HUMAN PAPILLOMAVIRUSES AND THEIR ASSOCIATION WITH CERVICAL DISEASE.

by

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ABSTRACT.

The overall aim of the project was to establish whether human papillomaviruses (HPV) are associated with cervical intraepithelial neoplasia (CIN) and cervical carcinoma and examine the use of HPV 16 as an indicator of cervical disease. For this purpose, a Southern blot hybridization system was developed to detect HPV DNA in cervical scrape samples. However, following the description of the polymerase chain reaction for detection of nucleic acid a PCR system for detection of HPV DNA was developed. The ability of Southern blot hybridization and PCR to detect HPV DNA in cervical scrape samples was compared to determine the most suitable method for use as a diagnostic test for HPV. The PCR method was 100,000 times more sensitive than Southern blot and was more accurate in identifying women with cervical disease.

The PCR system was used to analyse cervical scrape samples from two study groups for the presence of HPV 16 DNA. The first group (Study A) consisted of 200 women from a General Practice population who were expected to have normal cervical cytology. HPV 16 was present in 17% of women with no cervical abnormalities. In those women from Study A who had cervical disease (n = 22) the prevalence of HPV 16 increased with greater severity of disease from 15.4% in those with CIN 1, 40% in those with CIN 2, to 75% of those with CIN 3. The presence of HPV 16 DNA was significantly associated with CIN 2 and 3 ($p = 0.009$) and was therefore useful as an indicator of severe cervical disease in this population. The ability of PCR for HPV 16 to identify women with disease was compared with that of standard cytological analysis. There was no significant difference between the two methods, although a combination of screening by

cytology and PCR resulted in the identification of a higher proportion of women with disease and PCR was associated with a higher false positive rate.

The second group (Study B) consisted of 200 women who had been referred to the Royal Free Hospital colposcopy clinic with a smear report suggesting mild dyskaryosis. Within this group there were 54 women who were cytologically normal, 59 women who had CIN 1 or WVI and 66 women with severe cervical disease (CIN 2 or 3). The results of Study B concurred with Study A in demonstrating an increasing prevalence of HPV 16 with greater severity of disease from 53% in women with CIN 1, 64% of women with CIN 2 to 74% of patients with CIN 3. However, the prevalence of HPV 16 in the normal women in Study B was 63%, and this high value precludes the use of HPV 16 as an indicator of severe cervical disease in this population. Duplicate analysis of each cervical scrape sample from Study A and Study B allowed the reproducibility of the HPV 16 PCR system to be determined. The false positive rate was 0.1% and the false negative rate was 0.77%.

The long control region (LCR) of HPV 16 was cloned from a woman without cervical disease (C0) and a woman with CIN 3 (C3). The DNA sequence of each isolate was determined and compared with the prototype HPV 16 sequence. Nucleotide variations were evident in both isolates, but LCRC3 shared less homology with the prototype sequence than LCRC0. A single nucleotide mutation occurred within the glucocorticoid responsive element of LCRC3, which disrupts the palindrome of the protein binding domain. The level of expression from the HPV 16 LCR was determined using a chloramphenicol acetyltransferase assay and found to be 5-fold lower than that of the SV40 early promoter.

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ABBREVIATIONS.

A	absorbance
AP-1	activatorprotein-1
ATP	adenosine triphosphate
bp	base pair
BPV	bovine papillomavirus
Bq	Bequerel
CIN	cervical intraepithelial neoplasia
CRPV	cotton-tailrabbit papillomavirus
CTP	cytidine triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
EBV	Epstein-Barr virus
EDTA	ethylenediamine tetra-aceticacid
ELISA	enzyme-linked immunosorbant assay
EV	epidermodysplasia verruciformis
FISH	filter *in situ* hybridization
g	unit of relative centrifugal force
GRE	glucocorticoid responsive element
GTP	guanine triphosphate
HIV	human immunodeficiency virus
HPV	human papillomavirus
HSV	herpes simplex virus
LB	Luria broth
LCR	long control region
Mr	relative molecular mass
mRNA	messenger RNA
NCWVI	non-condylomatous wart virus infection
NF-1	nuclear factor-1
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
p105-Rb	retinablastoma tumour supressor protein
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SDW	sterile distilled water
SV40	simian virus 40
Taq	*Thermus aquaticus*
Tm	melting temperature of a DNA hybrid
TTP	thymidine triphosphate
UV	ultraviolet
WVI	wart virus infection

PAPILLOMAVIRUSES.

1.1 Classification.

Papillomaviruses are small, non-enveloped DNA viruses which, together with the polyomaviruses and vacuolating viruses, form the *Papovaviridae* family. The virus consists of an icosahedral protein particle of 52-55nm in diameter containing a single circular double stranded DNA molecule of approximately 7.9kbp in length. Papillomaviruses are species specific and the first to be described was the cottontail rabbit papillomavirus (CRPV) (Shope and Hurst, 1933). Many more papillomaviruses have since been characterized from cattle, sheep, elk, deer, horse and man, as well as avian species (Chen *et al.*, 1982; Giri *et al.*, 1985; Groff and Lancaster, 1985; Moreno-Lopez *et al.*, 1986). The discovery of papillomavirus DNA in penile carcinomas of rhesus and colobus monkeys provides the opportunity for development of a primate system which would have potential as a model for studying human papillomaviruses (Kloster *et al.*, 1988; Reszka *et al.*, 1991).

Classification of the papillomaviruses is based on the host range and the extent of nucleic acid homology with other papillomaviruses. Isolates are considered to be independent types if they show less than 50% cross-hybridization with heterologous viral DNA by reassociation in liquid phase (Coggin and zur Hausen, 1979). However, such analyses cannot be correlated with the extent of nucleic acid homology at the level of DNA sequence, as is demonstrated by the genital HPV types 6 and 11. These viruses are defined as distinct HPV species by

reassociation kinetics (Gissmann *et al.*, 1982), but share over 80% DNA sequence homology (Dartmann *et al.*, 1986). Papillomavirus sub-types have also been documented and these are defined as having reassociation values of greater than 50%, but incomplete homology. At least three sub-types of HPV 6 have been documented with HPV 6a and b isolated from wart tissue (de Villiers *et al.*, 1981; Schwarz *et al.*, 1983) and HPV 6vc isolated from a vulvar carcinoma (Rando *et al.*, 1986).

There are over sixty different human papillomavirus types which have been isolated so far, and the DNA sequence of several of these has been determined (Danos *et al.*, 1982; Seedorf *et al.*, 1985; Dartmann *et al.*, 1986; Cole and Streeck, 1986; Cole and Danos, 1987; Zachow *et al.*, 1987; Kirii *et al.*, 1991; Marich *et al.*, 1992). Several authors have attempted to sub-divide the HPV types into groups of related viruses. Pfister (1984) segregated the HPV types into groups according to their sequence homology, whereas Smith and Campo (1985) divided the HPVs according to the site of infection. The groupings are very similar due to the fact that viruses which are closely related in sequence are usually found to have similar properties and therefore infect the same anatomical sites.

The bovine papillomaviruses (BPV) have been separated, by similar criteria, into two sub-groups, comprising BPV 1, 2 and 5, which cause fibropapillomas of the dermis and BPV 3 and 4, which cause only epithelial proliferations (Jarrett, 1985). BPV type 6 does not appear to belong to either of these groups. There is also considerable cross-hybridization between deer fibropapillomavirus, European elk papillomavirus, reindeer papillomavirus and BPV types 1 and 2, all of which cause development of fibropapillomas (Moreno-

Lopez *et al.*, 1981; Lancaster and Sundberg, 1982).

Classification of papillomaviruses is difficult and unsatisfactory due to a lack of clear boundaries between the groups, but despite the enormous heterogeneity within the papillomaviruses there is no evidence for rapid genetic drift within the specific HPV types (Gassenmaier *et al.*, 1984; Kremsdorf *et al.*, 1984).

1.2 Specificity of Papillomaviruses.

Papillomaviruses are highly specific for their host species and there is no natural mechanism of transmission of papillomaviruses from one species to another, although transmission of BPV from cattle to horses and the production of tumours in hamsters, rabbits and mice transfected with BPV has been documented (Black *et al.*, 1963; Friedman *et al.*, 1963; Thomas *et al.*, 1964; Lancaster *et al.*, 1977; Meischke, 1979).

Papillomaviruses also have very marked specificity for tissue type and are tropic for epithelia of skin and mucus membranes. Papillomaviruses replicate only in differentiating keratinocytes, which are not easily cultured, therefore the *in vitro* production of papillomavirus particles is very difficult. However, certain types of papillomaviruses, such as BPV 1 and 2, European elk papillomavirus, deer papillomavirus and ovine papillomavirus, are able to infect fibroblasts where papillomavirus induced lesions develop into fibropapillomas in the dermal tissues. Since BPV types 1 and 2 are apparently able to cross both the species specific barrier to infect rodent and hamster cells (Lancaster and Olsen, 1982) and the tissue specific barrier, it is probable that these BPV types are less strictly

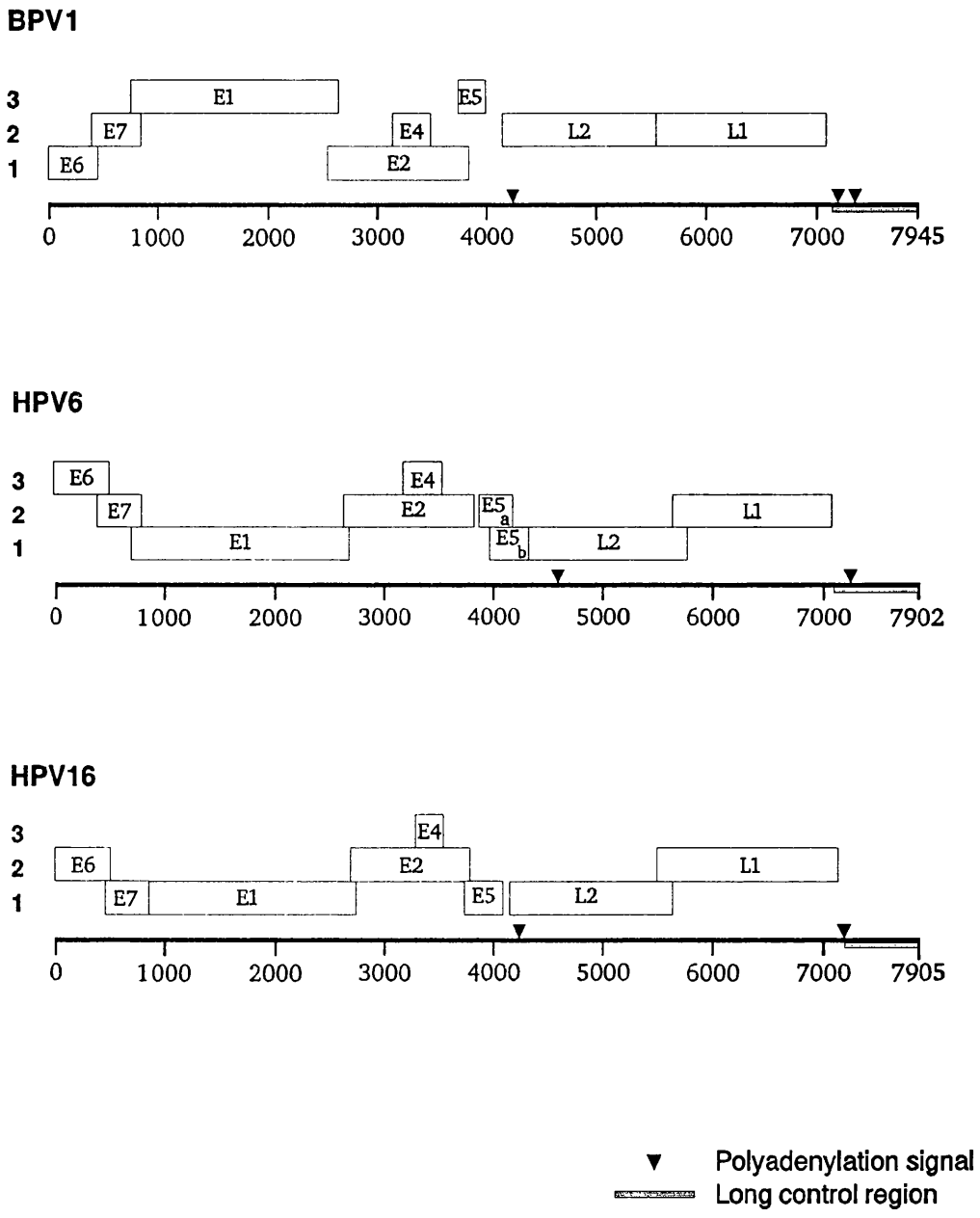
regulated than other papillomaviruses.

1.3 Papillomavirus Genome Organization.

The papillomavirus genome consists of a single, circular, double-stranded DNA molecule of approximately 7.9kbp in length (Chen *et al.*, 1982; Danos *et al.*, 1982; Schwarz *et al.*, 1983; Giri *et al.*, 1985), which complexes with cellular histone proteins (Pfister *et al.*, 1977). Except for the HPV types which are able to integrate into the host DNA, the HPV genome persists as an episome within the host cell nucleus (Durst *et al.*, 1985). The organization of the genome is similar in all papillomaviruses. There are ten major open reading frames (ORFs), although some papillomavirus types lack one or more of these. In contrast to the polyoma and SV40 viruses, in which the early and late genes are encoded on opposite strands of the DNA (Chen *et al.*, 1982; Danos *et al.*, 1982), all of the papillomavirus ORFs are encoded for on one strand of the DNA genome (Heilman *et al.*, 1980; Amtmann and Sauer, 1982; Chen *et al.*, 1982; Engel *et al.*, 1983; Pfister, 1984).

The papillomavirus genome can be separated into two regions. The early (E) region contains ORFs E1-E8 while the late (L) region contains ORFs L1 and L2. The division is based on location of the ORFs and from transcriptional analysis, rather than on the time of expression which has yet to be determined. The early gene region codes for transformation and plasmid replication functions (Lowy *et al.*, 1980). The late genes, L1 and L2, code for the structural proteins of the viral capsid and are only expressed in differentiated keratinocytes after

Figure 1.1. The genome organization of bovine papillomavirus type 1 and human papillomavirus types 6 and 16. The open reading frames are depicted by open boxes and the polyadenylation signals and long control regions are shown.



the onset of viral DNA replication (Amtmann and Sauer, 1982; Baker and Howley, 1987). The genome organization of BPV 1, HPV 6 and HPV 16 is shown in Figure 1.1. All papillomavirus genomes contain a non-coding region, called the long control region (LCR), of approximately 900bp in length which is situated between the 3' end of the L1 ORF and the 5' end of the E6 ORF. The sequence of the LCR is highly variable between individual papillomaviruses although conserved regions contain the transcriptional control signals for regulation of viral replication and gene expression. These include RNA polymerase II promoters, AT-rich regions, constitutive and inducible enhancers, transcription factor binding sites and the origin of replication (Waldeck *et al.*, 1984).

The HPV genome is usually retained as an episome in the nucleus of infected cells within benign lesions, but HPV 16 and 18 are found to be integrated into the cellular genome in malignant tissue and carcinoma derived cell lines. Integration disrupts the viral genome in the region of the E1/E2/E4 ORFs and loss of the E2 ORF may lead to deregulation of viral gene expression (Schwarz *et al.*, 1985; Lehn *et al.*, 1988; Cullen *et al.*, 1991).

1.4 Viral mRNA and Promoters.

The transcription patterns of papillomaviruses are very complex due to the presence of multiple promoters, intricate splicing patterns and the differential expression of mRNA in different cell types. The analysis of transcription has been hindered by this complexity and also by the low levels of virus specific mRNA found in cells harbouring papillomaviruses.

Transcription patterns have been most clearly defined for BPV-1, which has been used as a model for papillomavirus expression. The BPV-1 genome contains seven transcriptional promoters called P_{89}, P_{890}, P_{2443}, P_{3080}, P_{7185}, P_{7940} and P_{7250} (Ahola *et al.*, 1986; Baker and Howley, 1987; Stenlund *et al.*, 1987; Choe *et al.*, 1989). The nomenclature designates the promoter P while the subscript indicates the 5' end of the most abundant mRNA species expressed from that promoter. At least eighteen papillomavirus mRNA species have been detected (Lambert *et al.*, 1989a). P_{89} initiates expression of the E6 and E7 ORFs and, with P_{7940}, is *trans* activated by the E2 protein (Ahola *et al.*, 1986). P_{2443} initiates expression of the E2 protein and, by a splicing mechanism, the E5 protein. This promoter is also *trans* activated by the E2 protein so that expression of E2 is autoregulated (Spalholz *et al.*, 1991). P_{3080} is situated within the E2 ORF and initiates transcription of a truncated E2 protein called E2-TR (Choe *et al.*, 1989). P_{7250} is also known as P_L because it initiates transcription of the late genes L1, L2 and also E4, which is currently believed to code for a late protein (Baker and Howley, 1987). Approximately 10-100 times more mRNA is transcribed from P_L than any other promoter in the BPV-1 genome.

Most of the information concerning HPV transcription has been derived from mRNAs isolated from carcinoma cell lines in which the HPV genome is integrated into the host cell DNA (Baker *et al.*, 1987). The major HPV transcripts generated from integrated viral DNA are from the E6 and E7 ORFs. In CaSki cells, which contain multiple copies of integrated HPV 16, a promoter has been mapped to position 97 (Smotkin and Wettstein, 1986). P_{97} initiates transcription of at least three mRNA species of 4.5, 2.3 and 1.5kbp in size which code for the

E1, E7 and E6 ORFs. In HeLa cells, which contain integrated HPV 18 sequences, an equivalent promoter has been mapped to nucleotide 105 (P_{105}) (Schneider-Gadicke and Schwarz, 1986). Both P_{97} and P_{105} of HPV 16 and 18 are analogous to the P_{89} of BPV 1. The major transcripts in HeLa cells are 3.4 and 1.6kbp in size and code for the E6/E7/E1 and E6*/E7 proteins respectively.

In benign tumours the DNA remains episomal and northern blot analysis has identified mRNAs of 4.2, 2.6, 1.8 and 1.4kbp in size, which code for E6 and E7, as well as E1, E2, E4 and E5 (Schneider-Gadicke and Schwarz, 1986; Smotkin *et al.*, 1989).

The transcription patterns in cervical intraepithelial neoplasia (CIN) lesions and invasive cervical carcinoma are different from those of benign tumours and are highly variable. In some tumours the major transcript is 3.4kbp in size, which codes for all early genes except E1 and E2, whereas in other tumours the major transcript is of 1.8kbp and codes only for the E6 and E7 genes (Smotkin *et al.*, 1989). Additional mRNAs of 4.2, 2.2, 1.6 and 1.4kbp have also been identified. The general transcription pattern in carcinoma tissue is high levels of E6 and E7 transcripts and little or no transcription of the other early genes. Transcripts of the L1 and L2 ORFs have not been identified in samples derived from invasive cervical lesions.

1.5 Transcription Enhancers.

In addition to promoter sequences, the LCR of papillomaviruses contains several enhancer elements, both constitutive and inducible, which regulate viral gene expression. Each papillomavirus species appears to have several enhancer

domains which act in different ways and are responsive to cellular or viral factors. BPV 1 and HPV 11, 16 and 18 all have constitutive enhancer domains which function in the absence of viral proteins (Cripe *et al.*, 1987; Thierry *et al.*, 1987). These regions confer cell and tissue type specificity to viral gene expression. Within the constitutive enhancer region are motifs, with the sequence 5'-TTTGGCTT, which confer keratinocyte dependence and are found in all genital HPVs as well as BPV and CRPV (Schwarz *et al.*, 1983; Cole and Streeck, 1986; Dartmann *et al.*, 1986; Thierry *et al.*, 1987). A keratinocyte specific cellular factor has also been identified which binds to the HPV 18 constitutive enhancer (Garcia-Carranca *et al.*, 1988).

There is at least one inducible enhancer in the LCR of all papillomaviruses which is dependent on the binding of the E2 protein (Harrison *et al.*, 1987; Haugen *et al.*, 1987; Hirochika *et al.*, 1988). Regulation of HPV expression by this E2-dependent enhancer is described in detail in Section 1.8. In HPV 18 a second inducible enhancer has been mapped to the LCR and is *trans* activated by the HPV 18 E6 protein (Gius *et al.*, 1988). It has also been established that HPV 18 LCR sequences, when linked to the E6/E7 genes of either HPV 16 or 18, are more efficient in immortalization assays than the HPV 16 LCR in an equivalent construct. This difference is not due to the E6/E7 gene sequences, but has been mapped to the region of the LCR upstream of the E6 ATG codon (Romanczuk *et al.*, 1991). DNA footprinting analysis of the HPV genome using nuclear extracts from a variety of cells has demonstrated that a number of cellular proteins bind to sequences within the LCR, although very few of them have been characterized (Garcia-Carranca *et al.*, 1988; Gloss *et al.*, 1989). Several binding sites for the

transcription regulatory proteins, activator protein 1 (AP-1) and nuclear factor 1 (NF-1), have been identified in the enhancer region of HPV 16 and 18 (Garcia-Carranca *et al.*, 1988; Chin *et al.*, 1989). Beta transforming growth factors (TGFßs) 1 and 2 down regulate expression of the E6 and E7 mRNA of HPV 16 in several different cell lines (Pietenpol *et al.*, 1990; Woodworth *et al.*, 1990b). Cells which have been transformed by HPV show partial resistance to TGFß 1 regulation and acquisition of this resistance may contribute to carcinogenesis. TGFß 1 is known to interact with AP-1 and NF-1 and is therefore likely to function via enhancer elements to which these factors bind (Pietenpol *et al.*, 1990).

