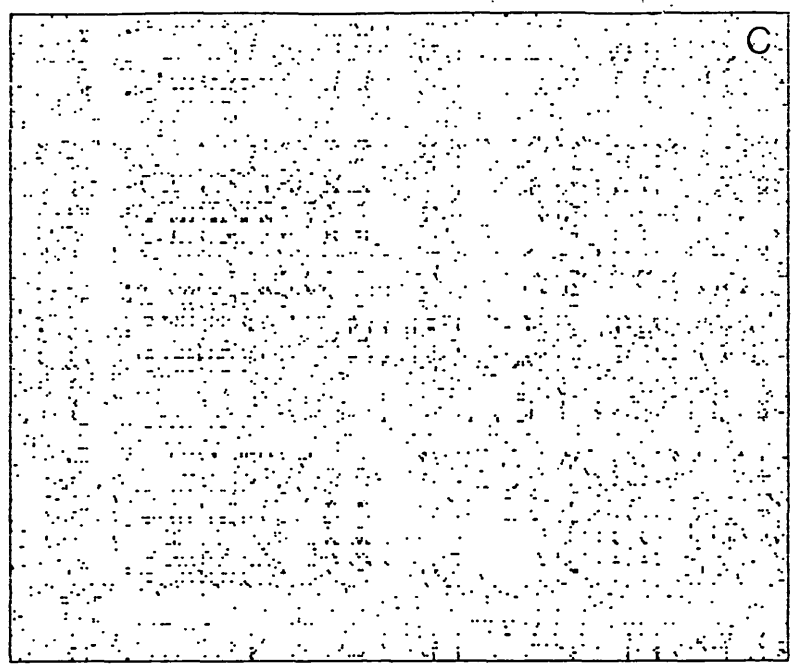
APPENDIX A: 'DIAGON' PLOTS TO ILLUSTRATE LACK OF HOMOLOGY BETWEEN HPV RESTRICTION FRAGMENTS.

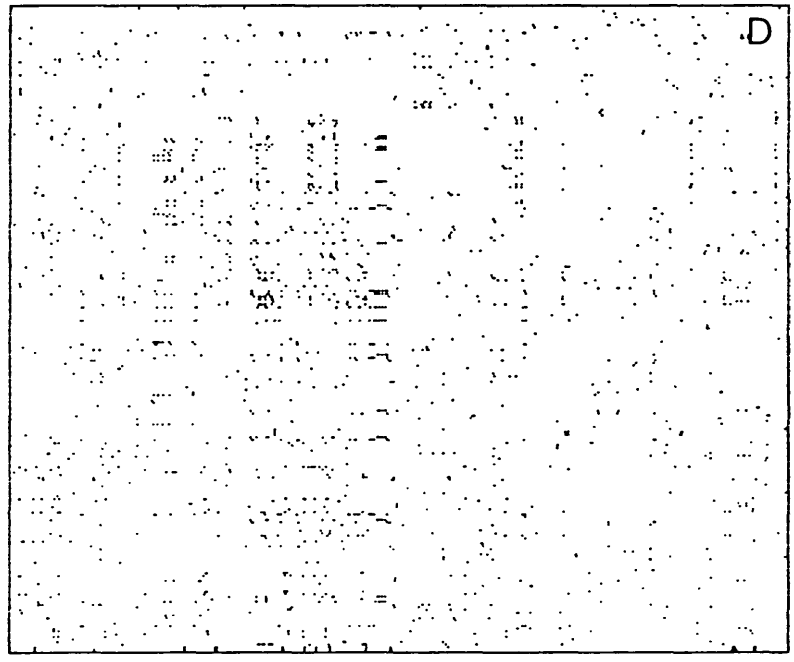




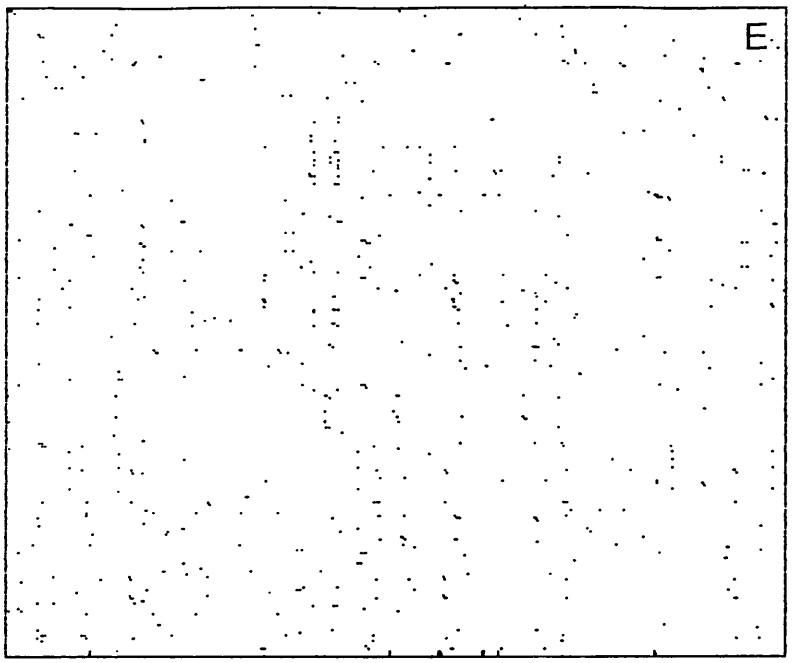
All plots were prepared using a scan length of five, and a score of five (nucleotide stretches five residues in length from the 'vertical' sequence were compared with the total 'horizontal' sequence; a dot was plotted on the diagram only at the position of an exact match). The diagrams clearly show that, at this stringency, there are no obvious regions of homology between the restriction fragment pairs chosen for use in the sandwich assay.