Within the enhancer region is a glucocorticoid responsive element (GRE) with the sequence 5'-TGTACAN$_3$TGTCAT, where N represents any nucleotide. The element is responsive to both progesterone and glucocorticoid hormones (Gloss *et al.*, 1987; Chan *et al.*, 1989). When the E6 and E7 genes of HPV 16 or 18 are under transcriptional control of their homologous promoters, full transformation of primary cells is only achieved in the presence of the *ras* oncogene and steroid hormones (Lambert *et al.*, 1987; Matlashewski *et al.*, 1987; Pater *et al.*, 1988). Thus, steroid hormones are necessary for full activity of the P_{97} and P_{105} promoters of HPV 16 and 18, respectively. Epidermal growth factor (EGF) induces down regulation of HPV 16 E6 and E7 transcription and this process is probably mediated by interaction with EGF responsive elements in the LCR of HPV 16 (Yasumoto *et al.*, 1991).

1.6 Viral Protein Function.

The function of the products of each ORF has not been fully defined due to the lack of cell systems for propagation of papillomavirus particles. Assignment of putative protein functions has been achieved by comparison of amino acid sequences of viral genes with protein sequence databases and by functional similarities. In addition, *in vitro* transformation of rodent cells with BPV 1 has generated a model of papillomavirus expression and mutational studies using this system have provided further information on protein structure and function (Berg *et al.*, 1986; Neary *et al.*, 1987; Schiller *et al.*, 1989).

1.7 The E1 Open Reading Frame and Viral Replication.

There are two modes of papillomavirus replication, plasmid and vegetative. During plasmid replication, which occurs in the basal cells of the epidermis, the viral DNA is maintained as an episome and replicated once per cell cycle. There are two phases of plasmid replication; establishment and maintenance. During the establishment phase, the infecting viral genome is amplified from low copy levels to moderate copy numbers of between 50 and 400 genomes per cell, after which the plasmid copy number remains constant for many cell divisions throughout the maintenance phase. Vegetative DNA replication occurs in the differentiated cells of the epidermis, where a burst of viral DNA synthesis generates many copies of the papillomavirus genome for packaging into viral progeny.

The E1 ORF is the only papillomavirus gene which codes for proteins directly involved in replication, although several of the other early papillomavirus genes such as E2, E5, E6 and E7 may regulate this process (Hermonat and

Howley, 1987; Neary *et al.*, 1987). The BPV 1 E1 ORF codes for two distinct, independent products and shares homology with the large T antigen of polyomavirus and an ATPase which has nucleotide binding capacity(Clertant and Seif, 1984). Mutations in either of the two genes result in a replication defective virus which integrates, at low copy number, into the host chromosome (Sarver *et al.*, 1984; Rabson *et al.*, 1986; di Maio *et al.*, 1987). The 5' region of the ORF codes for a modulator protein called E1-M which has a Mr of 23 000 (Thorner *et al.*, 1988) and is a negative regulatory protein which suppresses DNA replication to low copy number and links replication to host cell cycle control. The 3' region codes for E1-R which is directly responsible for replication and plays a role in the amplificationprocess which occurs during vegetativereplication (Lusky and Botchan, 1986a, 1986b).

Electron microscopic analysis of replicativeintermediates has mapped the origin of replication to a region in the LCR of BPV 1 (Waldeck *et al.*, 1984). Additional sequences necessary for plasmid replication, called plasmid maintenance sequences (PMS), have been identified between nucleotides 7116-7234 and 1515-1655 of the BPV genome (Lusky and Botchan, 1984; Waldeck *et al.*, 1984). The PMS alone are insufficientfor plasmid replication and require a *cis* enhancer element in order to function.

The life cycle of papillomaviruses is difficultto study due to the lack of a cell system for viral replication, although methods for the propagation of HPV 11 and 16 have been established (Kreider *et al.*, 1987; Sterling *et al.*, 1990). Fragments of human epithelium were extracted from a human vulvar condylomata, infected with HPV 11 and grafted beneath the renal capsule of

athymic mice. Virions were then purified from the resulting condylomatous cysts (Kreider *et al.*, 1987). This system has subsequently been used to analyse transcription and replication of the HPV within these lesions (Stoler *et al.*, 1990). Production of HPV 16 particles was carried out in a similar manner, but the virus was transfected into a keratinocyte cell line which maintains HPV 16 DNA in a monomeric episomal form (Stanley *et al.*, 1989). Cells were then grafted into nude mice and virus capsid antigen and particles isolated from the lesions (Sterling *et al.*, 1990).

1.8 The E2 Open Reading Frame and Transcriptional Regulation.

The E2 ORF codes for a *trans*activator protein which is also able to act as a repressor. E2 mediates transcriptional regulation by binding to *cis* elements within the viral LCR (Spalholz *et al.*, 1985; Harrison *et al.*, 1987). The consensus sequence to which the E2 protein binds is a palindrome of $ACCN_6GGT$ or $ACCN_6GTT$ (Androphy *et al.*, 1985; Hawley-Nelson *et al.*, 1988; McBride *et al.*, 1988; Li *et al.*, 1989). Two of these motifs, when situated close together, form an E2-dependent enhancer (Harrison *et al.*, 1987; Haugen *et al.*, 1987; Spalholz *et al.*, 1988) which enhances transcription from yeast and mammalian promoters when linked in *cis* (Gius *et al.*, 1988; Spalholz *et al.*, 1988; Hawley-Nelson *et al.*, 1988; Lambert *et al.*, 1989b). The E2 enhancer is independent of orientation, as are most enhancers, but the strength of enhancement is influenced by the distance between the two palindromic sequences and the distance of these from the promoter. Therefore, it has been postulated that the E2-dependent enhancer acts via a looping mechanism (Ptashne, 1986; Chin *et al.*, 1989).

There is considerable variation in the number, position and affinity of E2 binding sites within the papillomaviruses (Li *et al.*, 1989; Harrison *et al.*, 1987). The affinity for binding is dependent on the fidelity of the E2 consensus motif, composition of the N_6 region and sequence of the surrounding DNA. There are four E2 binding sites within the LCR of human papillomaviruses. Two of these form an enhancer element 42 bp upstream of the ATG initiation codon of the E6 ORF, a third motif is situated 100 nucleotides upstream of the E6 ORF and the fourth is positioned 400 nucleotides upstream of the third one (Garcia-Carranca *et al.*, 1988; Hirochika *et al.*, 1988).

The full length transcript from the E2 ORF of BPV 1 codes for a protein with a Mr of 48 000, but two additional E2 proteins have been identified (Lambert *et al.*, 1987; Hubbert *et al.*, 1988). A fusion protein of Mr 28 000 is expressed from a transcript generated by a splicing mechanism which joins 11 amino acids of E8 to the C-terminal region of E2 (Choe *et al.*, 1989; Lambert *et al.*, 1989b). Transcription from an internal promoter within the E2 ORF of BPV 1 generates a third protein (Mr: 31 000) from the 3' C-terminal of the E2 ORF. This truncated E2 protein, called E2-TR, acts as a repressor of E2 *trans*activation and inhibits viral mediated transformation (Lambert *et al.*, 1987, 1989b).

The extent of homology of the E2 ORF between different papillomavirus types is only 35%, but several conserved motifs exist which define three distinct structural domains, the N-terminal and C-terminal domains and an internal region. Separate functions have been ascribed to each domain (Giri and Yaniv, 1988). The N-terminal 220 amino acids code for the *trans*activation function and contains two negative amphipathic α-helices. The latter are thought to be directly

involved in protein-protein interactions with transcription factors such as TFIID, or RNA polymerase itself (Giniger and Ptashne, 1987). The C-terminal region, consisting of 100 amino acids, mediates dimerization of E2 molecules and is responsible for specific binding to the E2 consensus sequences (Mc Bride *et al.*, 1988). The mode of DNA binding is unknown, because there are no recognized DNA binding structures, such as zinc finger or helix-turn-helix motifs, predicted for the C-terminal region. Dimerization is mediated by conserved hydrophobic amino acid residues (Dostatni *et al.*, 1988; Mc Bride *et al.*, 1989) which have the potential to form a coiled coil structure (O'Shea *et al.*, 1989) resembling a leucine zipper motif (Landshultz *et al.*, 1988). The internal region of the E2 ORF varies in length and composition, although it is always proline rich and believed to be a hinge structure connecting the two functional domains. Both truncated forms of the E2 protein lack the N-terminal *trans*activation domain, but retain the C-terminal DNA binding and dimerization functions (Haugen *et al.*, 1989; McBride *et al.*, 1989).

Transcriptional repression by E2 may be mediated in one of two ways; either the E2-TR and E8/E2 act by competitive binding to the consensus sequence, thereby preventing E2 *trans*activator binding, or inactive heterodimers may be formed between the repressor and *trans*activator species which are unable to activate papillomavirus promoters.

Truncated repressor type E2 proteins have not yet been detected in human papillomaviruses despite the presence of transcription controls which would enable such proteins to be expressed (Hirochika *et al.*, 1988). However, the full length E2 protein of HPV is able to carry out both *trans*activation and repression

functions (Thierry and Howley, 1991). The repression of viral transcription is dependent on the location of E2 binding sites with respect to the promoter and is probably mediated by steric hindrance to binding of TFIID and RNA polymerase to promoter sequences (Thierry and Howley, 1991). Regulation by HPV E2 may be important with respect to the development of cervicalcarcinoma. In benign lesions the HPV genome is episomal and expression of the E6 and E7 genes is regulated by E2 repression. Integration of HPV into cellular DNA precedes the development of malignant lesions and results in disruption of the E2 ORF. Expression of E6 and E7 is then released from E2 repressor regulation leading to higher levels of HPV transforming proteins and hence to malignancy. However, as with BPV 1, the mode of action of HPV E2 has yet to be established experimentally.

1.9 The E3 and E4 Open Reading Frames.

The E3 ORF is absent from many papillomaviruses and in those papillomaviruses in which the ORF is present the function of E3 is unknown.

The E4 ORF overlaps the E2 ORF, but is translated from a different reading frame and is under transcriptional control of P_L (Baker and Howley, 1987). The E4 of HPV 1 is a cytoplasmicprotein of Mr 17 000 which is probably involved in viral maturation. The E4 of HPV 16 has been detected in CIN lesions in which it is localized to the nucleus, but has not been detected in carcinoma tissue (Palefsky *et al.*, 1991a).

1.10 Transforming Proteins of Papillomaviruses.

Certain of the papillomaviruses have been found to have transforming potential *in vitro* and assays have been established to examine this activity for both BPV and HPV (Dvoretzky *et al.*, 1980; Bedell *et al.*, 1987).

BPV 1 morphologically transforms established rodent cells such as C127 and NIH 3T3 *in vitro* (di Maio *et al.*, 1986; Rabson *et al.*, 1986; Schiller *et al.*, 1986). The cells become spindle shaped, lose contact inhibition, become anchorage independent and are tumorigenic when transplanted into nude mice (Dvoretzky *et al.*, 1980). BPV 1 is maintained as a multicopy plasmid in transformed rodent cells, whereas the BPV 4 genome is lost during development of malignancy in naturally occurring cancers of cattle (Campo *et al.*, 1985). BPV encodes two independent transforming proteins, E5 and E6.

Certain types of HPV are able to transform established rodent cells in a similar manner to BPV 1 (Bedell *et al.*, 1987; Laimins *et al.*, 1987; Watanabe and Yoshiike, 1988; Yasumoto *et al.*, 1986) and to transform primary rodent cells, but only in cooperation with the *ras* oncogene (Matlashewski *et al.*, 1987; Pater *et al.*, 1988). Human primary fibroblasts and keratinocytes are also transformed by HPV (Durst *et al.*, 1987; Pirisi *et al.*, 1987; Sedman *et al.*, 1991), but the resulting cells are non-tumorigenic in nude mice (Schlegel *et al.*, 1988). The conclusion reached from these studies is that HPV is less efficient than BPV 1 in inducing transformation. Only a few of the human papillomaviruses, such as HPV 16 and 18 which are prevalent in high-grade clinical lesions and able to integrate into host DNA, have transforming potential (Schlegel *et al.*, 1988; Storey *et al.*, 1988). The types of HPV normally associated with benign lesions, such as 6 and 11, are only able to transform keratinocytes at a greatly reduced efficiency (Storey *et al.*,

1990). The transforming potential of HPV 16 and 18 has been mapped to the E6 and E7 genes (Watanabe *et al.*, 1989). The E5 protein of HPV may also have a role to play in the transforming process of HPV (Leptak *et al.*, 1991) and, thus, as with CRPV, HPV may code for three transforming proteins (Meyers *et al.*, 1992).

1.11 The E5 Open Reading Frame.

The E5 protein of BPV 1 is able to transform rodent fibroblast cell lines such as C127 and NIH 3T3 *in vitro* (diMaio *et al.*, 1986; Rabson *et al.*, 1986; Schiller *et al.*, 1986) and with a Mr of 7 000 is the smallest viral transforming protein so far described (Schlegel *et al.*, 1986). The BPV E5 protein is localised in the Golgi apparatus and other intracellular membranes (Burkhardt *etal.*, 1989).

The sequence of E5 is very similar in those papillomaviruses which cause both dermal and epidermal proliferations so E5 may play a role in the stimulation of dermal proliferation. The BPV-1 E5 protein contains two structural domains: an N-terminal hydrophobic domain which mediates insertion into the cellular membrane (Schlegel *et al.*, 1986) and a C-terminal hydrophilic region which induces cellular DNA synthesis (Green and Loewenstein, 1987). The protein is active as a dimer and causes transformation by interference with growth regulatory proteins. The ß type receptor for platelet-derived growth factor (PDGF) is constitutively activated in E5 transformed cells and stimulation of the receptor may be mediated via a short region of the E5 protein which is homologous with sequences in PDGF (Petti *et al.*, 1991). The E5 protein also associates with a 16kD subunit of the vacuolar H^+-ATPase (Goldstein *et al.*,

1991).

Many, but not all, human papillomaviruses are predicted to code for short hydrophobic E5 proteins and HPV6 codes for two proteins, E5a and E5b. The E5a protein of HPV 6c is able to induce morphologic transformation in NIH 3T3 cells (Chen and Mounts, 1990). The E5 protein of HPV 16, when under control of retroviral LTR promoters, is also able to induce tumorigenic transformation, but only of established keratinocytes and not C127 or NIH 3T3 cells (Leptak *et al.*, 1991). Comparison of the amino acid sequence of the E5 ORF of the different HPV types reveals little homology between them, but conserved regions of hydrophobic residues may be involved in anchoring the protein to intracellular membranes.

1.12 The E6 Open Reading Frame.

The exact function of the E6 gene has not been elucidated, although it is necessary for development of the fully transformed phenotype in all transforming papillomaviruses (Sarver *et al.*, 1984; Neary and diMaio, 1989).

The E6 gene product of BPV-1 has a Mr of 15 000 and is found in both nuclear and membraneous compartments. The E6 protein of CRPV is twice as large as that of BPV-1 due to a duplicated region of the ORF.

The complete HPV E6 gene codes for a protein of 158 amino acids with a Mr of 18-19 000, but the size of the protein product detected in carcinoma cell lines is highly variable. An internal splicing event in the E6 ORF of HPV 16 and 18 can generate a truncated version of E6, known as E6* (Schwarz *et al.*, 1985), which may correspond to a papillomavirus protein detected in CaSki cells with a

Mr of 11 000 (Seedorf *et al.*, 1987). Other types of papillomavirus, including BPV-1 and CRPV, are unable to code for the E6* protein.

The E6 protein contains four Cys-X-X-Cys motifs, which form two zinc finger domains ($Cys-X_2-Cys-X_{29}-Cys-X_2-Cys$ where X = any amino acid) characteristic of DNA binding proteins which bind zinc *in vitro* (Barbosa *et al.*, 1989). Both motifs are necessary to retain the E6 transforming ability (Vousden *et al.*, 1989). The E6 protein has been found to induce anchorage independence in immortalized keratinocytes and to transform primary human fibroblasts in conjunction with the E7 protein (Munger *et al.*, 1989; Sedman *et al.*, 1991). It is only the full length E6 protein product which has transforming ability (Munger *et al.*, 1989; Sedman *et al.*, 1991).

The E6 protein of HPV 16 and 18 associates with p53 (Scheffner *et al.*, 1990) which possesses tumour suppressor properties and is known to reduce the efficiency of transformation by oncogenes (Scheffner *et al.*, 1991). The SV40 large T antigen and adenovirus E1B protein both bind to p53, resulting in an increased half-life, but inactivation of the tumour suppressor protein. However, E6, on binding to p53, promotes degradation of the p53 in a process believed to involve the ATP-dependent ubiquitin protease system. Degradation of p53 is mediated by the E6 protein of HPV 16 and 18, but not HPV 6 and 11. Thus, degradation of p53 may reduce tumour suppression and be an additional mechanism by which HPV 16 and 18 induce malignant conversion (Scheffner *et al.*, 1990, 1991).

1.13 The E7 Open Reading Frame.

The E7 ORF codes for the major transforming protein of oncogenic type human papillomaviruses. The protein is a nuclear phosphoprotein which acts as a transcriptional modulator (Phelps *et al.*, 1988). It is the most abundant protein found in cervical carcinoma tissue and carcinoma cell lines. The E7 proteins of HPV 16 and 18 are able to transform established cell lines and to co-operate with the activated *ras* oncogene to transform primary cells such as baby rat kidney cells (Pater *et al.*, 1988; Phelps *et al.*, 1988; Storey *et al.*, 1988). E7 alone is insufficient for transformation of primary human fibroblasts or keratinocytes and co-operation with E6 is necessary to alter these cells to the immortalized phenotype from which they can no longer differentiate. The E7 proteins of the genital HPV types associated with benign lesions, e.g. HPV 6 and 11, are able to transform primary keratinocytes, but only at a greatly reduced level compared with HPV 16 and 18 (Storey *et al.*, 1990).

The E7 protein of HPV 16 is comprised of 98 amino acids. The N-terminal 37 amino acids share homology with domains 1 and 2 of adenovirus E1A protein and the large T antigen of SV40 (de Caprio *et al.*, 1988; Phelps *et al.*, 1988). These sequences are necessary for the transformation function of all three viruses and amino acid changes in E7 confer loss of transforming ability in the same manner as similar mutations in E1A (Edmonds and Vousden, 1989).

E7 binds the retinoblastoma tumour suppressor protein p105-Rb *in vitro* and sequences mediating this binding are highly conserved in all genital HPV types (Whyte *et al.*, 1988; Dyson *et al.*, 1989). Indeed, all E7 proteins from genital HPV types which were analysed have been found to associate with p105-Rb

(Munger *et al.*, 1989). It is believed that p105-Rb may be involved in the control of cell proliferation, consequently the binding of E7 to p105-Rb may interfere with normal cellular processes. It is interesting to note that both HPV 16 and 18 bind to p105-Rb with a 5-20 fold higher affinitythan HPV 6, which has virtually no transforming ability (Barbosa *et al.*, 1990; Gage *et al.*, 1990). Adenovirus E1A and SV40 large T proteins are also able to associate with p105-Rb (de Caprio *et al.*, 1988, Whyte *et al.*, 1988) and it has therefore been postulated that all three viruses use the same mechanism for cell transformation. The E7 of HPV is also a substrate for Casein Kinase II (CK II) which phosphorylates two serine residues at amino acids 31 and 32. Replacement of these serines with amino acids which cannot be phosphorylated leads to a reduction in E7 transforming activity,but does not affectits ability to bind p105-Rb (Barbosa *et al.*, 1990). The E7 of HPV 18 is phosphorylated more rapidly than that of HPV 16, which in turn is phosphorylated twice as fast as that of HPV 6. Thus, p105-Rb binding and phosphorylation of E7 by CK II are independent events which are both required for efficient transformation by E7. These activities correlate directly with the relative oncogenicity of the genital HPVs.

The C-terminal domain of the E7 ORF contains one zinc-finger motif similar to those present in the E6 ORF. These motifs are also found in domain 3 of the E1A protein and since this region of the adenovirus genome is implicated in *trans*activationof transcription,the Cys-X-X-Cysmotifs in E7 may confer DNA binding properties to this protein and hence, modulate transcription(Phelps *et al.*, 1988).