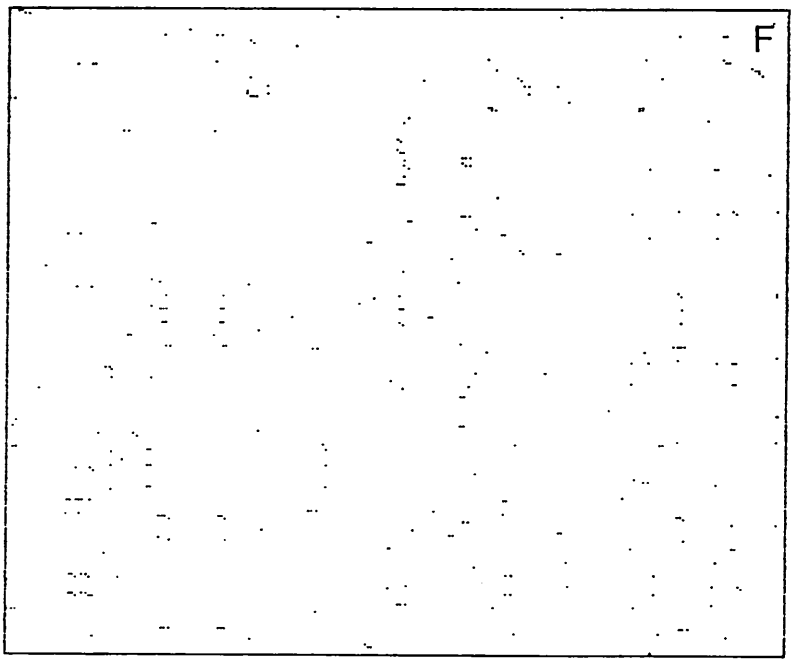
The diagrams above are comparisons of the following restriction fragments ('vertical' sequence first): **A**, HPV 6b 1,081 bp fragment vs. HPV 6b 686 bp fragment; **B**, HPV 11 1,042 bp fragment vs. HPV 11 435 bp fragment.