In contrast to the E7 of certain papillomaviruses, the BPV-1 E7 protein

product has no transformation or transcriptional regulation functions, but is involved in the control of viral replication to high copy number (Berg *et al.*, 1986b; Lusky and Botchan, 1986b). The regions of the HPV E7 which are homologous with the E1A domains are absent from BPV-1 E7 protein and it is unable to bind to the p105-Rb gene product.

1.14 The L1 and L2 Open Reading Frames.

The late structural proteins of the papillomavirus capsid are encoded by the L1 and L2 open reading frames and are only expressed in terminally differentiated keratinocytes following viral DNA replication.

The L1 ORF codes for the major capsid protein which is highly conserved between the papillomaviruses (Chen *et al.*, 1982; Schwarz *et al.*, 1983; Seedorf *et al.*, 1985; Dartmann *et al.*, 1986). The L1 proteins of BPV, HPV and CRPV have a Mr of 54-56,000 (Feitelson *et al.*, 1985; Meinke and Meinke, 1981; Roseto *et al.*, 1984; Tomita *et al.*, 1987) and contain the genus-specific epitopes. Antibodies to L1 proteins are cross-reactive with those of other papillomaviruses, including BPV (Jenson *et al.*, 1980; Nakai *et al.*, 1986). The L1 product is highly glycosylated which increases the stability of the virus particle structure.

The L2 ORF (Mr: 70-76 000) codes for the minor capsid protein which is very poorly conserved between papillomaviruses (Danos *et al.*, 1984). The antigenic determinants within L2 are HPV type specific (Komly *et al.*, 1986).

1.15 Diagnosis of HPV Infection and Cervical Disease.

In this section, the techniques for detection and treatment of both HPV infection and cervical lesions are described.

The cervical smear test was developed by George Papanicolaou to detect cell changes in the cervix and vagina and has become the major method for the early detection of cellular abnormalities of the uterine cervix (Papanicolaou, 1949). The process involves the collection of cells from the cervix, which are smeared onto a glass slide, stained and examined by light microscopy. Dyskaryotic cells may be present, showing nuclear changes indicative of cervical disease such as enlargement, hyperchromasia and multinucleation. HPV infection may also be indicated by the presence of koilocytes, in which vacuolization of the cytoplasm surrounding the nucleus has occurred (Meisels and Fortin, 1976). In cytological screening programmes of women from the general population 2-3% of cervical smears show abnormalities, although this value is generally higher in women attending sexually transmitted diseases clinics (Meisels *et al.*, 1982; Kiviat *et al.*, 1989).

Colposcopy is a method by which a magnified view of the cervix and vagina can be obtained and any lesions examined. The visibility of lesions can be enhanced by the application of dilute acetic acid or Lugols' iodine to the cervix. Acetic acid causes the areas of abnormality to become white, called acetowhite areas, and iodine stains the normal tissue around the lesion. A punch biopsy may be taken under colposcopic direction to ensure that the tissue obtained is from the area of abnormality.

Histological analysis can be carried out on punch biopsies obtained during colposcopic examination, or cone biopsies taken during treatment of cervical lesions. The tissue samples are fixed in formalin, paraffin embedded, sectioned, stained and examined by light microscopy. The abnormalities associated with cervical disease are dyskeratosis, nuclear atypia, basal layer hyperplasia and also koilocytosis as evidence of HPV infection. Histological analysis can be used to define the severity of cervical disease and grade of cervical intraepithelial neoplasia (CIN).

Until recently, the most common method used for the detection of HPV infection was nucleic acid hybridization. This process can be carried out using cervical scrape material or biopsy samples. The hybridization techniques available include Southern and northern blot, dot blot, *in situ* hybridization and filter *in situ* hybridization (Crum *et al.*, 1985; Campion *et al.*, 1986; Toon *et al.*, 1986; Shirasawa *et al.*, 1986; Burns *et al.*, 1987; Melchers *et al.*, 1988). The development of DNA amplification procedures has allowed more sensitive detection of HPV in clinical samples (Saiki *et al.*, 1988; van den Brule *et al.*, 1989; Young *et al.*, 1989). Additional methods for detection of HPV infection include electron microscopy, by which wart tissue can be examined for the presence of viral particles. The standard virological procedures of serology and virus isolation are not routinely used for the detection of HPV, due to the problems associated with culture of HPV *in vitro*.

1.16 Immune Response to Papillomavirus Infection.

The immune response to papillomavirus infection has not been elucidated in detail due to the lack of a cell culture system to propagate these viruses. Clinical observations suggest that the T-cell response is important since there is an increased frequency of warts, and other HPV related symptoms, in patients with T-cell immunosuppression e.g. patients with acquired immune deficiency syndrome and those undergoing transplantation or immunosuppressive chemotherapy (Rudlinger *et al.*, 1986; Feingold *et al.*, 1990; Matorras *et al.*, 1991). On improvement of cell-mediated immunity the warts and HPV infections disappear. Trauma to, or removal of, a wart can lead to the regression of others probably by activation of immune responses by antigens released from the wart tissue. Cells which have been transfected with the E7 gene of HPV 16 demonstrate cytolytic susceptibility to macrophages, which are able to destroy associated neoplastic lesions (Banks *et al.*, 1991).

The L1 and L2 capsid proteins are described in section 1.14. L1 is the most immunogenic papillomavirus protein, although antibodies against L2, E1, E2, E4 and E7 have been detected in human sera (de Martynoff *et al.*, 1989; Dillner *et al.*, 1989; Jochmus-Kudielka *et al.*, 1989; Kochel *et al.*, 1991a).

Screening for HPV by serological methods is not routinely used as a diagnostic technique. There are several factors which may account for this situation. The immune response to papillomavirus proteins is poor due to the major antigens, L1 and L2, being expressed only in fully differentiated keratinocytes. There are few blood vessels in keratinocytes and therefore HPV particles within these cells are protected from detection by the immune system.

In addition, most of the available serological assays have been established using bacterially expressed HPV proteins (Tomita *et al.*, 1987a, 1987b; Jenison *et al.*, 1988; Jochmus-Kudielka *et al.*, 1989) which may differ from the authentic proteins and thus alter antigen-antibody interactions (Kochel *et al.*, 1991b).

Future developments in the treatment of papillomaviruses and related disease may include immunization against HPV. Some of the B- and T-cell epitopes of viral proteins, such as L1 and E7, have recently been identified and may have potential for use in vaccine development (Dillner, 1990; Tindle *et al.*, 1990; Comerford *et al.*, 1991; Suchankova *et al.*, 1991). The L1 and L2 proteins of BPV 2 expressed in *E. coli* have been used to vaccinate calves. Vaccination has been found to promote tumor rejection and also prevent tumor formation when administered prior to challenge with BPV (Jarrett *et al.*, 1991).

1.17 Warts, Condyloma and Papillomas.

Transmission of papillomaviruses occurs by direct contact with infected tissue and, indirectly, by contact with infected objects. Infection is probably mediated through minor trauma to the skin, when the basal cells of the epithelium are exposed to virus.

On initial infection, papillomaviruses cause minor proliferations of epithelium and mucosal tissue, which are called warts or papillomas. Warts are benign, self-limiting, proliferative lesions which are monoclonal in origin, consist of localized epithelial hyperplasia and retain a defined boundary with an intact basal membrane. All of the normal layers of epithelium are represented within wart tissue, although several cellular changes may occur within these layers.

Vacuolated koilocytes are usually present within the granular layer, and cell keratinization and multinucleation are typical of cells infected with HPV (Meisels and Fortin, 1976).

Each of the cells within wart tissue contain the viral genome, but expression of viral genes is not uniform and very closely linked to the state of differentiation of the cells. The early genes are expressed in the cells of the lower epidermis and the viral genome only replicates in the basal cells. In the keratinizing layers of cells, both late gene expression and viral synthesis take place (Stoler *et al.*, 1990).

The human papillomaviruses cause a variety of clinical symptoms at different anatomical sites of the body. The major HPV types associated with skin lesions are HPV 1, which causes plantar warts of the feet (Heilman *et al.*, 1980), HPV 2 which causes common warts of the hands (Orth *et al.*, 1977), HPV 7 which causes butchers' warts (Orth *et al.*, 1981; Oltersdorf *et al.*, 1986) and HPV 3 and 10, which result in flat warts of the face and arms (Orth *et al.*, 1978; Kremsdorf *et al.*, 1983). Treatment of skin warts involves the local application of caustic agents, liquid nitrogen and in certain circumstances, interferon.

Infection of the respiratory tract with papillomaviruses can lead to the growth of laryngeal papillomas. The site of initial infection is usually the transformation zone, between the squamous and ciliary epithelium, which is analogous to the transformation zone of the uterine cervix. The HPV types which are most common in the larynx are HPV 6, 11 and 30, with 11 being the most prevalent type. Papillomaviruses can also spread to the trachea and lung where growth of the papillomas can lead to obstruction of the air passages. Surgical

removal of the growths is necessary after which recurrence of the papillomas is common, although development of severe dysplasia and carcinoma is rare. Papillomas may also occur in the nasal passages, but tumours at this site are very rare and usually associated with HPV 16 or HPV 18 (Furuta, 1990). There is a greater variation in the type of HPV associated with lesions of the oral mucosa. Focal epithelial hyperplasia is caused by HPV 13 and 32, whereas squamous papillomas, condyloma acuminatum and verruca vulgaris contain types 4, 6, 11, 16 and 18. Warty lesions of the lips are usually associated with HPV 2, and HPV 7 is most frequent in oral warts of HIV positive patients (Greenspan *et al.*, 1988)

Lesions of the conjunctiva and cornea of the eye are also rare, but are associated with HPV 6 and 11, except for dysplastic lesions and carcinoma of the conjunctiva which usually contain HPV 16 (McDonnell *et al.*, 1989).

There are a large number of human papillomaviruses which infect the genital tract and these are listed in Table 1.1. The benign lesions caused by genital papillomaviruses are condyloma acuminatum and non-condylomatous wart virus infection (NCWVI) (Laverty *et al.*, 1978) which are also known as flat condyloma (Meisels *et al.*, 1977) or subclinical papillomavirus infection (Reid *et al.*, 1982). These lesions are indistinguishable from low grade CIN under colposcopic analysis (Walker *et al.*, 1983b). In men, condyloma usually occur on the penis, anus, perineum and very rarely on the scrotum, whereas in women condyloma are found on the vagina, vulva, perineum, anus and cervix. The papillomavirus types which are most strongly associated with condyloma, NCWVI and mild dysplasia are HPV 6 and 11 (McCance *et al.*, 1985a; Burk *et al.*, 1986; Toon *et al.*, 1986). HPV 16 and 18 are also frequently detected in cervical

samples, but are usually associated with higher grade CIN lesions and are found in only 10-30% of all condyloma acuminatum specimens (Campion *et al.*, 1986; Shirasawa *et al.*, 1986).

1.18 Epidermodysplasia Verruciformis.

Epidermodysplasia verruciformis (EV) is a rare, familial disease in which genetic factors are involved. The nature of the genetic defect is as yet unknown, but is believed to be associated with a homozygous autosomal recessive gene which results in an immunological malfunction. All patients with the disease exhibit a marked inability to resolve wart virus infection and some have symptoms of mental retardation. The wart-like lesions, which are prolific in these patients, are mainly found on the face, trunk and hands. Both flat warts and red macular plaques are frequently seen in EV patients and tend to develop into confluent lesions covering the skin. However, common warts and genital warts are generally rare.

The most common types of HPV detected in the lesions of EV patients are 5, 8, 17 and 20, although there are large numbers of rare HPVs found only in EV patients (Orth, 1987; Ostrow *et al.*, 1982; Kremsdorf *et al.*, 1984) and these are listed in Table 1.1. Progression of macular plaques to malignancy is frequent, but only in association with HPV types 5, 8 and 17 and in the areas of skin which are exposed to sunlight. However, these tumours are usually non-metastasizing and slow growing.

Table 1.1. Anatomical sites of human papillomavirus infection and the HPV types most frequently detected in associated lesions.

SITE OF LESION	HPV TYPE
Skin Lesions	1, 2, 3, 7, 10
Larynx	6, 11, 30, 31
Nasal	6, 11, 16
Oral	2, 4, 6, 11, 13, 16, 18, 32
Conjunctiva/cornea	6, 11, 16
Genital: Benign associated	6, 11, 34, 40, 42, 43, 44, 53, 54, 55, 57, 58, 59
Genital: CIN/Cancer associated	16, 18, 30, 31, 33, 35, 39, 45, 51, 52, 56
Epidermodysplasia verruciformis (EV)	5, 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, 46, 47,

1.19 Cervical Intraepithelial Neoplasia and Cervical Carcinoma.

Cervical carcinoma has been defined as a sexually transmitted disease (Kessler, 1976; zur Hausen, 1977) which is monoclonal in nature (Lehn *et al.*, 1985) and takes between 10 and 40 years to develop. There are approximately 500,000 new cases of invasive carcinoma of the uterine cervix per year worldwide (Peto, 1986) and over 4,800 deaths annually in the USA. Cervical cancer is most frequent in developing countries, accounting for 24% of all cancers in women. In developed countries, squamous cell carcinoma accounts for over 85% of cervical cancers and 7% of all cancers in women. In 1910, Rubin described the neoplastic changes found in cervical epithelium and suggested that these may be the precursor of invasive carcinoma. The abnormal cells were first termed carcinoma *in situ* (Broders, 1932) and later dysplasia (Reagan *et al.*, 1953), which was defined as lesions in which part of the epithelium was replaced with cells showing atypia (Poulsen *et al.*, 1975). The current concept of cervical disease is of a lesional continuum and cervical intraepithelial neoplasia (CIN) has now been introduced as a collective term encompassing both dysplasia and carcinoma *in situ* of the cervix (Richart *et al.*, 1967). There are three stages of cervical disease as defined by histological criteria; preinvasive, microinvasive and invasive carcinoma. Pre-invasive disease encompasses CIN which develops in the epithelium of the transformation zone of the cervix in the region of the squamocolumnar junction. In young women the squamocolumnar junction is in the vicinity of the external *os* or on the endocervical surface, whereas in older women it is usually within the endocervical canal. The neoplastic cells within CIN lesions possess varying degrees of differentiation including undifferentiated basal and parabasal cells. The

cell growth within the lesion is disorganized and the mitotic activity is altered and no longer confined to the basal layers. The nuclei of the undifferentiated cells are large and irregular with a high chromatin content and the nucleo:cytoplasmic ratio is considerably higher than that of normal cells. CIN is separated into three grades by histological criteria according to the proportion of epithelial thickness occupied by the basal and parabasal neoplastic cells and the degree of nuclear abnormality (Buckley *et al.*, 1982; Ferenczy, 1982). Lesions in which less than 1/3 of the epithelium is affected are graded CIN 1 or mild dysplasia, those with between 1/3 and 2/3 affected are graded CIN 2 or moderate dysplasia and those with greater than 2/3 of the epithelial layers affected are called CIN 3 or severe dysplasia.

Cytological criteria for the designation of severity of cervical disease is based on nuclear abnormalities such as enlargement, variation in size and shape, hyperchromasia and clumping of chromatin, multinucleation and the presence of mitotic figures. The collective term for the nuclear abnormalities is dyskaryosis (Evans *et al.*, 1986), which is also separated into three grades of severity; mild moderate and severe. The grade of dyskaryosis does not necessarily correspond with the grade of CIN diagnosed for the same lesion. Treatment of pre-invasive disease is carried out using electrodiathermy, in which the lesion is destroyed by heat, or by carbon dioxide laser ablation of the lesion. In severe cases surgery may be necessary.

Microinvasive cancer is defined as a tongue of malignant cells pushing into the stroma beneath the epithelium in which no vascular or lymphatic vessels have been broken. In invasive carcinoma the cells have advanced to uncontrollable

growth and have breached the vascular and lymphatic vessels. The carcinoma cells are able to penetrate and invade the surrounding tissues and metastasize via lymph and blood systems to other organs where they may develop into secondary tumours. Methods for the treatment of invasive cancer include surgical removal of the tumour, hysterectomy and chemotherapy.

Cervical disease is presumed to progress from preinvasive, through microinvasive, to invasive carcinoma. Evidence for this sequence of events is derived from studies of women with cervical disease in which the age distribution is consistent with a progression from CIN to cancer (Meanwell *et al.*, 1987). Studies in which CIN patients were left untreated have shown that 57% of untreated CIN lesions will develop into carcinoma after 10 years (Harris and Peterson, 1956) and 19% will progress to cancer within 2 years (Kinlen and Spriggs, 1978). However, a proportion of CIN lesions will remain unchanged, or regress if left untreated (Reagen *et al.*, 1953; Fox, 1967; Richart and Barron, 1969; Spriggs, 1984; Evans and Monaghan, 1985; Weaver *et al.*, 1990). Unfortunately, the behaviour of a specific CIN lesion cannot be predicted from morphological data (Kottmeier, 1961; Fidler *et al.*, 1968).

1.20 The Association of HPV with Cervical Disease.

Considerable data have accumulated from both clinical and scientific research to indicate the involvement of some types of HPV in the aetiology of CIN and carcinoma. Cervical cancer behaves in the manner of a sexually transmitted disease (Kessler, 1976) and the risk factors associated with the development of CIN are identical to those for the transmission of HPV i.e. early

age of first coitus, history of venereal disease and low socioeconomic status (Rotkin, 1973; Herrero *et al.*, 1990; Ley *et al.*, 1991). Disease is rare in virgins, but occurs frequently in women who have multiple sexual partners or whose partner is promiscuous (Christopherson and Parker, 1965; Buckley *et al.*, 1981; Brinton *et al.*, 1986).

The presence of morphological changes such as koilocytes, keratinization and multinucleation, which are indicative of HPV infection, in large numbers of abnormal cervical smears, but few normal smears (Meisels and Fortin, 1976; Purola and Savia, 1977) led to the prediction of an association of HPV with cervical neoplasia (Meisels and Fortin, 1976). The presence of HPV in cervical lesions was also demonstrated by electron microscopy (Stanbridge *et al.*, 1981; Ferenczy *et al.*, 1981) and immunocytochemistry (Ferenczy *et al.*, 1981; Walker *et al.*, 1983a). HPV-induced lesions are indistinguishable from low grade CIN by colposcopy (Walker *et al.*, 1983b) and during culture of epithelial tissue in raft type systems, which allows stratification and differentiation to take place, transfection with HPV 16 leads to inhibition of differentiation consistent with CIN (McCance *et al.*, 1988) and development of cellular morphology similar to that of premalignant lesions (Blanton *et al.*, 1991).

Nucleic acid hybridization and DNA amplification techniques have both been used to determine the prevalence of HPV in different populations. The advantage of these methods is that they are able to distinguish the type of HPV present in a given lesion. There are many types of HPV which infect the genital tract, but not all of these are associated with cervical carcinoma lesions. HPV 6 and 11 are found in 20-65% of benign condyloma acuminatum, (Gissmann *et al.*,

1983; Crum *et al.*, 1985; McCance *et al.*, 1985a; Burk *et al.*, 1986; Campion *et al.*, 1986; Fuchs *et al.*, 1987) and in fewer than 30% of cancerous lesions (Gissmann *et al.*, 1983; Wagner *et al.*, 1984; McCance *et al.*, 1985a). The converse is true of HPV 16 and 18 which are found in 50-90% of high grade CIN lesions and 80-100% of malignant lesions (Durst *et al.*, 1983; McCance *et al.*, 1985b; Meanwell *et al.*, 1987; Webb *et al.*, 1987; Cornelissen *et al.*, 1989; van den Brule *et al.*, 1989, 1991; Young *et al.*, 1989; Riou *et al.*, 1990; Fujinaga *et al.*, 1991), but in only 5-38% of low grade CIN samples (Campion *et al.*, 1986; Cox *et al.*, 1986; Shirasawa *et al.*, 1986; Meanwell *et al.*, 1987; Kyo *et al.*, 1991). HPV 16 is the most common of the two viruses, but HPV 18 appears have a greater malignant potential than HPV 16 (Barnes *et al.*, 1988; Kurman *et al.*, 1988).

The genes which confer this oncogenic potential to HPV 16 and 18 are the E6 and E7 ORFs which have been described in sections 1.12 and 1.13. E6 and E7 are the major viral genes to be transcribed in carcinoma cell lines and elevated levels of these proteins have been detected in malignant tumours. These findings support the hypothesis that HPV induces malignant conversion by the action of the E6 and E7 oncoproteins. Human cervical and foreskin cells which have been immortalized by HPV exhibit dysplastic differentiation and the morphology of premalignant cells when analysed in raft cultures (Sedman *et al.*, 1991) or transplanted into athymic mice (Woodworth *et al.*, 1990a). The transformation potential of HPV 16 E6 and E7 proteins *in vivo* has been demonstrated by the development of transgenic mice in which the E6 and E7 genes were expressed. Testicular enlargement and tumour formation were frequent in the male offspring of transgenic lineage, but no tumour development was observed in the female

offspring (Kondoh *et al.*, 1991).

However, cancer is a rare consequence of infection with HPV and the disease has a very long latency period of 10-40 years. It must therefore be recognized that infection of the uterine cervix with HPV alone may be insufficient to cause the development of cervical cancer and other agents and molecular events are probably necessary to alter normal cell growth to malignancy.