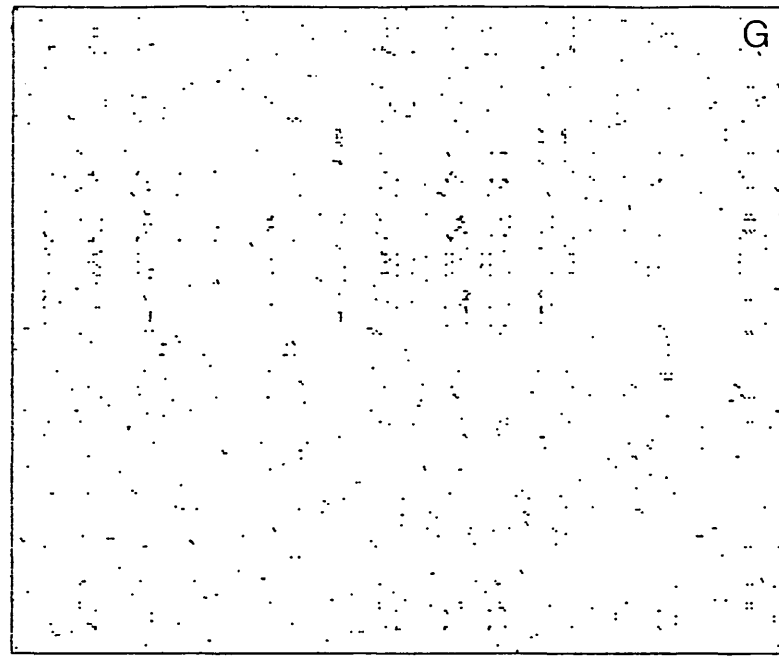


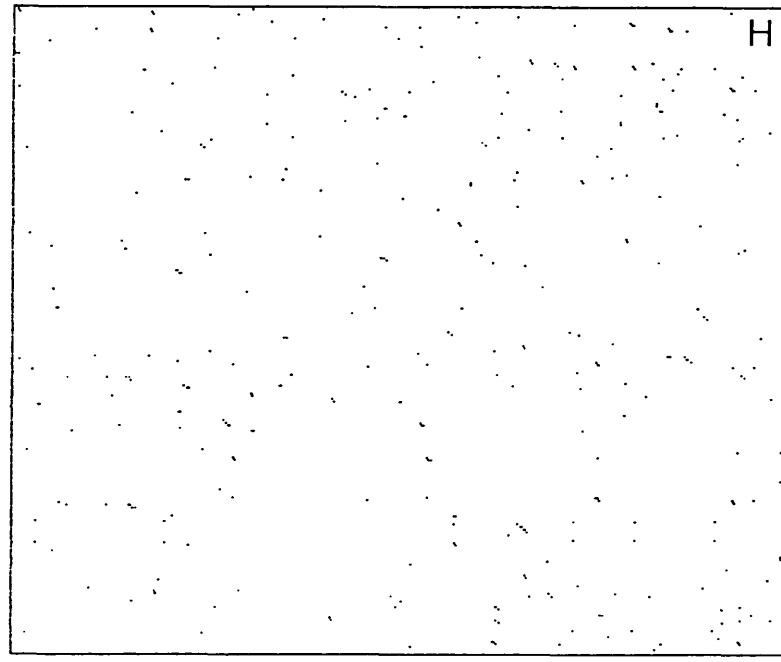
The diagrams above are comparisons of the following restriction fragments ('vertical' sequence first): **C**, HPV 16 2,817 bp fragment vs. HPV 16 1,776 bp fragment; **D**, HPV 16 1,776 bp fragment vs. HPV 16 1,063 bp fragment.





The diagrams above are comparisons of the following restriction fragments ('vertical' sequence first): **E**, HPV 16 1,776 bp fragment vs. HPV 16 483 bp fragment; **F**, HPV 16 1,776 bp fragment vs. HPV 16 216 bp fragment.





The diagrams above are comparisons of the following restriction fragments ('vertical' sequence first): **G**, HPV 16 1,063 bp fragment vs. HPV 16 483 bp fragment; **H**, HPV 18 555 bp fragment vs. HPV 18 441 bp fragment.

APPENDIX B: DNA HYBRIDIZATION KINETICS.

The kinetics of solution (single-phase) hybridization (Wetmur & Davidson, 1968), and mixed-phase hybridization (in which one of the two associating fragments is immobilized on a solid support, and the other is free in solution [Flavell et al., 1974; Cannon et al., 1985]) have been extensively investigated.

The rate at which two DNA strands (each with an overall negative charge) hybridize in solution is dependent upon temperature, salt concentration and the size and base composition of the molecules involved; in addition, the presence of certain macromolecules (for example, dextran sulphate), or hydrogen bond donors and acceptors (for example, urea or formamide) can have a significant effect on the rate of formation and stability of the DNA duplex.

The rate of renaturation of fully denatured DNA in solution is kinetically a second order reaction. The reaction rate increases as the temperature decreases below Tm (the temperature at which 50% of the paired strands are denatured), reaching a broad flat maximum from 15 to 30°C below Tm, and then decreases with a further decrease in temperature (Wetmur & Davidson, 1968). If the complexity of the DNA is represented by N, and L is the average number of nucleotides per denatured strand, the second order renaturation rate constant is approximated by the expression:

$$K_2 = \frac{3.5 \times 10^5 . L^{0.5}}{N} \qquad \text{-------- A]}$$

The units of this constant are moles/second, and the formula must be applied at (Tm-25)°C, and at $[Na^+]$ = 1.0 mole/litre. Note that the expression only applies for DNA:DNA hybridization, where the two strands are of identical length, and are present in equal concentrations.

DNA reassociation in biphasic systems follows a first order reaction, because the concentration of the free probe is in vast excess

over the target sequence. Thus:

$$t_{1/2} = \frac{\ln 2}{k.c} \qquad \text{-------- B]}$$

Where k is the pseudo-first order rate constant (litres/mole of nucleotide/second), and c is the probe concentration (moles of nucleotide/litre). Assuming that equation A holds for mixed-phase reactions, then:

$$t_{1/2} = \frac{N.\ln 2}{3.5 \times 10^{5}.L^{0.5}.c} \qquad \text{-------- C]}$$

It is important to remember that these equations are only applicable for a biphasic reaction in which the probe is single-stranded. The kinetics of mixed phase reactions in which the probe is double-stranded will deviate from those described above. Clearly, the kinetics of a biphasic hybridization in which there are three components (for example, the two-step sandwich assay) will be complex. It is possible that, if the immobilized and labelled probes are in a vast excess over the target DNA, the reaction will follow first order kinetics.

8. ERRATA.