1.21 Co-factors Involved in Carcinogenesis with HPV.

There are many co-factors which may play a role in the development of carcinoma and there are two mechanisms by which these factors may act. The first is by direct action on the general state of the cell, leading to malignant changes and the second is by indirect action on the papillomavirus affecting the expression and regulation of viral genes and enhancing the transformation process.

The group of agents which act directly on the cell includes carcinogens and mutagens which affect normal cellular mechanisms. Smoking has long been considered to be a factor in the development of cancers, particularly squamous cell carcinoma, and Winkelstein (1977) suggested that cigarette smoking may be a cause of cervical squamous carcinoma. This is supported by several studies demonstrating a significant association of cigarette smoking with carcinoma and dysplasia of the cervix (Clarke *et al.*, 1982; Trevathan *et al.*, 1983; Barton *et al.*, 1989) This association is still valid after adjustment for age, number of sexual partners, age at first coitus and oral contraceptive use. The relative risks of carcinoma and severe dysplasia in women who smoked, with respect to those who never have, were 3.6 and 3.3 and the relative risk increases with cumulative

exposure to smoke. A 5-10 times greater risk of cervical cancer was found in women with either HPV 16 or 18 who smoked, compared with those who only had the viral infection (Trevathan *et al.*, 1983). There is also a comparable association of higher incidence of oral carcinoma in those with HPV infection who smoke or chew Betal Quid (Chang *et al.*, 1989).

Sunlight is also known to be associated with the development of cancers and it is the UV component which causes mutations in cellular DNA, leading to malignancy. Patients with EV are prone to develop skin cancers, but only in those HPV-containing lesions which are exposed to sunlight (Orth, 1987). UV irradiation may be another independent factor in the development of HPV associated lesions, but is unlikely to be involved in any other tumours than those of EV patients. In the 1940s, laryngeal papillomas were irradiated with X-rays in an attempt to reduce growth of these tumours, but instead accelerated the development of carcinoma, thus demonstrating a similar effect to UV irradiation on papillomavirus associated lesions (Galloway *et al.*, 1960).

Chemical carcinogens may also be co-factors in the development of papillomavirus associated cancers. Shope papillomavirus lesions in rabbits can be accelerated to cancer by application of coal tar or methylcholanthrene to benign lesions (Rous and Kidd, 1936). Cattle in Scotland, which feed on bracken fern, are more susceptible to carcinoma of the alimentary canal due to a radiomimetic substance present in bracken, which acts synergistically with BPV-4 to cause malignancy (Jarrett, 1980). Bracken fern also contains immunosuppressants which allow increased growth of BPV in cattle.

An important factor in the development of any type of cancer is the

genetic composition of the individual, which can influence the rate and extent of malignancy. For example, CRPV is associated with naturally occurring cancers in cottontail rabbits, but the frequency of progression to squamous carcinoma is higher in domestic rabbit (Rous and Beard, 1935). The T-cell response is also important in controlling papillomavirus infection and there is a high incidence of HPV infection and associated disease in immunocompromised hosts. Recently, a link between the human leucocyte antigen (HLA) genes and squamous cell carcinoma has been found. The HLA DQw3 allele is only expected to be present in 50% of the general population, but is found in 88% of Caucasian patients with squamous cell carcinoma of the cervix. In the control groups of this study the prevalence was 41-50%. The relative risk of cervical disease in women with the DQw3 allele was 7.1 (Wank and Thomssen, 1991).

There are several factors which have been found to interact with the HPV genome to cause an increase in the expression of E6 and E7 transforming genes and thus, malignant development. The steroid hormones have been found to act in such a manner (Crook *et al.*, 1988; Chan *et al.*, 1989). Oral contraceptive users have a significantly higher incidence of cervical cancer as compared with those who have never used them (Vessey *et al.*, 1983), although the former have a lower incidence of endometrial and ovarian cancer (Beral *et al.*, 1988). There is also an increase in the prevalence of HPV and a faster development rate of malignancy during pregnancy when levels of the hormone progesterone are elevated. Glucocorticoid responsive elements (GREs) have been found within the LCR of HPV and these elements are responsive to both progesterone and glucocorticoid hormones, e.g. dexamethasone, and have been shown to regulate expression of the

E6 and E7 genes of HPV 16, 18 and 11 (Gloss *et al.*, 1987; Chan *et al.*, 1989). Oestrogen has also been shown to increase expression of E6 and E7 in SiHa cells (Mitrani-Rosenbaum *et al.*, 1989). Binding of steroid hormone to the GRE is necessary for the *in vitro* transformation of primary cells with HPV 16 and activated *ras* oncogene (Pater *et al.*, 1988) and the transforming activity of HPV 16 in this assay system is inhibited by the hormone antagonist RU486 (Pater and Pater, 1991). Increased levels of progesterone lead to elevated expression of the transforming genes E6 and E7 which may be important in the development of cervical carcinoma. The effects of progesterone may also provide a molecular explanation for the higher frequency of HPV lesions and associated cancer in women than men, since women have very high levels of progesterone during pregnancy and at specific times during the ovulation cycle.

It is also apparent that oncogenes can interact with HPV to transform cells *in vitro*. The activated *ras* (EJ c-Ha-*ras*) and v-*fos* oncogenes are able to cooperate with the E6 and E7 genes of HPV 16 in the presence of steroid hormones to transform baby rat kidney cells (Crook *et al.*, 1988; Pater *et al.*, 1988; Storey *et al.*, 1988). Further analysis has shown that it is only HPV 16 and 18 which are able to co-operate with oncogenes to induce transformation (Crook *et al.*, 1989). The c-*myc* oncogene does not interact with HPV in this assay (Storey *et al.*, 1988), but appears to have a marked effect on cells containing HPV in the development to malignancy (Crook *et al.*, 1989). This is due to the direct effect of c-*myc* increasing the growth rate of transformed cells. A role for c-*myc* and Ha-*ras* as co-factors in the development of cervical disease is supported by the discovery of rearrangements and amplification of these oncogenes in CIN and carcinoma

samples (Ocadiz *et al.*, 1987; Pinion *et al.*, 1991).

Prior to HPV being considered as the etiological agent associated with cervical disease, other microorganisms were investigated as possible candidates including *Trichomonas vaginalis, Chlamydia trachomatis* and herpes simplex virus type 2 (HSV-2) (Bertini and Hornstein, 1971; Alexander, 1973; zur Hausen, 1982; Woods *et al.*, 1984; Prakesh *et al.*, 1985). Currently, HPV is believed to be involved in the initiating process, but additional agents are being considered as possible co-factors in the development of cervical disease. DNA amplification analysis for the detection of Epstein-Barr virus (EBV) and HSV-2 has been carried out on women with cervical abnormalities. All patients studied were infected with HPV, 30% were infected with HSV-2 and 33% were infected with EBV DNA. A further 11% were infected with all three virus types (Bevan *et al.*, 1989). Serological analysis for HSV has also been carried out and women with cervical disease have a higher prevalence of antibodies against HSV-2 when compared with women with no cervical disease (Rawls *et al.*, 1976, 1980; Hildesheim *et al.*, 1991). The presence of HSV-2 or EBV infection in women with cervical disease may be coincidental, or these viruses may be assisting in the development of cervical neoplasia by interaction with HPV. HSV has been shown to have oncogenic potential in rodent cells *in vitro* and has *trans* inducing factors (TIF) which can activate non-HSV promoters. Several of these have been tested for ability to activate HPV 18 LCR and the TIF and infected cell protein 0 (ICP0) of HSV-1 are both able to function in this assay, whereas ICP4 and ICP27 cannot (Gius and Laimins, 1989). Only *trans*activation by the oral type HSV-1 have been analysed, but since the activators of HSV-1 and HSV-2 are

interchangeable in other assays, it is believed that TIF and ICP0 of genital HSV-2 would function in the same manner.

HPV induced cervical and anogenital lesions are more common and aggressive in human immunodeficiency virus (HIV) positive patients and are more likely to recur (Feingold *et al.*, 1990) indicating that HIV may be able to *trans*activate HPV expression (Matorras *et al.*, 1991). However, the *tat* genes of human T-cell leukaemia virus (HTLV-1) and HIV have not been found to interact with HPV in this manner. The higher prevalence of disease in HIV patients is likely to be the result of the HIV induced immunosuppression which exacerbates HPV mediated cellular abnormalities (Feingold *et al.*, 1990) and increases the risk of disease (Palefsky *et al.*, 1991b).

Interaction of any possible co-factors with HPV and cellular mechanisms is complex, but a simplified model of the synergism between HPV and additional factors in the development of cervical cancer has been proposed (zur Hausen, 1982, 1991). HPV is presumed to be the initiating event altering the cell growth to cause the development of papillomas and CIN 1. At this stage the immune system may intervene to cause the lesion to regress, or further mutagenic events may take place causing progression to CIN 2 or 3. At these later stages the lesion may still regress, but this event is less likely as the severity of disease increases and additional factors may be necessary for development of the lesion to carcinoma.

AIMS OF THE PROJECT.

The aim of this study is to investigate further the association between human papillomaviruses and cervical disease and to determine whether HPV 16 may be of use in the identification of women with cervical disease. There is considerable epidemiological data available indicating that HPV is associated with cervical disease. However, there are shortcomings in the design of many of the studies, which include insufficient information concerning the source of cases within a study, small study populations and variations in cervical sampling methods. Clinical diagnosis is often based solely on cytological analysis, which is associated with a high false negative rate. There is also enormous variation in the techniques used to detect HPV DNA in cervical samples and differences in sensitivity of the methods used.

The initial approach of the project is to compare the current techniques for detection of HPV DNA and develop an assay system for HPV 6, 11 and 16, which is sufficiently sensitive to detect HPV DNA sequences in cervical scrape samples and specific enough to distinguish between the different papillomavirus types. This will then be used to examine the prevalence of HPV 16 in women with and without disease.

The results of several studies have suggested that detection of HPV 16 may be useful as an indicator of women with cervical disease, but the value of detecting HPV has not been examined in suitable screening populations. Therefore, two clinical studies have been designed to address some of the problems encountered in other epidemiological studies. The first study consists of women from a General Practice population who volunteered for cervical

screening. The results of this study will allow the prevalence of HPV 16 in women with no cervical abnormalities to be determined and the use of HPV 16 as an indicator of cervical disease in the General Practice population to be evaluated. The second study consists of women sequentially attending the Royal Free Hospital colposcopy clinic with a smear report suggesting mild dyskaryosis. The results will be used to assess the association of HPV with cervical disease and the use of HPV 16 as an indicator of women with severe disease who may require urgent treatment.

In addition to the clinical analysis, the project includes an investigation into sequence divergence within the LCR of HPV 16. The HPV 16 LCR will be cloned from cervical scrape samples from women with and without cervical disease and the sequences determined and compared with the prototype HPV 16 sequence. Nucleotide variations between the isolates will be identified, particularly those within defined protein binding sequences which may affect viral transcription. The functional significance of any changes and the possible role of additional factors, such as steroid hormones and viral transactivators, will be analysed using a chloramphenicol acetyltransferase expression assay system.

CHAPTER 2: DEVELOPMENT OF A NUCLEIC ACID HYBRIDIZATION METHOD FOR THE DETECTION OF HPV DNA IN CLINICAL MATERIAL.

INTRODUCTION.

2.1 Nucleic Acid Hybridization.

The double helical structure of DNA allows the two strands to separate without disrupting covalent bonds, and to reform at a rapid rate under suitable conditions. This feature makes it possible for DNA to be manipulated *in vitro* by denaturation, using either heating to temperatures of 95°C and above, or by the addition of alkali. Both methods work by destroying the inter-strand hydrogen bonds. This effect is easily reversed by altering the conditions to those under which the DNA can re-anneal to form hydrogen bonded molecules. When nucleic acids from different sources are involved in this reaction, it is termed hybridization.

A measurement of the ease with which a DNA species can be denatured and re-annealed is represented by the Tm value. This is the temperature, or conditions, at which 50% of the molecules of a particular DNA species is single stranded and is characteristic of that DNA. Several factors affect the Tm of a duplex, e.g. the G+C content of the DNA, the sodium chloride concentration and the pH of the solution.

The G+C content of the DNA sequence affects the Tm by the increased hydrogen bonding capacity of guanine:cytosine bases, which have three potential hydrogen bonds, compared with two for thymine:adenine bases. The strands of a DNA species with a high G:C content will be less easily denatured due to the

greater energy required to break the extra bonds and will therefore have a higher Tm value. The G+C content of the DNA can only be regulated in hybridization analysis by selecting probes to an area of DNA with a high or low G+C content as required.

The Tm value of DNA is influenced by the concentration of sodium chloride, because the positively charged sodium ions neutralize the negative charge of the DNA backbone. Thus, an increase in the salt concentration reduces the repulsion forces between the two molecules and facilitates hybridization.

The temperature at which hybridization is carried out directly affects annealing of DNA molecules. At temperatures greater than the Tm value of the DNA species more than 50% of the molecules will be single-stranded and at temperatures below the Tm value, homologous DNA can re-anneal. However, temperatures which are much lower than the Tm of the duplex, allow annealing of heterologous DNA.

DNA hybridization is a powerful technique, which can be applied to the detection of DNA or RNA species of interest. This is achieved by labelling DNA with chemicals such as biotin, or radioisotopes e.g. ^{32}P, to produce probes which can identify any complementary sequences with which they can anneal. The denaturation and annealing of DNA molecules can be controlled by altering the conditions, or stringency, of the hybridization reaction. Under high stringency (between 10 and 20°C below the Tm value) only those molecules which are homologous to the probe DNA will be detected, whereas under lower stringency (between 30 and 40°C below the Tm value) non-homologous binding can occur and hence, allow the detection of related DNA species.

2.2 Detection of Human Papillomaviruses using Hybridization Methods.

There is currently no cell culture system for papillomaviruses, because the only cells which are permissive for HPV replication are fully differentiated keratinocytes (Ferenczy *et al.*, 1981; Pfister, 1984), which cannot easily be cultured *in vitro*. DNA hybridization has therefore been the most efficient technique for the detection of papillomavirus infection in clinical specimens. Since the human papillomaviruses share considerable sequence homology, high stringency conditions are necessary in order to distinguish between the viral types.

Hybridization studies on the prevalence of HPV infection in women are normally carried out on cervical biopsy material rather than on cervical scrape samples, since larger quantities of DNA are available from biopsy samples (Durst *et al.*, 1983; Boshart *et al.*, 1984; Crum *et al.*, 1985; McCance *et al.*, 1985b; Lorincz *et al.*, 1986; Meanwell *et al.*, 1987; Williamson *et al.*, 1989). Biopsy tissue can also be sampled specifically from the region of abnormality, when taken under colposcopic direction (Meanwell *et al.*, 1987; Hallam *et al.*, 1989), although it may be important for the detection of HPV to sample cells from the entire transformation zone (Cornelissen *et al.*, 1988; Hallam *et al.*, 1989). Biopsy sampling is an invasive technique and therefore, cannot be used for screening purposes (Vermund *et al.*, 1989). Hybridization methods would be of greater value when applied to cervical scrapes, since increased numbers of women could be investigated if the samples were taken at the time of a routine cervical smear test. This approach would also be more suitable for development as a diagnostic test should HPV be found to be of use as a prognostic indicator of cervical disease, as several authors have suggested (Burk *et al.*, 1986; Campion *et al.*, 1986; Fuchs

et al., 1987; Walker *et al.*, 1989). The problems associated with the analysis of cervical scrapes include the extraction of sufficient DNA from the specimen and attaining high enough sensitivity in the hybridization reaction in order to detect the HPV DNA, if present.

An alternative method of non-invasive tissue sampling is cervicovaginal lavage, where the cervix is washed with normal saline and the cells collected by aspiration (Burk *et al.*, 1986; Vermund *et al.*, 1989; Morris *et al.*, 1990). However, this technique samples cells from the entire cervicovaginal region, therefore HPV infection detected by cervicovaginal lavage alone may not be relevant to the cervical transformation zone (Vermund *et al.*, 1989). The quantity of tissue sampled in both scrape and cervicovaginal samples is smaller than from biopsies, but several studies have shown a good correlation of HPV detection in cervical scrapes with those in biopsies derived from the same patient (Burk *et al.*, 1986; McCance *et al.*, 1986). It has been suggested that scrapes may confer greater sensitivity than biopsies, if the exfoliated cells harbour greater quantities of viral DNA and hence, contain a higher HPV DNA: cellular DNA ratio (Cornelissen *et al.*, 1988).

Having considered the alternatives, cervical scrapes were the sampling method of choice for this project, because they provide samples specifically from the uterine cervix and are currently used for the routine screening of large numbers of women.

There are several different hybridization methods suitable for analysis of HPV DNA in clinical specimens. The technique which has been most widely used is Southern blot hybridization, in which the DNA is digested with restriction

enzymes, electrophoresed in agarose gels and the DNA transferred to nylon membranes. The sample DNA is then probed with radiolabelled DNA to detect homologous sequences. This has been shown to be more sensitive than other methods (Schneider, 1987; Schneider *et al.*, 1987; Caussy *et al.*, 1988; Brandsma *et al.*, 1989), gives high specificity (Crum *et al.*, 1987) and can provide additional information on the state of the HPV DNA within the lesion, i.e. whether the DNA is episomal or integrated into the host cell genome (Durst *et al.*, 1985; Lehn *et al.*, 1985, 1988; Shirasawa *et al.*, 1986; Wagatsuma *et al.*, 1990; Cullen *et al.*, 1991). Differentiation of the viral types can also be accurately performed by restriction analysis of the sample DNA prior to electrophoresis (Crum *et al.*, 1985; McCance *et al.*, 1985a; Brandsma *et al.*, 1989). The major disadvantage associated with Southern blotting is the length of time required to complete all of the processes involved.

Dot blot hybridization is an alternative method, in which the length of time required to complete the procedure is reduced by spotting the DNA directly onto membranes. A method of dot blotting has been described in which the DNA is spotted into an agarose gel prior to transfer to a membrane by the standard Southern technique (Wickenden *et al.*, 1985). However, any advantages conferred by the latter process have not been substantiated. The standard dot blot system is quicker and easier to perform than Southern blot, but DNA must still be purified from tissue specimens and, after hybridization, very high background signals may result in false positive results (Cornelissen *et al.*, 1988; Morris *et al.*, 1990).

An alternative technique, in which the DNA extraction process is

unnecessary, is filter in situ hybridization (FISH), because the cervical cells are filtered directly onto the membrane. This does not reduce the non-specific signals and the sensitivity of the system is highly variable, with certain studies demonstrating greater sensitivity than Southern blot hybridization (Caussy *et al.*, 1988; Melchers *et al.*, 1989a) and others lower sensitivity than dot blot (Cornelissen *et al.*, 1988; Morris *et al.*, 1990). The problem of interpretation of results with this technique, including the inability to determine whether intermediate signals are due to non-specific hybridization, or low copy number of virus genomes, is acknowledged by several authors (de Villiers *et al.*, 1987; Caussy *et al.*, 1988; McNicol *et al.*, 1989; Schneider, 1987).

Two recently developed alternatives to Southern blotting have been described for use in detecting HPV sequences. The first is reverse blotting, where labelled cellular DNA is used to hybridize a range of membrane-bound HPV samples (Webb *et al.*, 1987). The second is sandwich hybridization whereNA sample DNA and radiolabelled HPV DNA are combined in the hybridization solution, which is used to probe additional recombinant HPV DNA attached to a membrane. The HPV sequences are non-homologous and therefore a signal is only obtained when HPV DNA is present in the sample to bind to the radiolabelled DNA and then with the membrane-bound DNA (Ranki *et al.*, 1983; Parkkinen *et al.*, 1986). The latter methodologies were designed to increase the sensitivity of HPV detection, but are unsuitable for the detection of large numbers of samples, because only one sample can be analysed in any one hybridization reaction.

An additional method for detecting DNA sequences of interest is *in situ*

hybridization. However, this is a lengthy process requiring biopsy material and is much less sensitive than the alternative methods (Crum *et al.*, 1986; Nagai *et al.*, 1987). The major use for this technique is in providing information concerning the specific cells in which the virus is present (Burns *et al.*, 1987; Nagai *et al.*, 1987; Caussy *et al.*, 1988).

Southern blot hybridization is the most sensitive and specific technique available and was therefore considered to be the most accurate method for analysis of HPV infection. However, the processes involved take considerably longer than other available techniques, such as dot blot hybridization which is a more suitable method for development as a diagnostic test for HPV. Since no single method fulfilled all of the requirements for an accurate and rapid test for detection of HPV DNA, both the dot blot and Southern blot techniques were developed in order to compare their ability to detect HPV sequences in cervical scrape samples. The development of these techniques is described and their relative merits discussed.

2.3 Sample Collection and Preparation.

Cervical scrapes were obtained by rotating an Ayre spatula through 360° over the transformation zone of the uterine cervix of each patient. The spatula was placed in 10ml of cold phosphate buffered saline (PBS) and stored at 4°C. All samples were processed, as follows, within 24 hours of taking the scrape. The cells were dislodged from the spatula by vortex and, after removing the spatula, the cells were collected by centrifugation at 1700g for five minutes. The resulting cell pellet was washed twice with 5ml of PBS, resuspended in 100-200µl of PBS and stored at -70°C until required for analysis.

The number of cells obtained by this method was estimated in a random sample of 40 scrapes. After the cells were dislodged from the spatula, and prior to the centrifugation step, 20µl of the cell suspension were mixed with 20µl of 4% trypan blue and the cells counted using a Neubauer Haemocytometer.

2.4 DNA Extraction.

The frozen cervical scrape samples were thawed on ice and the cells centrifuged for 60 seconds at 13 000 rpm in a microcentrifuge. The supernatant was discarded and each pellet resuspended in 500µl of 50mM Tris.HCl pH 7.5, 10mM ethylenediamine tetra-acetic acid (EDTA) pH 8.0, 50mM NaCl, 2% sodium dodecyl sulphate (SDS). Proteinase K was added to a final concentration of 100µg/ml and the samples incubated at 37°C for 4-16 hours. The DNA was purified with an equal volume of phenol mix (75% phenol, 10% m-cresol, 15% chloroform, 0.05% 8-hydroxyquinoline, equilibrated with 0.5M Tris.HCl pH 8.0)

and then with an equal volume of a 25:24:1 (v:v:v) mixture of phenol:chloroform:isoamylalcohol. The phenol:chloroform extraction was repeated until no proteinaceous material could be seen at the phase interface. The DNA was precipitated by the addition of 1/10 volume of 3M sodium acetate and 2-3 volumes of 100% ethanol, followed by incubation at -70°C for a minimum of one hour. The DNA was collected by centrifugation for 20 minutes in a microcentrifuge at 13 000 rpm, washed with 70% ethanol and vacuum dried. The DNA from each sample was resuspended in 100µl of TE buffer (10mM Tris.HCl pH 8.0, 1mM EDTA) and stored at -70°C.

The absorbance (A) of the DNA solution from each sample was measured at 260nm using a UV spectrophotometer and the DNA concentration and yield calculated using the standard equation of, $A_{260} = 1 = 50\mu g/ml$ of double stranded DNA. To obtain an estimate of the purity of the samples, the absorbance at 280nm was also measured and ratio between the readings at 260nm and 280nm (A_{260}/A_{280}) calculated. Pure preparations of DNA have a ratio of between 1.8 and 2.0 and any samples with a lower value were considered impure and were subsequently repurified as before.

2.5 Dot Blot Hybridization.

The DNA samples (1-5µg) to be analysed by dot blot hybridization were diluted to a final volume of 10-100µl with TE and heated to 95°C for five minutes. The samples were chilled on ice and applied to a nylon membrane (Hybond-N, Amersham) using a micropipette or minifold dot blot apparatus (Schleicher and Schuell) and air dried. The membrane was floated on denaturing

solution (1.5M NaCl, 0.5M NaOH) with the DNA uppermost, for 5-10 minutes at room temperature. The membrane was then transferred in the same manner to neutralizing solution (1.5M NaCl, 0.5M Tris.HCl pH 7.2, 0.001M EDTA) for 5-10 minutes. The membrane was air dried, wrapped in cling film and UV irradiated for 2-5 minutes to bind the DNA.

2.6 Agarose Gel Electrophoresis.

Prior to gel electrophoresis, the cervical scrape DNA was digested with *Eco*R1 restriction endonuclease in 1 x high salt buffer (6mM Tris.HCl pH 7.4, 6mM $MgCl_2$, 150mM NaCl, 0.1mM dithiothreitol), or with *Bam*H1 restriction endonuclease in the presence of 1 x medium salt buffer (6mM Tris.HCl pH 7.4, 6mM $MgCl_2$, 50mM NaCl, 1mM dithiothreitol) in a final volume of 20µl and incubated at 37°C for 2-4 hours.

Agarose gels were made by dissolving an appropriate quantity of powdered agarose in 1 x TBE (0.089M Tris, 0.089M boric acid, 0.002M EDTA) using a microwave oven. When the agarose solution was cooled to 50°C, ethidium bromide was added to a final concentration of 0.1µg/ml. The agarose was poured into a sealed frame and allowed to set for a minimum of 30 minutes. After setting, the well-forming comb was removed and the gel placed in an electrophoresis tank and covered with 1 x TBE. Loading buffer (1/6 volume; 30% glycerol, 0.1% bromophenol blue dye) was added to each sample, which were applied to the gel and electrophoresed for 30-180 minutes with a constant voltage of 30-100 volts. The DNA was visualized using a UV transilluminator and photographed if required.

2.7 Southern Transfer of DNA to Nylon Membranes.

After electrophoresis, the agarose gel was immersed in 200ml of denaturing solution and incubated for 15 minutes with gentle shaking. The buffer was replaced with fresh denaturing solution and incubated for a further 30 minutes. This procedure was repeated with neutralizing solution. Both the denaturing and neutralizing solutions are described in section 2.5. When transferring high molecular weight DNA, the gel was incubated at room temperature in depurination solution of 0.25M HCl, prior to denaturation.

The DNA transfer apparatus was formed as described by Southern (1975). A filter paper wick was placed over a glass plate with the ends dipping in a buffer reservoir. The agarose gel was placed on the filter paper with a nylon membrane, cut to the same size as the gel, on top of the gel. Three pieces of 3MM filter paper and a stack of absorbent towels, 5-6cm in height, were placed on top of the membrane the whole apparatus compressed with a 1kg weight. The buffer reservoir was filled with 20 x SSC (3M NaCl, 0.3M tri-sodium citrate) or phosphate transfer buffer (0.5M sodium di-hydrogen orthophosphate, 0.5M di-sodium hydrogen orthophosphate pH 6.5) and the transfer allowed to proceed for 16-24 hours.

At the end of the transfer the membrane was washed in 2 x SSC, air dried and wrapped in cling film. The DNA was bound to the membrane by exposure to UV irradiation as described in section 2.5.

2.8 Preparation of Cloned HPV DNA.

Clones of HPV 6, 11, 16 and 31 were obtained in vectors pAT153, pSV2,

pAT153 and pBR322, respectively. Each plasmid had previously been transformed into *E. coli* cells and a single, plasmid-containing colony of each HPV type was inoculated into 5ml of luria broth (LB) (0.5% NaCl, 0.5% yeast extract, 1.0% tryptone w/v) with 50μg/ml filter-sterilized ampicillin and incubated at 37°C for 20 hours, with shaking. The cultures were then inoculated into 500ml of LB with ampicillin and incubated as before.

The cells were harvested by centrifugation at 2 500g for five minutes and the supernatant discarded. The cells were then resuspended in 10ml of solution I (50mM glucose, 25mM Tris.HCl pH 8.0, 10mM EDTA, 5mg/ml lysozyme) and incubated at room temperature for five minutes. To this were added 20ml of solution II (0.2M NaOH, 1% SDS) and the solutions gently mixed and incubated on ice for five minutes. Solution III (15ml; 60ml 5M potassium acetate, 11.5ml glacial acetic acid in 100ml final volume) was added, mixed vigorously and incubated on ice for a further five minutes. The precipitated debris was removed by centrifugation at 1 700g for five minutes and the supernatant filtered through sterile gauze. To precipitate the DNA, propan-2-ol (0.6 volumes) was added and incubated at room temperature for 15 minutes, after which, the DNA was collected by centrifugation at 3 600g for ten minutes. The pellet was washed with 70% ethanol and dried in a vacuum desiccator.

2.9 Caesium Chloride Gradient.

The caesium chloride gradients were made by resuspending the plasmid DNA in 8ml of TE buffer in 14ml polycarbonate tubes. CsCl (8.48g) and ethidium bromide (480μl) were added to a final concentration of 1g/ml of CsCl and

600μg/ml of ethidium bromide and the remaining volume of each tube filled with paraffin oil. The gradients were centrifuged at 140 000g for 48 hours in a MSE 65 ultracentrifuge. The covalently closed circular plasmid bands, which were visible under natural light, were collected using Pasteur pipettes and the ethidium bromide extracted with 80% propan-2-ol saturated with CsCl, after which the DNA samples were dialysed overnight against TE buffer.

The absorbance of each HPV DNA solution was measured at 260nm as described in 2.4 and the concentration calculated.

2.10 Purification of HPV DNA from Vector Sequences.

Plasmid DNA (20μg) was digested with the relevant endonuclease to remove the HPV DNA insert from the vector (see Table 2.1). This was carried out in 1 x medium salt buffer and incubated at 37°C for three hours. After one hour, a 0.5μg aliquot of each digest was electrophoresed on a 0.7% agarose gel to confirm the size of each vector and insert and to ensure the digest had gone to completion.

The remaining digested DNA was electrophoresed on a 0.7% low melting point agarose gel as described in 2.6, except that 1 x TAE buffer (0.04M Tris-acetate, 0.001M EDTA) was used. The regions of the gel containing the HPV DNA were excised and the DNA purified using Geneclean (Stratagene, USA) according to the manufacturers' instructions. Briefly, sodium iodide solution (3 volumes) was added to each gel section and the mixture incubated at 55°C for ten minutes, or until all of the agarose had melted. Glassmilk (5μl) was added, the solution mixed and incubated at room temperature for five minutes to allow

binding of the DNA to the glassmilk. The glassmilk-DNA complex was centrifuged for one minute at 13 000 rpm in a microcentrifuge and the pellet washed three times, with NEW buffer (NaCl, Ethanol, Water). After the final wash, all traces of buffer were aspirated and the DNA eluted into 20-50µl TE buffer by incubation at 45°C for 2-3 minutes. The glassmilk was removed by centrifugation and the supernatant, containing the eluted DNA, retained.

After purification, a 0.5µg aliquot of each DNA preparation was examined by agarose gel electrophoresis to ensure that no contaminating vector DNA remained.

2.11 Radiolabelling of Probe DNA.

Radiolabelled nucleotide, deoxycytidine 5' [α-^{32}P] triphosphate, was incorporated into HPV DNA using the random primer method (Amersham, multiprime) as described by Feinberg and Vogelstein (1983). Between 50 and 200ng of HPV DNA was denatured by heating to 96°C for ten minutes and cooled on ice. To this, were added a mixture of hexamer primers, dATP, dGTP, dTTP and 50µCi of [α-^{32}P] dCTP; specific activity 100 000 MBq/mmol. One unit of the Klenow fragment of *E. coli* DNA polymerase I was added and the reaction incubated at 37°C for one hour. The radiolabelled DNA was purified using Geneclean as described in 2.10.

2.12 Hybridization of Radiolabelled DNA to HPV Sequences.

The membrane, from either Southern transfer or dot blot, was first wetted in 2 x SSC and incubated in 20ml pre-hybridization solution (6 x SSC, 5 x

Denhardts' solution [100 x Denhardts' = 2% bovine serum albumin, 2% ficoll, 2% polyvinylpyrrolidone], 0.5% SDS, 0.5mg denatured, sonicated salmon sperm DNA) for a minimum of one hour at 65°C. The membrane was then incubated in 20ml of hybridization solution, identical to the pre-hybridization solution, but to which had been added the denatured, radiolabelled HPV DNA. The membrane was incubated for 16-20 hours at 65°C and, following hybridization, washed with 20ml of 2 x SSC, 1% SDS at 42°C for 30 minutes. The stringency wash was repeated twice and the membrane wrapped in cling film and exposed to X-ray film for 1-48 hours in a cassette with intensifying screens. If high stringency conditions were required, the membrane was washed in 20ml of 2 x SSC, 0.1% SDS, and then in 0.1 x SSC, 1% SDS at 65°C for 30 minutes and autoradiographed as before. The film was developed in an automatic X-ray film processor.

2.13 Removal of Radiolabelled DNA from Nylon Membranes.

To remove radiolabelled DNA prior to re-hybridization, membranes were incubated at 45°C in 10ml of 0.4M NaOH for 30 minutes and then transferred to 20ml of 0.1% SDS, 0.1 x SSC, 0.2M Tris.HCl pH 7.5 for a further 30 minutes. After incubation, each membrane was wrapped in cling film and stored at 4°C.

2.14 Determination of the Sensitivity of Hybridization for HPV DNA.

The sensitivity of the two hybridization methods was determined by titrating cloned HPV 16 DNA from 1ng to 5pg on both dot and Southern blot. The two membranes were hybridized with radiolabelled HPV 16 DNA under high stringency conditions and the lowest quantity of HPV DNA detectable was

determined from the autoradiograph.

2.15 Modification of the Hybridization Protocol for Detection of HPV DNA.

Labelling of HPV DNA.

The quantity of DNA labelled in each reaction was reduced from 50-200 ng to 20-50ng and the incubation period extended to 16 hours at room temperature. Sephadex chromatography was used to purify the radiolabelled DNA, instead of the Geneclean process. The sephadex column was made by plugging the end of a Pasteur pipette with glasswool and filling the shaft with Sephadex G-50 in TE buffer. The column was washed with TE buffer and the labelled DNA applied to the column and eluted with TE. Fractions (100µl) were collected and the radioactivity of each aliquot was assayed using a hand held ß-counter. The labelled DNA was eluted first, whilst the second peak contained unincorporated [α-^{32}P] dCTP. The fractions containing the labelled DNA were pooled and used as the probe in hybridization reactions. The specific activity of the probe was calculated as shown:

Specific activity of the labelled DNA = (B) ÷ (A) x 10^3 dpm.

(A) Total amount of DNA =

$$\frac{\text{Total number of } \mu\text{Ci added x 13.2 x \% incorporation.}}{\text{Number of dNTPs x average specific activity of dNTPs}} + 25$$

(B) Amount of radioactivity incorporated in the reaction (dpm) =

Total number of µCi added x 2.2 x 10^4 x % incorporation.

Hybridization of Radiolabelled HPV DNA.

The protocol for DNA hybridization was modified to include 50% deionized formamide in the pre-hybridization and hybridization solutions, in which the membrane was incubated at 42°C, instead of 65°C. The stringency washes were altered to include a single room temperature wash in 2 x SSC, 1% SDS, followed by a 37°C and 42°C wash in fresh buffer. When high stringency conditions were required a 65°C wash was carried out in 1 x SSC, 1% SDS and the membranes exposed to X-ray film as described in section 2.12.

2.16 Sample Quantification.

The number of cells present in 40 cervical scrapes was estimated using a Neubaurer Haemocytometer. Each sample contained between 1×10^6 and 1×10^9 cells. The yield of nucleic acid from each cervical scrape sample was estimated from the DNA absorbance at 260nm and the mean quantity extracted was 28 ± 21 μg (range 5-100μg).

2.17 Preparation of Cloned HPV DNA.

After purification of the plasmid DNA by caesium chloride gradient, the four preparations of HPV 6, 11, 16 and 31 DNA were digested with the enzyme by which the HPV had been restricted prior to cloning. The size of both vector and insert were verified by agarose gel electrophoresis and all fragment sizes were identical to those predicted for each clone. The enzymes used and the fragment sizes are shown in Table 2.1 and the results are shown in Figure 2.1.

After excision of the HPV insert from the vector, each preparation was analysed by agarose gel electrophoresis to ensure recovery of the DNA and that no contaminating plasmid sequences, which may cross-hybridize with cellular DNA, were present. As can be seen in Figure 2.2 all four HPV preparations were of 7.9kbp in length and did not contain residual plasmid sequences.

Table 2.1: Details of vector size, insert size and cloning enzyme for HPV 6, 11, 16 and 31 DNA.

HPV Type	Vector	Enzyme	Insert length	Vector length
HPV 6	pAT153	*Bam*H1	7900bp	3600bp
HPV 11	pSV2	*Bam*H1	7900bp	5700bp
HPV 16	pAT153	*Bam*H1	7900bp	3600bp
HPV 31	pBR322	*Eco*R1	7900bp	4300bp

Figure 2.1. Restriction analysis of cloned HPV DNA to verify the vector and insert sizes. Plasmid DNA containing HPV 6, 11 and 16 was digested with *Bam*HI (B) and HPV 31 DNA was digested with *Eco*RI (E) enzymes. Undigested plasmid DNA is also shown (U). M = size marker of lambda DNA digested with *Eco*RI enzyme, fragment sizes are shown in base pairs.

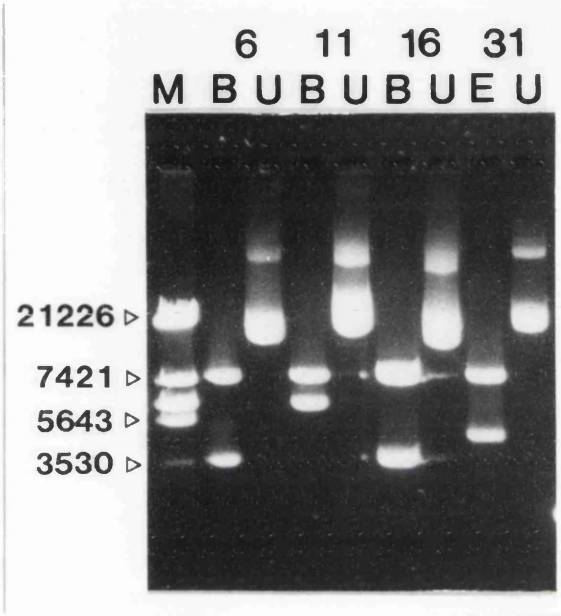
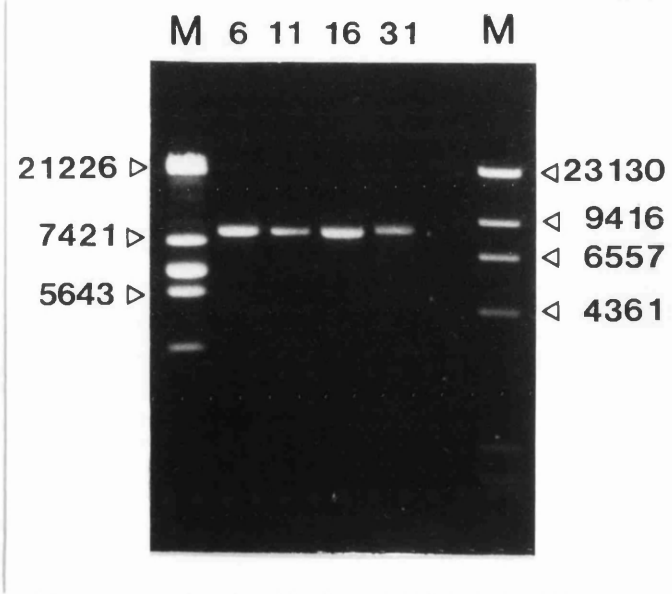


Figure 2.2. Analysis of purified HPV 6, 11, 16 and 31 DNA after excision from vector DNA. M = size markers of lambda DNA digested with *Eco*RI and *Hind*III enzymes, fragment sizes are shown in base pairs.



2.18 Sensitivity of the Dot and Southern Blot Hybridization Systems.

The sensitivity of dot blot and Southern blot hybridization systems for detection of HPV DNA was determined by titrating cloned HPV 16 DNA from 1ng to 5pg. The dot blot system was able to detect 20pg of cloned HPV 16 DNA as shown in Figure 2.3, whereas the Southern blot system could detect 10pg as shown in Figure 2.4.The Southern blotting method was therefore marginally more sensitive than the dot blotting system.

2.19 Detection of HPV DNA in Cervical Scrapes by Dot Blot Hybridization.

Sixty cervical scrape samples, derived from patients with CIN 2 or 3, were analysed by dot blot, of which 10/60 (16%) were positive for HPV with 6/60 (10%) containing HPV 16 and 4/60 (6%) containing HPV 6/11 sequences. The results of analysis of 17 samples for HPV 6/11 and HPV 16 DNA are shown in Figure 2.5. Within this subset, only two samples were found to be positive, one for HPV 6/11 and one for HPV 16, which were samples 3A and 2D respectively. The results of hybridization with the HPV 16 labelled DNA are shown at both high (Tm - 10°C) and low (Tm - 30°C) stringency.

2.20 Detection of HPV DNA in Cervical Scrapes by Southern Blot Hybridization.

The cervical scrape samples analysed by dot blot were also subjected to Southern blot hybridization and 14/60 (23%) were positive for HPV, of which 9/60 (15%) contained HPV 16 and 5/60 (8%) were positive for HPV 6/11. The 17 samples for which the dot blot results are shown were electrophoresed in two separate agarose gels. The results of the Southern hybridization analyses are

Figure 2.3. Sensitivity of the dot blot hybridization system for detection of HPV 16 DNA. HPV DNA was titrated from 1ng, 100pg, 50pg, 20pg, 10pg to 5pg, which correspond to samples 1-6. The system was capable of detecting 20pg of cloned HPV DNA.

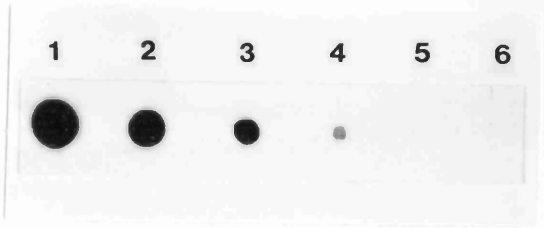


Figure 2.4. Sensitivity of Southern blot hybridization for detection of HPV 16 DNA. HPV DNA was titrated from 1ng, 100pg, 50pg, 20pg, 10pg to 5pg, which correspond to lanes 1-6. The system was capable of detecting 10pg of HPV DNA.

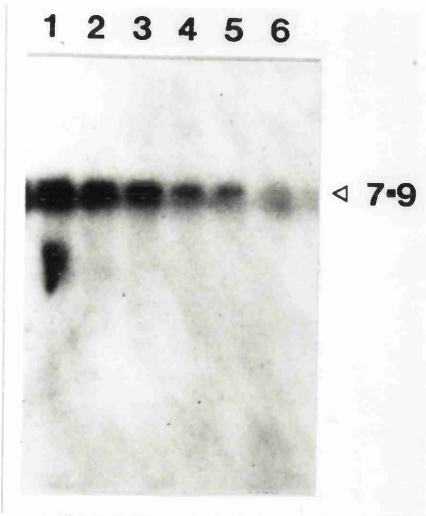
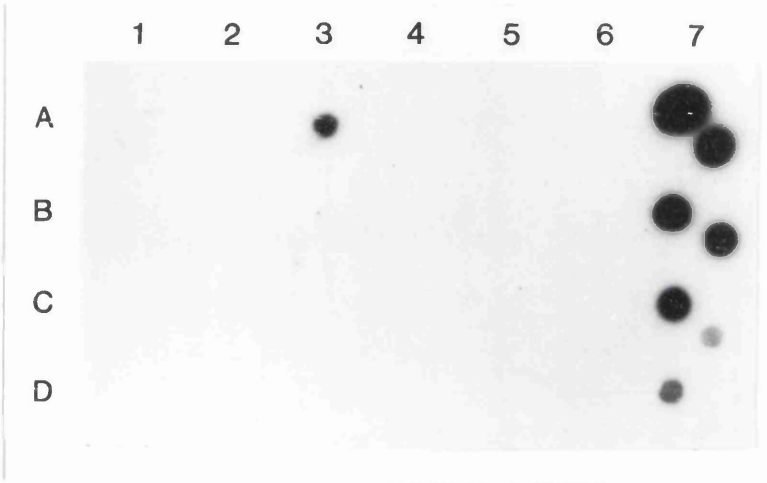
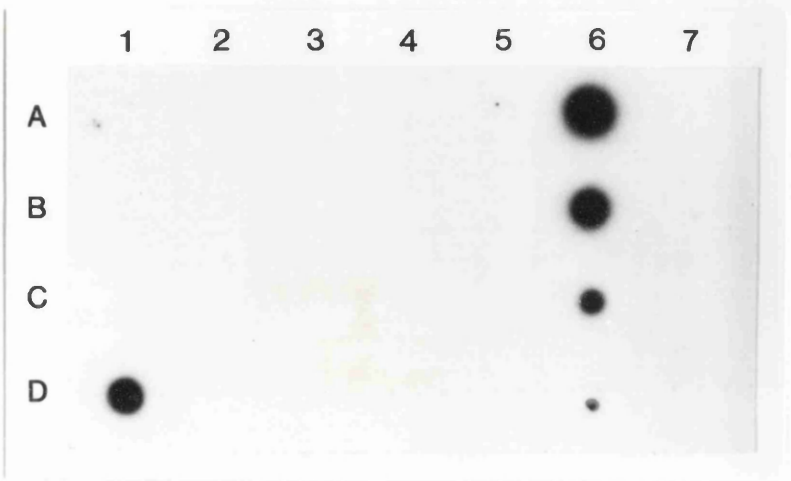


Figure 2.5. Dot blot hybridization of 17 cervical scrape samples with HPV 6/11 (a) and HPV 16 (b) radiolabelled probes under high stringency conditions (Tm - 10°C). The results with the HPV 16 probe are also shown at low (Tm -30°C) stringency (c). Column 6 contains cloned HPV 16 marker DNA (1ng, 100pg, 50pg, 20pg) and column 7 contains cloned HPV 6 (1ng, 100pg, 50pg, 20pg) and cloned HPV 11 (100pg, 50pg, 20pg) marker DNA.

a)



b)



c)

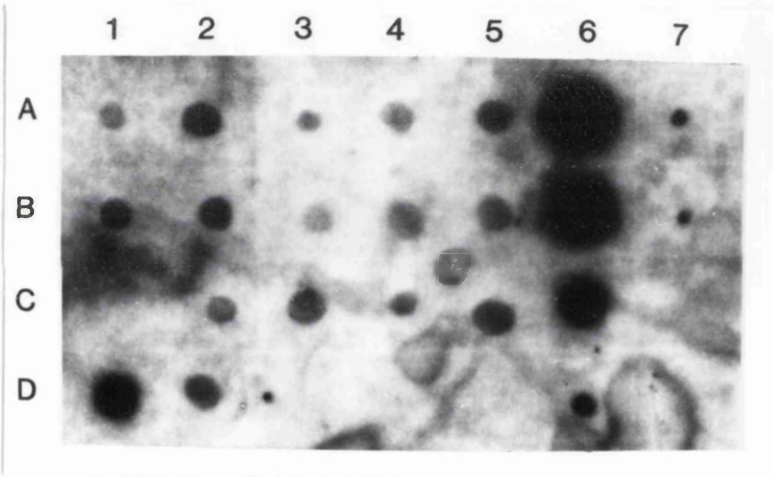
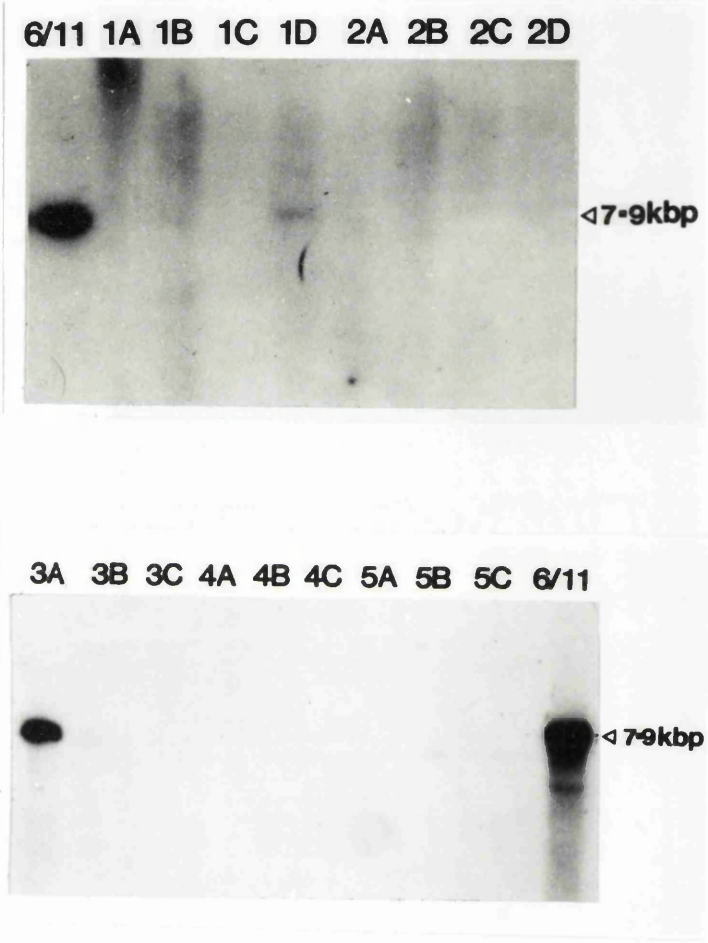
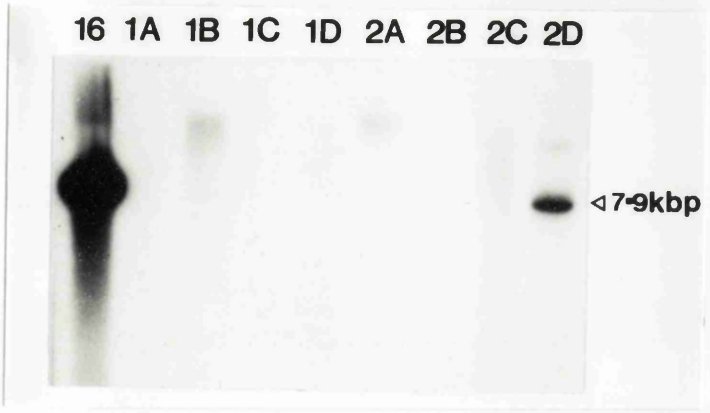


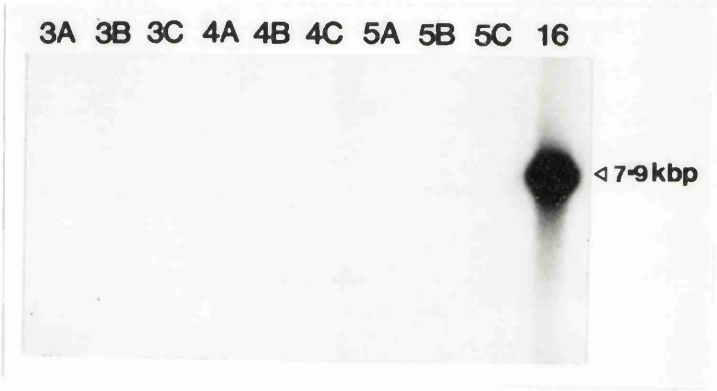
Figure 2.6. Southern blot hybridization of 17 cervical scrape samples with HPV 6/11 (a) and HPV 16 (b) radiolabelled probes. Markers of 50pg of cloned HPV 6/11 and HPV 16 are shown.

a)



b)





shown in Figure 2.6. One sample was positive for HPV 16, sample 2D, and 2 samples were found to be positive for HPV 6 and 11, samples 1D and 3A, only one of which was also found to be positive by dot blot.

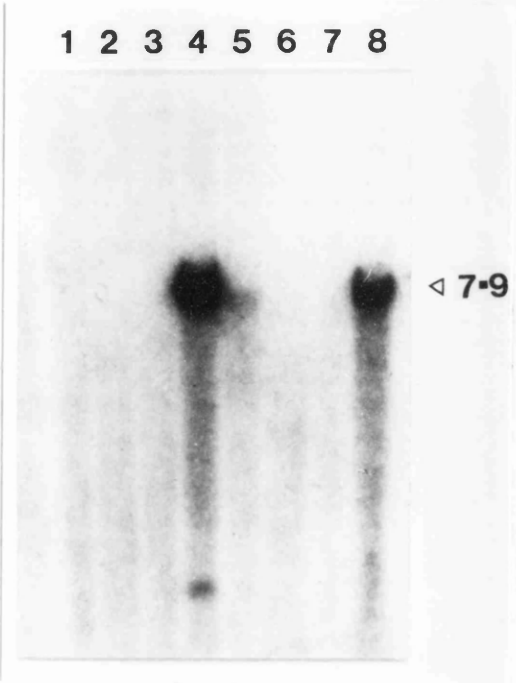
2.21 Detection of HPV DNA by Optimized Southern Blot Hybridization.

Southern blot hybridization was superior to dot blot hybridization in the levels of sensitivity and of non-specific binding of the probe. This method was therefore, adapted and optimized for use in detecting HPV DNA in clinical samples. The specific activity of the radiolabelled HPV probe was calculated and found to be 1×10^9 dpm/ μg.

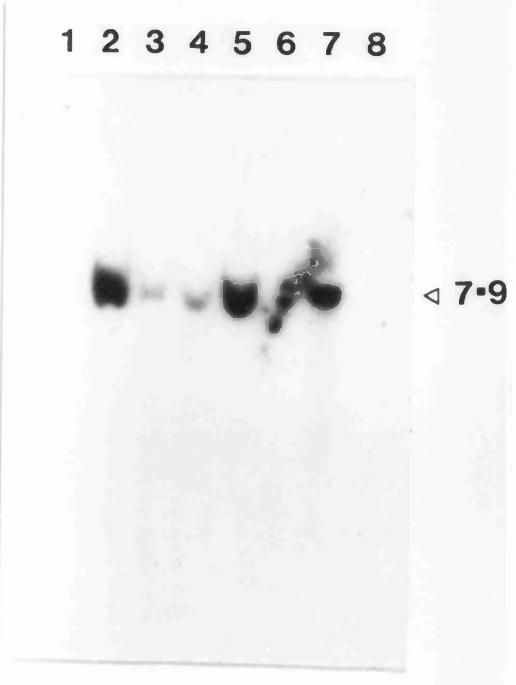
After optimization of the Southern blotting technique, a further 30 cervical scrape samples from women with CIN 2 or 3 were analysed for HPV 6/11 and 16 DNA. The overall detection rate in these samples was 19/30 (63%) with 8/30 (26%) positive for either HPV 6 or 11 and 14/30 (46%) positive for HPV 16. There were three samples in which both HPV 16 and either HPV 6 or 11 were present. The results of analysis for HPV 16 and HPV 6/11 in eight of these samples are shown in Figure 2.7.

Figure 2.7. Detection of HPV 6/11 and HPV 16 DNA in 8 cervical scrape samples from patients with CIN 2 and 3 using the optimized Southern hybridization system.

a) HPV 6/11



b) HPV 16



2.22 DISCUSSION.

Much of the evidence supporting the association of specific types of HPV with cervical carcinoma is derived from DNA:DNA hybridization studies (Durst *et al.*, 1983; McCance *et al.*, 1983, 1985a; Boshart *et al.*, 1984; Lehn *et al.*, 1985; Meanwell *et al.*, 1987). The objective of the work described in this chapter was to develop a reliable, sensitive and specific test for HPV DNA in cervical scrape samples, which could then be applied to specific patient groups.

There is contention over whether the number of cells collected for analysis is critical for accurate diagnosis of HPV. Some groups suggest that the minimum number of cells required is 1.6×10^6 (McCance *et al.*, 1986; Parkinnen *et al.*, 1986), whilst others find no correlation between the number of cells within the scrape and the HPV positivity, and successfully detect HPV DNA in samples containing between 1×10^4 and 8×10^5 cells per analysis (Schneider *et al.*, 1986; Caussy *et al.*, 1988; Cornelissen *et al.*, 1988; Vermund *et al.*, 1989). Hence, to determine whether the samples contained sufficient material for the analysis of HPV as designated by McCance *et al.* (1986), the number of cells was determined in a random selection of 40 samples. All samples tested contained in excess of 1×10^6 cells and were therefore presumed to contain sufficient material for HPV analysis. The mean quantity of DNA extracted from each cervical scrape was higher than previously reported for scrape samples (Webb *et al.*, 1987). However, this result was not unexpected since the number of cells in each scrape was greater than in those of other studies (McCance *et al.*, 1986; Parkinnen *et al.*, 1986; Schneider *et al.*, 1986; Caussy *et al.*, 1988; Cornelissen *et al.*, 1988). The DNA extraction procedure was improved by reducing the duration of the

proteinase K digestion from 16 hours to four hours without affecting the purity of the resulting DNA. This effect may be the result of a reduction in cellular nuclease activity during the shorter incubation time.

The hybridization methods most suitable for this study were Southern blot and dot blot hybridization due to the high sensitivity of the former and the rapidity of the latter. The sensitivity of hybridization for detection of HPV was determined for both methods using cloned HPV DNA. Dot blot hybridization detected 20pg and Southern blot detected 10pg of cloned HPV 16 DNA. The levels of sensitivity are comparable with those achieved in other studies (di Luca *et al.*, 1986; Webb *et al.*, 1987; Caussy *et al.*, 1988; Melchers *et al.*, 1988), although some studies have claimed detection limits of 1pg of HPV or BPV DNA by filter *in situ* hybridization (Wagner *et al.*, 1984; Caussy *et al.*, 1988).

The specificity of hybridization was controlled by the stringency of the reaction and, under low stringency conditions, related papillomavirus types can be detected (Brandsma *et al.*, 1989; Melchers *et al.*, 1989b; Williamson *et al.*, 1989). However, when using dot blot hybridization, or FISH, low stringency signals can be indistinguishable from non-specific hybridization. Such problems were not associated with Southern blot hybridization, but non-specific binding of labelled DNA was evident in dot blot analysis of cervical scrape samples in this study. Errant results may also be obtained by cross-hybridization of plasmid sequences with bacterial DNA which may be present in cervical scrapes (Ambinder *et al.*, 1986). Several authors have overcome this problem by re-hybridizing all membranes with radiolabelled pBR322 DNA (di Luca *et al.*, 1986; Cornelissen *et al.*, 1988; Reeves *et al.*, 1989). One study found 4-6% of samples were positive for

bacterial sequences, thus negating the HPV results in these samples (Reeves *et al.*, 1989). Excision of HPV DNA from plasmid vectors is a simpler and more effective method of preventing hybridization with bacterial sequences (Melchers *et al.*, 1988) and has been included in the present study. Gel electrophoresis analysis of the purified HPV DNA demonstrated the absence of plasmid sequences and, therefore, all positive signals on hybridization with these probes were assumed to be of HPV origin, rather than bacterial.

The ability of Southern blot and dot blot hybridization to detect HPV DNA was assessed by analysis of cervical scrapes from women with CIN 2 or 3. The dot blot method detected HPV 6, 11 or 16 in 16% of the samples, whilst 23% were positive for these HPV types by Southern blot hybridization.

Southern blot hybridization was chosen as the technique to be used to analyse further cervical scrape samples since it has greater sensitivity than dot blot and, even under low stringency conditions, does not result in non-specific hybridization signals. However, the detection rate achieved with Southern blot was considerably lower than that of other studies identifying HPV DNA in 50-90% of women with CIN (Crum *et al.*, 1982; Gissmann *et al.*, 1983; Burk *et al.*, 1986; di Luca *et al.*, 1986; Meanwell *et al.*, 1987; Riou *et al.*, 1990). The Southern blot hybridization protocol was therefore optimized by reducing the quantity of DNA to be labelled by random primer extension, increasing the incubation period and incubating the labelling reaction at a lower temperature. These changes were designed to allow maximum incorporation of radiolabelled nucleotides into the DNA and to increase the specific activity of the resulting probe. Purification of labelled DNA by sephadex chromatography conferred several advantages over the

Geneclean method, including more efficient separation of the labelled DNA from unincorporated nucleotides. Addition of 50% formamide, which destabilizes hydrogen bonds, to the hybridization solution enabled the hybridization temperature to be reduced to 42°C, whilst retaining high stringency conditions. The advantage of a lower hybridization temperature is that the DNA does not suffer damage, such as strand breakage, that can result from exposure to high temperatures (Lewin, 1990).

After optimization, the ability of the Southern hybridization system to detect HPV sequences in cervical scrapes from women with CIN 2 or 3 was assessed and the HPV 16 positivity in these samples increased from 23% to 63%. With greater sensitivity, not only was the rate of detection of HPV increased, but the system was also capable of detecting dual infection with two different types of virus in three of the cervical samples.

Having optimized the Southern blot hybridization, the technique was to have been used for the analysis of HPV DNA in cervical scrape samples from women from a General Practice population. However, the polymerase chain reaction was documented by Saiki *et al.* (1988) with the use of a thermostable DNA polymerase, and due to the extremely high potential of the method for detecting DNA sequences of interest, it was considered to be superior to any of the hybridization techniques. The project was therefore modified to include the development of a polymerase chain reaction system for the detection of HPV DNA in cervical scrape samples from women with and without disease. The development of the PCR assay system is described and discussed in chapter 3.

CHAPTER 3: THE DEVELOPMENT OF A POLYMERASE CHAIN REACTION SYSTEM FOR THE DETECTION OF HUMAN PAPILLOMAVIRUS DNA IN CERVICAL SCRAPE SAMPLES.

INTRODUCTION.

3.1 The Polymerase Chain Reaction.

The polymerase chain reaction (PCR) is an *in vitro* method for primer directed enzymatic amplification of specific target DNA, developed by Saiki and colleagues (1985, 1986). The original method involved two oligonucleotide primers which flank the DNA sequence of interest and repeated cycles of heat denaturation, primer annealing and extension of the annealed primers by a DNA polymerase enzyme. This results in amplification of target DNA between the primers and, because the two primers are complementary to the newly synthesized DNA, each successive cycle potentially doubles the quantity of DNA present from the previous cycle. Thus, an exponential increase (2^n where n = number of cycles) of the specific target sequence is achievable with PCR. The reaction, however, slows to a linear increase in DNA when larger quantities of template have been synthesized than the amount of enzyme present can use as substrate. This 'plateau' occurs after approximately twenty cycles have been completed (Saiki *et al.*, 1988).

PCR was originally carried out using the Klenow fragment of DNA polymerase I to extend from the primers, but denaturation at temperatures above 90°C destroys Klenow polymerase activity, making it necessary to add new enzyme every cycle. The utilization of a thermostable DNA polymerase from the

bacterium *Thermus aquaticus* (*Taq*), which can survive temperatures of 95°C and above, has simplified the PCR methodology considerably, because only one addition of *Taq* polymerase is necessary. The specificity of the reaction is increased due to the elevated temperature of 72°C, at which *Taq* polymerase is active. As the temperature is raised to 72°C any primers bound to non-homologous sequences dissociate, leaving only primers bound to the correct site intact for primer extension. An additional advantage of using *Taq* polymerase is that the sensitivity of the PCR is improved, because fewer non-specific products are synthesized and the stage at which the plateau occurs is delayed until 25-30 cycles have been completed (Saiki *et al.*, 1988).

3.2 Detection of Human Papillomavirus DNA by PCR.

The detection of human papillomavirus (HPV) infection in cervical tissues has relied, almost entirely, on DNA hybridization techniques due to the lack of any suitable cell culture system for these viruses. The development of PCR has provided a new technique with which to study papillomaviruses and allows detection of HPV DNA in samples which have too few genome copies to be detectable by hybridization. As with the hybridization techniques, the stringency of the PCR must be high in order to distinguish between different HPV types.

In this chapter, the development of a PCR system to specifically detect the three most common genital papillomavirus types, HPV 6, 11 and 16 in cervical scrape samples is described and discussed. During the time of development of this PCR system there were no reports published using PCR to amplify sequences from HPV DNA. Therefore, the methods were based on two studies using *Taq*

polymerase for the amplification of human gene sequences (Saiki *et al.*, 1988; Kogan *et al.*, 1987).

The area of the HPV genome selected as a target for the PCR system was a section of the early region containing the E6 and E7 genes. This is the region of least homology between the papillomaviruses and, therefore, the primers to this region would be less likely to anneal to the DNA of heterologous HPV types. In addition, the E6/E7 gene region remains intact when certain of the HPVs, e.g. HPV 16 and 18, integrate into the cellular genome of the host. Amplification of these genes has the potential to enable both integrated and episomal viral forms to be detected by the PCR system.

3.3 Primer Design, Synthesis and Purification.

The E6 and E7 gene sequences of all papillomavirus types whose sequences are known were compared using the alignment program developed by the Protein Information Resource in order to find the regions of least homology. These data were then used to design three sets of oligonucleotide primers, one for each of the three papillomavirus types of interest. The primers were 24bp in length and included regions of high G+C content (55-70%). The primers for HPV 6, 11 and 16, called PV 6, PV 11 and PV 16 respectively, amplified different sized fragments in order to allow the virus types to be distinguished when the products were separated by gel electrophoresis. The primer sequences are shown in Table 3.1.

Two additional oligonucleotides, homologous to the internal portions of the amplified fragments, were designed for use as probes to verify the results obtained by gel electrophoresis of the PCR products. The oligonucleotides were 20bp in length, with one homologous to an internal region of the HPV 16 fragment and the other homologous to an internal region of both the HPV 6 and 11 fragments. The sequences of these oligonucleotides are shown in Table 3.1.

The primers and oligonucleotides were synthesized using a cyclone DNA synthesizer, Biosearch Inc. in the Department of Haematology, Royal Free Hospital.

Table 3.1. Oligonucleotide primer sequences, primer location within the HPV genome and predicted size of amplified product generated by PCR with PV 6, PV 11 and PV 16 primers. Primer sequence and location of Southern blot hybridization oligonucleotide probes PS6/11 and PS16.

Primer	Sequence (5'-3')	Location	Product Size (bp)
PV6 (A)	GTACGTGGAAGGGTCGCTGCCTAC	495-518	167
PV6 (B)	TCTTGTCCGTCCACTTCGTCCACC	639-662	
PV11 (A)	ACCAGTGGAAGGGTCGTTGCTTAC	495-518	251
PV11 (B)	GTGCACTCCACAACCAGTCGGACG	723-747	
PV16 (A)	GGGGTCGGTGGACCGGTCGATGTA	490-514	223
PV16 (B)	GGGCTCTGTCCGGTTCTGCTTGTC	690-714	
PS 6/11	ACCCTGTAGGGTTACATTGC	587-606	
PS 16	TGCATGAATATATGTTAGAT	586-605	

Oligonucleotide DNA was eluted from each synthesis column by incubation with 1ml of 35% ammonia. The ammonia was introduced into the column using two 1ml syringes, one attached at each end. After 45 minutes the ammonia was washed through the column using the syringes and incubated for a further 90 minutes. The ammonia solution, containing the oligonucleotide DNA, was transferred to a screw capped 1.5ml microcentrifuge tube and incubated at 55°C for 16 hours to remove the amine protection groups present during the oligonucleotide synthesis. The ammonia was then allowed to evaporate in a fume cupboard for six hours and the resulting solution freeze dried (Edwards, modulyo freeze drier). The pellet was resuspended in 60µl of TE buffer.

3.4 Purification of Oligonucleotides.

The oligonucleotides were purified by excision from a polyacrylamide gel or by chromatography through Sephadex G-25.

Purification by Polyacrylamide Gel Electrophoresis.

The oligonucleotides were heated to 90°C for ten minutes with an equal volume of formamide dye mix (80% formamide, 0.1M EDTA, 0.01mg/ml bromophenol blue, 0.01mg/ml xylene cyanol). After cooling on ice, the oligonucleotides were applied to a 20% polyacrylamide gel and electrophoresed at 35mA for four hours. The DNA was visualized by laying the gel on autoradiography intensifier screens under UV irradiation (254nm). The oligonucleotides could be seen as a shadow on a light background. The position of each oligonucleotide was marked and the sections excised from the gel. The

resulting gel slices were placed in dialysis membrane tubing with 1.5ml of sterile distilled water (SDW) and dialysed for 16 hours against water. The membrane had previously been sterilized by boiling in 1% sodium carbonate and washed with SDW. The eluted DNA was filtered through a 0.45μm filter (Flowpore) and the absorbance of each oligonucleotide sample was measured at 260nm as described in Chapter 2, section 2.4.The concentration of purified oligonucleotides was calculated using the standard equation of A_{260} = 1 = 20μg/ml of primer DNA.

Purification by Chromatography through Sephadex G-25.

A Sephadex G-25 column was prepared for each oligonucleotide by plugging the bottom of a 1ml disposable syringe with glass wool and packing the column with sterile Sephadex G-25 buffered with TE. The column was centrifuged at 200g for three minutes and washed by the addition of 100μl of sterile TE buffer and centrifugation as before. The oligonucleotide primers were diluted with TE buffer to a final volume of 100μl, applied to separate columns and centrifuged with microcentrifuge tubes at the base of each column in which the eluted DNA was collected. The absorbance of the oligonucleotide solutions were measured in the same manner as described for the polyacrylamide gel purification.

3.5 Development of the PCR System for the Detection of HPV DNA.

The primers, described above, were used to amplify cloned HPV DNA in order to develop and optimize the PCR system for detection of HPV 6, 11 and 16. A basic PCR system was used for all experiments and the conditions were

modified according to the results of each experiment. HPV DNA was used as target for PCR with 100pg of each of the relevant primers and 1.5mM of each deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, dTTP), 1 x buffer and 1 unit of *Taq* polymerase (Cetus). All samples were overlaid with 100µl of mineral oil to prevent evaporation and PCR was carried out on a programmable heat-block (Perkin Elmer-Cetus). Each sample was subjected to 25 cycles, where one cycle consisted of 95°C for 30 seconds to denature the DNA, 50°C for 30 seconds to allow primer annealing to occur and 72°C for 60 seconds for extension of the annealed primers.

3.6 Development of the PCR Buffer.

The components of the PCR buffer are vital for the functioning of the *Taq* polymerase enzyme and hence, to the success of the PCR. The buffers first used with the HPV primers were those described by Kogan *et al.* (1987) and Saiki *et al.* (1988) and will be referred to as Kogan buffer and Saiki buffer respectively. 1 x Kogan buffer consisted of 16.6mM ammonium sulphate, 67mM Tris.HCl pH 8.8, 6.7mM magnesium chloride, 6.7µM EDTA, 10mM ß-mercaptoethanol, 170µg/ml bovine serum albumin, 10% dimethyl sulphoxide (DMSO). 1 x Saiki buffer consisted of 10mM Tris.HCl pH 8.4, 2.5mM magnesium chloride, 50mM potassium chloride and 200µg/ml gelatin.

The basic PCR system was used to determine the most effective buffer for PCR amplification of cloned HPV DNA, but no detectable amplification resulted with either of the two buffers. A third buffer was developed by combining the two recipes; the resulting 1 x buffer consisted of 25mM Tris.HCl pH 8.4, 17mM

ammonium sulphate, 10mM magnesium chloride, 10mM ß-mercaptoethanol and 0.02% gelatin. This will be referred to in the following experiments as 1 x Taq buffer.

The activity of *Taq* polymerase in the three buffers was compared using 1pg of HPV 16 DNA as the target for the PV 16 primers. The Klenow fragment of *E. coli* was also used to amplify the same target DNA, in buffer conditions of 50mM sodium chloride, 10mM Tris.HCl pH 7.6, 10mM magnesium chloride and 10% DMSO. The 25 PCR cycles utilizing Klenow DNA polymerase were carried out in water baths at the correct temperatures; one cycle consisted of 95°C for 30 seconds followed by 37°C for 30 seconds. At this stage of each cycle, 1 unit of Klenow DNA polymerase was added and incubation continued at 37°C for one minute.

Following amplification, 25µl of each sample was electrophoresed on a 1% agarose gel, the DNA stained with ethidium bromide and visualised on a UV transilluminator (302nm).

3.7 Determination of the Optimal Magnesium Chloride Concentration.

The optimal concentration of magnesium ions required for the PCR system was determined empirically with a series of Taq buffers containing decreasing concentrations of $MgCl_2$: 10.0mM, 9.0mM, 8.0mM, 7.0mM, 6.0mM, 5.0mM, 4.0mM, 3.0mM, 1.5mM and 0.5mM. PCR for HPV 6, 11 and 16 was carried out in the presence of each Taq buffer and the PCR products analysed on a 1.8% agarose gel. The optimal $MgCl_2$ concentration for each primer set was established by visual inspection of the ethidium bromide stained agarose gel.

3.8 Determination of the Optimal Primer Concentration.

PCR experiments were carried out as described previously, but with varying concentrations of the PV primer sets as follows: 0.2μM, 0.5μM, 1μM, 2μM, 5μM and 10μM. 1pg of the relevant DNA was used as substrate and the products were analysed on a 1.8% agarose gel. The optimal primer concentration for use with the HPV PCR systems was determined from the amplification patterns obtained.

3.9 Further Development of the PCR Cycle Conditions.

A primer annealing step of 50°C for 30 seconds was initially introduced to the PCR and used throughout the developmental procedures. However, the annealing temperature was later increased to 60°C and the duration of this step extended to 75 seconds. Further modifications of the cycling conditions included an increase in the denaturation step from 30 seconds to six minutes at 95°C prior to cycling and a ten minute extension step of 72°C during the final two cycles. The final cycling conditions selected for amplification of HPV DNA were as follows:

Heating to 95°C for six minutes to denature the target DNA, followed by amplification with 35 PCR cycles. One cycle consists of denaturation at 94°C for 75 seconds, primer annealing at 60°C for 75 seconds and extension of annealed primers at 72°C for 90 seconds.

3.10 Analysis of the Specificity of PCR for the Detection of HPV DNA.

The specificity of the PCR system, developed to detect HPV 6, 11 and 16, was determined by using 1μg of Raji cell DNA and 1pg of cloned HPV and

pBR322 plasmid DNA as target. In addition to these controls, the PV 16 primers were further tested using 1pg of cloned HPV 31 DNA and 1μg of HeLa cell DNA, which contains approximately ten copies of integrated HPV 18 DNA per cellular genome. All analyses were carried out using the methods described previously.

3.11 Labelling of the 5' Termini of Oligonucleotides.

The 5' termini of 1μg (10 pmoles) of PS 16 and PS 6/11 oligonucleotides were labelled with 1.5MBq of adenosine [γ-^{32}P] triphosphate in 1 x kinase buffer (50mM Tris.HCl pH 7.5,10mM magnesium chloride, 10mM dithiothreitol, 0.1mM spermidine, 0.1mM EDTA) with two units of T4 polynucleotide kinase. After incubation for one hour at 37°C, the labelled oligonucleotide was purified from unincorporated radiolabelled nucleotides by separation on a Sephadex column. The methods for this procedure are described in Chapter 2, section 2.15, except that Sephadex G-25 was used instead of Sephadex G-50.

3.12 Southern Blot Hybridization of PCR Amplified DNA.

The DNA amplified by PCR, was transferred from agarose gels to nylon membranes (Amersham, Hybond N) by Southern transfer, as described in Chapter 2, section 2.7. The membranes were hybridized with radiolabelled PS 16 and PS 6/11 oligonucleotides, according to the protocol described in Chapter 2, section 2.15, but without formamide in the hybridization solution. The membranes were washed twice with 2 x SSC, 1% SDS at room temperature for ten minutes, once at 42°C for 30 minutes and then exposed to X-ray film for 18-48 hours. Prior to

re-hybridization with the HPV 6/11 oligonucleotide, any remaining HPV 16 probe was removed using the method described in Chapter 2, section 2.13.

3.13 Analysis of the Sensitivity of PCR for the Detection of HPV DNA.

In order to determine the sensitivity of the PCR system for the detection of HPV 6, 11 and 16, cloned HPV DNA was titrated from 10pg to 0.01fg in samples of 1μg of Raji cell DNA. The titration was carried out for all three primer sets on samples containing 10pg, 1pg, 100fg, 10fg, 1fg, 0.1fg and 0.01fg of the relevant cloned HPV DNA. The PCR amplification and sample analysis were carried out using the methods described previously.

3.14 PCR for the Analysis of Cervical Scrape Samples.

DNA extracted from cervical scrape samples from women with CIN 2 or 3, was analysed for HPV 16 DNA by PCR. 1μg of each sample was used and the PCR products were analysed by electrophoresis through a 1.8% agarose gel. 1pg of HPV 16 DNA was then added to 1μg of DNA from eight of the cervical scrape specimens which were then used as target DNA for the PCR system with PV 16 primers.

3.15 Procedures for the Elimination and Prevention of Contamination.

The major problem associated with PCR is the false positive results which arise from contamination of the system. Due to the sensitivity of PCR, any contaminating DNA which is homologous to the primers, will be amplified to detectable levels and cause erroneous results. Several simple measures were

introduced to the PCR system to minimise this problem.

Firstly, the area of work and all equipment, including pipettes, were cleaned thoroughly with dilute SDS and/or 70% ethanol prior to use. All microcentrifuge tubes and pipette tips were packed under clean conditions and sterilized using an autoclave. Sterile water (Baxter Healthcare Ltd.) was used for the preparation of all reagents used in the reactions and these were stored in aliquots at -20°C until required. In addition, meticulous laboratory techniques were implemented and sterile gloves were worn when carrying out any procedure associated with the PCR.

Positive controls of cloned HPV DNA and DNA extracted from SiHa and CaSki cell lines, which contain integrated HPV 16 DNA, were implemented to ensure that the PCR was working efficiently. When necessary, negative controls of Raji cell and pBR322 DNA, which contain no HPV sequences, and HeLa cell DNA, which contains integrated HPV 18, were analysed to ensure that non-specific priming was not occurring. In order to control for contamination in each experiment, the PCR reagents with one unit of *Taq* polymerase, but no target DNA, were subjected to the PCR cycling.

3.16 Comparison of the PCR Buffers for the Amplification of HPV DNA.

The ability of *Taq* polymerase to PCR amplify HPV 16 DNA was analysed in three buffers, Kogan, Saiki, and Taq buffer and compared with the levels of amplification achieved with Klenow DNA polymerase using the PV 16 primers and HPV 16 DNA. The results of these analyses are shown in Figure 3.1.

The HPV 16 DNA was amplified by *Taq* polymerase in the presence of Taq buffer to levels visible on an agarose gel, but no fragment was amplified when either Kogan or Saiki buffers were used. A small quantity of HPV 16 DNA was amplified by Klenow DNA polymerase, but this was not comparable with the high levels achieved with *Taq* polymerase in 1 x Taq buffer. It was later established that DMSO inhibits *Taq* polymerase and that EDTA reduces the activity by chelating magnesium ions required by the enzyme. Although both of these components were present in Kogan buffer, neither reagent was included in the Taq buffer.

3.17 Confirmation of PCR Product Size.

The sizes of the PCR products of HPV 6, 11 and 16 were determined and compared with the expected fragment sizes as designated by the primer binding sites. Each of the primer sets amplified a fragment of the expected size as shown in figure 3.2. HPV 16 primers amplified a fragment of 223bp, HPV 11 primers amplified a fragment of 251bp and the HPV 6 primers amplified a fragment of 167bp.

Figure 3.1. Comparison of buffers for use with *Taq* DNA polymerase for the amplification of HPV 16 DNA. K = Kogan buffer, T = Taq buffer, S = Saiki buffer and Kl = amplification of HPV DNA using Klenow DNA polymerase. M = size marker of lambda DNA digested with *Hind*III enzyme, fragment sizes are shown in base pairs.

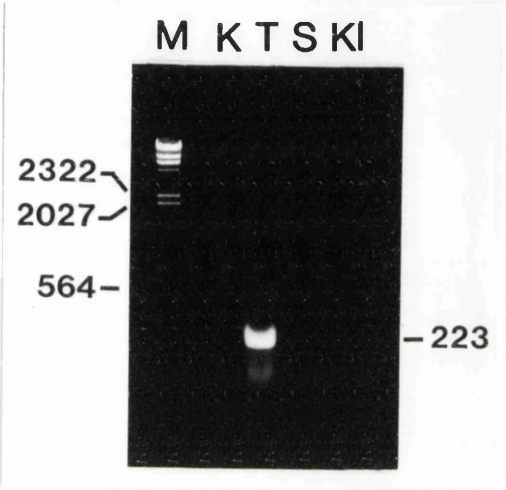
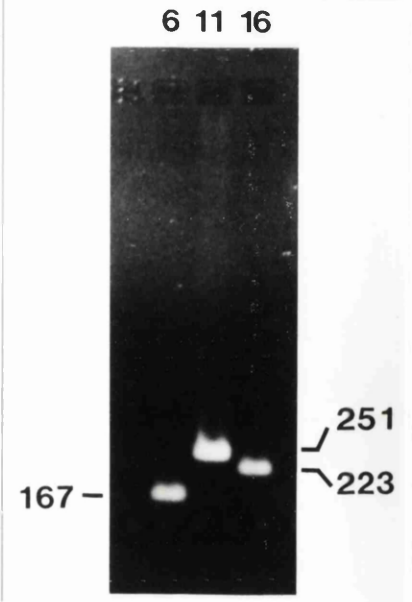


Figure 3.2. Analysis of the size of PCR products obtained after amplification of HPV DNA with PV 6, PV 11 and PV 16 primers. Amplification of HPV 6 DNA resulted in a fragment of 167bp, HPV 11 in a fragment of 251bp and HPV 16 in a fragment of 223bp.



3.18 Determination of the Optimal Magnesium Chloride Concentration.

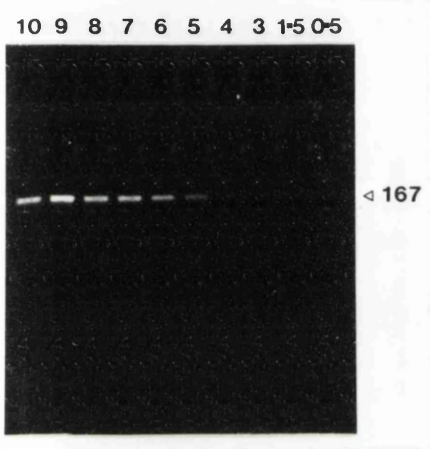
The optimal magnesium chloride concentration was determined for each primer set and the results are shown in Figure 3.3. The HPV 16 PCR system amplified HPV 16 DNA when the magnesium ion concentration was between 0.5mM and 4.0mM, with maximum amplification at 3.0mM. Therefore, 10 x Taq buffer containing 30mM magnesium chloride was used for all future experiments. The HPV 6 and HPV 11 PCR systems functioned within the range of 10.0 to 5.0mM magnesium chloride with an optimal concentration of 9.0mM for PV 6 and 8.0mM for PV 11 primers. 10 x Taq buffer containing 80mM magnesium chloride was used in subsequent experiments with both primer sets.

3.19 Determination of the Optimal Primer Concentration.

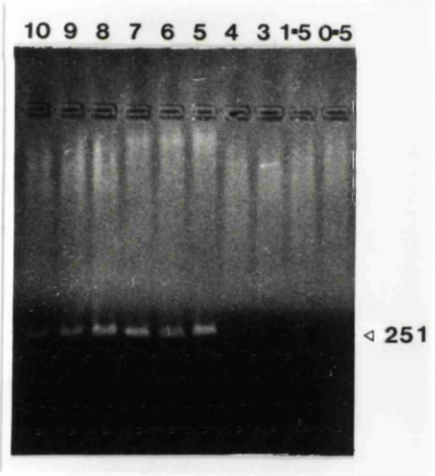
The concentration of oligonucleotide primers used in a PCR system is critical for the specificity of the reaction and therefore the optimal primer concentration for use with the HPV PCR system was determined for each of the primer sets. The results are shown in Figure 3.4. At primer concentrations of 5μM and above, non-specific amplification occurred resulting in fragments of varying sizes in addition to the fragment of the expected size. However, at primer concentrations between 0.2-0.5 μM, the amplification of the target DNA was greatly reduced, compared with that achieved at higher concentrations. Thus, the primer concentration chosen for the HPV PCR system was 1μM which resulted in high levels of amplified DNA without non-specific products.

Figure 3.3. Determination of the optimal magnesium ion concentration for PCR amplification of HPV DNA. PV 6 (a), PV 11 (b) and PV 16 (c) primers were used to amplify HPV DNA in Taq buffer containing decreasing quantities of magnesium chloride from 10mM to 0.5mM. Fragment sizes are shown in base pairs.

a)



b)



c)

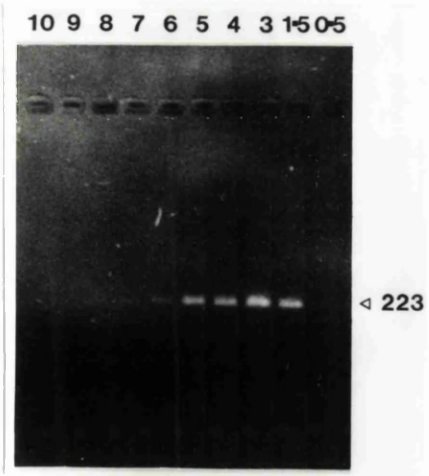
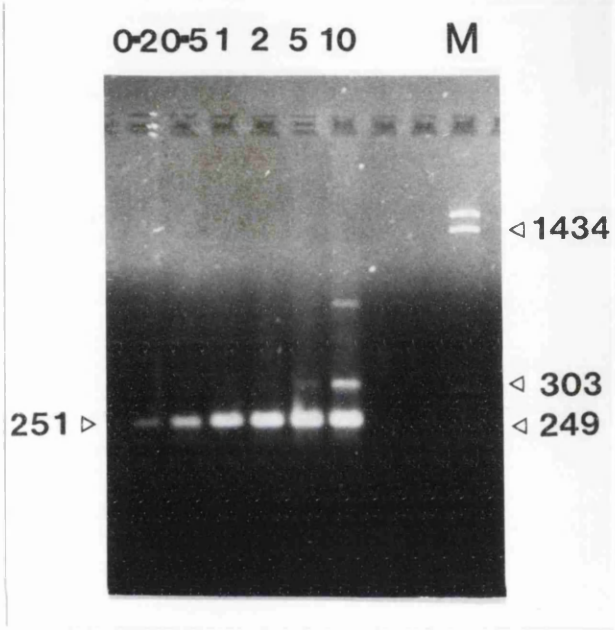


Figure 3.4. Determination of the optimal primer concentration for PCR amplification of HPV DNA with PV 11 primers. Primer concentrations were titrated from 0.2 to 10μM. M = size marker of pBR322 digested with *Ava*II enzyme, fragment sizes are shown in base pairs.



3.20 Development of the PCR Cycling Conditions.

The PCR cycling conditions described by Saiki and colleagues (1988) include a primer annealing step at 40°C over two minutes. When annealing the PV 6, PV 11 and PV 16 primers at this temperature, a fragment of the correct size was amplified. However, when the three primer sets were used to amplify each of HPV 6, 11 and 16 DNA in separate reactions, the low annealing temperature allowed HPV DNA other than the specific type for which the primers were designed, to be amplified.

To overcome this problem, a primer annealing step of 50°C was introduced and this modification was used throughout the developmental procedures. However, after optimization for $MgCl_2$ and primer concentration, the cross-amplification with other HPV types was still apparent. Therefore, the annealing temperature was raised to 60°C and the duration of this step increased to 75 seconds. This modification eliminated all non-specific primer binding, but allowed amplification of the target sequence. Further modifications to the PCR cycling conditions included a denaturation step of six minutes at 95°C prior to cycling to ensure that all target DNA was fully denatured and to maximise amplification. An extension step of ten minutes at 72°C was also included in the final two cycles, to ensure that all amplified DNA was of full length.

3.21 Specificity of PCR for the Detection of HPV DNA.

The specificity of the PCR system was determined by testing the three primer sets against target DNA of Raji cell and pBR322 DNA, neither of which contained any HPV sequences. No amplification occurred with any combination

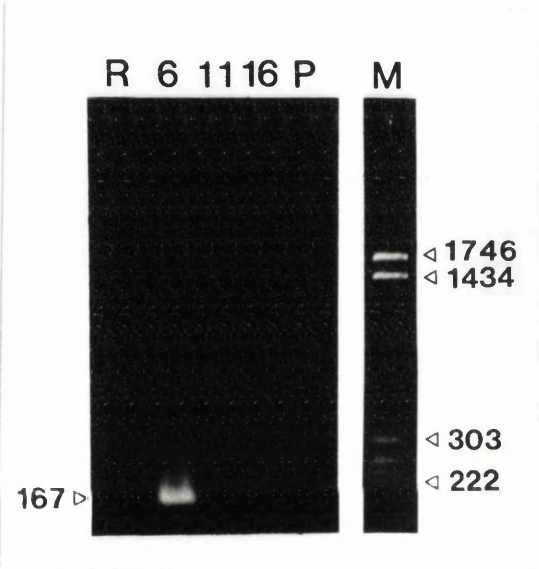
of primers and target DNA in this analysis.

To ensure that each primer set was specific for the type of HPV for which it was designed, the primers were included in PCR reactions containing additional types of HPV. The PV 6 primers amplified only HPV 6 DNA and the PV 11 primers amplified only HPV 11 DNA when cloned HPV 6, 11 and 16 DNA was used as target. In addition to cloned HPV 6 and 11, the PV 16 primers were tested further using HPV 31 DNA and HeLa cell DNA as targets. The PV 16 primers were specific for HPV 16 DNA and did not amplify any of the other HPV types included in this analysis. The results are shown in Figure 3.5.

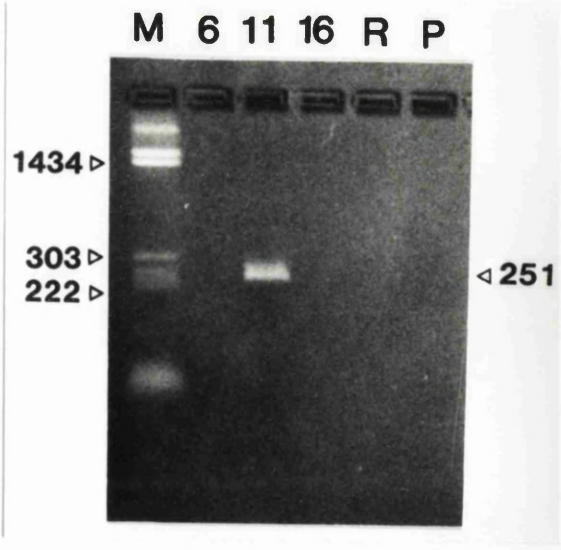
The specificity of the PCR in detecting HPV 6, 11 and 16 DNA was confirmed by hybridization with oligonucleotides PS 16 and PS 6/11, which were homologous with an internal region of the amplified fragment of HPV 16 and both HPV 6 and 11, respectively. The PS 16 hybridized only with the HPV 16 DNA fragment of 223bp and in the same manner, the PS 6/11 probe hybridized with both the 251bp HPV 11 fragment and the 167bp HPV 6 fragment, but not with the HPV 16 DNA. It is unnecessary for the latter oligonucleotide to distinguish between HPV 6 and 11, because the differences in the size of fragments obtained by PCR will be sufficient for that purpose.

Figure 3.5. Specificity of the PV 6, 11 and 16 primers for HPV DNA. All primers were tested using target sequences of pBR322 plasmid DNA (P), Raji cell DNA (R) and HPV 6, 11 and 16 DNA. The PV 16 primers were tested further with HPV 31 DNA and HeLa cell DNA (H). M = size marker of pBR322 DNA digested with *Ava*II enzyme, fragment sizes are shown in base pairs.

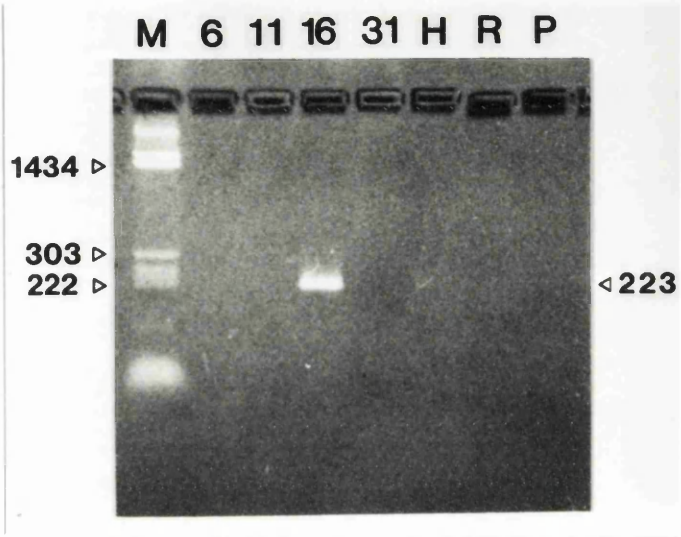
a) PV 6 Primers



b) PV 11 Primers



c) PV 16 Primers



3.22 Sensitivity of PCR for the Detection of HPV DNA.

The sensitivity of the optimized PCR system was determined by titrating samples of cloned HPV 16 DNA from 10pg to 0.01fg and using this as the target DNA for the PV 16 primers. The results of this analysis are shown in figure 3.6. The lowest quantity of DNA which could be amplified to detectable levels on an ethidium bromide stained gel was 0.1fg, which corresponds to five copies of the entire HPV 16 genome.

3.23 Use of PCR for the Detection of HPV DNA in Cervical Scrapes.

PCR was carried out on cervical scrape specimens from women with either CIN 2 or 3 in order to ensure that, when analysing clinical specimens, the PCR system would function to the high specificity and sensitivity achieved when amplifying cloned DNA. The results of PCR for HPV 16 of eight cervical scrape samples are shown in figure 3.7a. In this analysis samples 5 and 6 were found to contain HPV 16. It is possible that impurities, which may be present in the DNA samples isolated from cervical scrapes, could inhibit the PCR reaction and prevent detection of HPV DNA in samples which are positive, so yielding false negative results. To examine this possibility, 1pg of HPV 16 DNA was added to each scrape sample and the PCR repeated. The results are shown in figure 3.7b and all samples were positive in the subsequent analysis, demonstrating that inhibition of the PCR system with contaminants was not a common occurrence.

Figure 3.6. Sensitivity of the PCR system for detection of HPV 16 DNA using PV 16 primers. HPV 16 DNA was titrated from 10pg to 0.01fg and the smallest quantity of HPV DNA detectable was 0.1fg which corresponds with five copies of the HPV genome. N = negative control with no target DNA.

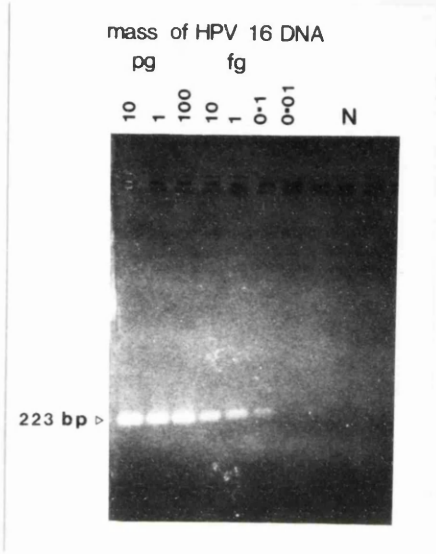
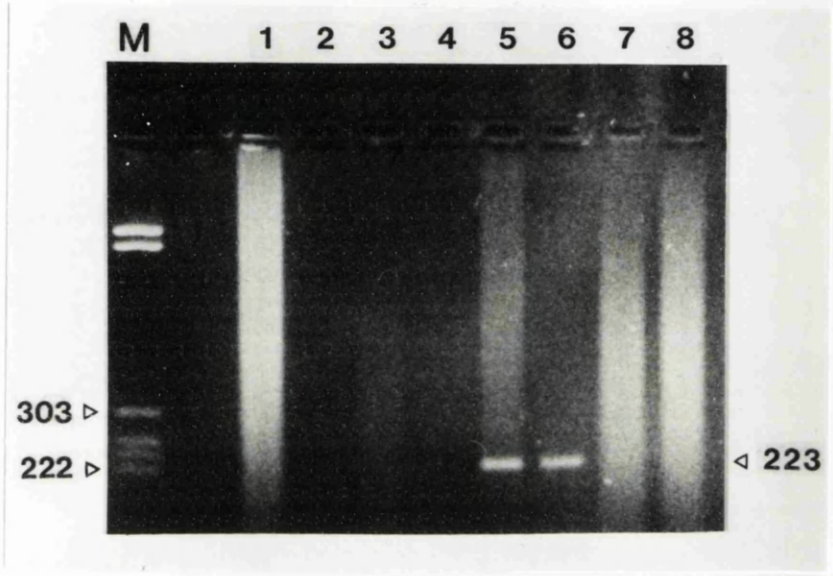
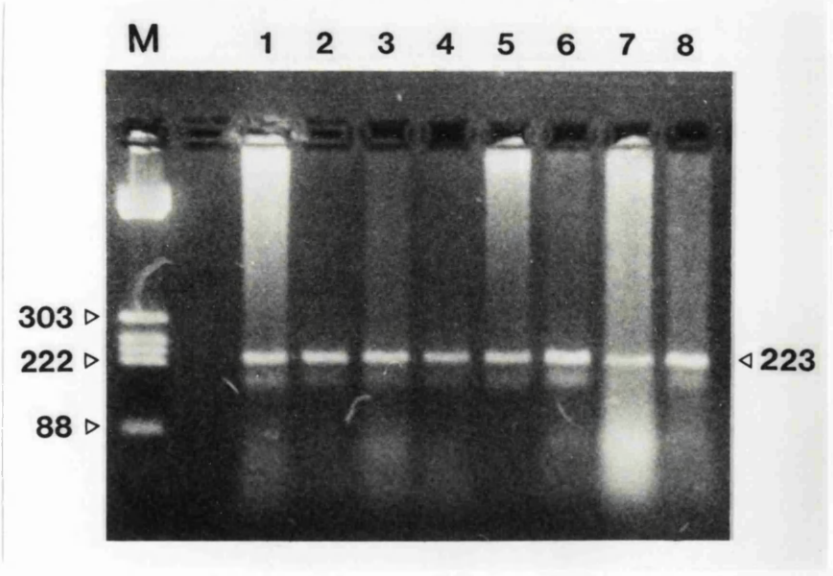


Figure 3.7. PCR analysis for HPV 16 DNA in 8 cervical scrape samples from women with CIN 2 or 3 (a). The PCR analysis was repeated with 1pg of cloned HPV 16 DNA added to each sample (b). M = size marker of pBR322 DNA digested with *Ava*II enzyme, fragment sizes are shown in base pairs.

a)



b)



3.24 DISCUSSION.

DNA:DNA hybridization has been the major method for analysis of HPV infection in clinical samples, due to the lack of a suitable cell culture system. The application of PCR to this area of study has provided a highly efficient technique which can be used to identify HPV DNA, even when the quantity of target DNA available is very small. This is particularly important for the detection of HPV in cervical scrape samples which frequently have insufficient material for analysis by Southern blot or dot blot hybridization (McCance *et al.*, 1985b; Lorincz *et al.*, 1986; Meanwell *et al.*, 1987). PCR is also applicable to other samples such as paraffin embedded tissue (Griffin *et al.*, 1990) on which *in situ* hybridization is usually carried out.

In this Chapter, the development of a PCR system for detection of HPV 6, 11 and 16 DNA in cervical scrape samples has been described. The primers were designed to amplify a section of the E6 and E7 genes and to have a high G+C content in order to allow for stringent conditions to be implemented. Many other studies have selected this region as the target for PCR, because it remains intact when certain HPV types, e.g. HPV 16 and 18, integrate into the cellular genome (Dallas *et al.*, 1989; Tidy *et al.*, 1989; Chow *et al.*, 1990; Griffin *et al.*, 1990; Pao *et al.*, 1990). Alternatively, researchers have concentrated on the late region, amplifying a section of the L1 ORF (Cornelissen *et al.*, 1989; Gregoire *et al.*, 1989; van den Brule *et al.*, 1989; Snijders *et al.*, 1990). General oligonucleotide primers have also been designed, which are capable of amplifying a target sequence from a wide range of papillomavirus types (Gregoire *et al.*, 1989; Snijders *et al.*, 1990). It has been suggest that these primers may be useful for the

detection of previously unidentified HPV types, but are, however, unsuitable for the routine detection of either HPV 16 or 18, because the integrated viral forms may not be detected, as discussed earlier.

The PCR system to detect HPV 6, 11 and 16, described in this chapter, was specific for each virus type. The sizes of the amplified DNA fragments, obtained by PCR with the HPV primers were consistent with the size predicted from the primer positions. DNA from HPV types, other than those for which the primers were designed, was not amplified by this PCR system and no non-specific amplification was detectable when either Raji cell or pBR322 plasmid DNA was used as the target. The specificity of the system was confirmed by Southern blotting with radiolabelled oligonucleotide probes homologous with an internal portion of the amplified fragments.

The PCR system was able to detect as little as 0.1fg of cloned HPV DNA in 1μg of cellular DNA, which corresponds with five HPV genome equivalents. With the additional sensitivity of Southern blot hybridization with radiolabelled oligonucleotide probes, the PCR system was potentially capable of detecting a single copy of HPV DNA. This level of sensitivity is comparable with that achieved by other workers (Dallas *et al.*, 1989; Young *et al.*, 1989). PCR systems have been described in other studies, whose limit of detection is between 10fg and 1pg (Chow *et al.*, 1990; Pao *et al.*, 1990; Snijders *et al.*, 1990). The latter quantity is close to the range sensitivity of Southern blotting (Chan *et al.*, 1985; Cornelissen *et al.*, 1988) and therefore offers little improvement on conventional hybridization detection methods.

Due to the very high sensitivity achievable with PCR, any contaminating

target DNA can be amplified to detectable levels and this is of particular importance when PCR is to be used for diagnosis. Several authors have acknowledged this problem and suggested methods by which this may be reduced (Bell, 1989; Tidy and Farrell, 1989; van den Brule *et al.*,1989; Young *et al.*,1989). Many of the procedures described for minimising contamination are similar to those adopted during the development of the PCR system described in this chapter. These include the storing of all PCR reagents in aliquots, the wearing of gloves for all PCR related work and the use of suitable positive and negative controls.

Methods for prevention and identification of contamination in PCR systems have been described. Physical separation of PCR work from all other DNA manipulation has been suggested (Bell, 1989; Pao *et al.*, 1990), but this measure is not feasible for most groups with limited space and positive control DNA, such as plasmids and cell lines containing HPV sequences, could still be a source of contaminating target DNA.

Selection of amplification primers to the region of HPV which includes the cloning site enabled contaminating plasmid DNA to be identified in one study (van den Brule *et al.*, 1989). PCR amplification using these so-called "anti-contamination primers" resulted in a fragment of 350bp, except when cloned HPV plasmid DNA was present, when a larger DNA fragment (3 kbp) was amplified. After gel electrophoresis of the PCR samples, the two products were clearly distinguishable from each other. There are, however, two drawbacks to this method. The system is only capable of detecting contaminating HPV sequences contained within plasmid DNA. The most frequent source of contamination is

believed to be previously amplified material (Gibbs and Chamberlain, 1989; Kwok and Higuchi, 1989; Tidy and Farrell, 1989; Williams, 1989; Kitchin *et al.*, 1990) and products from amplified HPV DNA would be indistinguishable from the *bona fide* product. This method also limits the choice of primers to the cloning region, which is usually the *Bam*HI site, situated in the L1, E5 and E2 region of most papillomavirus types (van den Brule *et al.*, 1989). When papillomavirus DNA integrates into cellular DNA, as described in Chapter 1, section 1.3, the E1/E2/E4 region is disrupted and the late genes are often lost (Choo *et al.*, 1987; Lehn *et al.*, 1988). Therefore, the PCR system described may be incapable of detecting integrated HPV and would have limited use for the routine detection of HPV. In order to overcome the latter problem, Cornelissen *et al.* (1989) employed primers to the L1 region of HPV 16 in conjunction with primers to the E7 region and were able to detect integrated and non-integrated HPV 16 with the E7 primers and eliminate plasmid contamination with the L1 primers (Cornelissen *et al.*, 1989).

Contaminating DNA sequences can be destroyed by irradiation of reagents with UV light prior to the addition of the target DNA to prevent amplification of these sequences (Sarkar and Sommer, 1990). The procedure involves exposure of buffer, primers and deoxynucleotides to UV irradiation (254nm and 300nm) for 5-20 minutes. The effect of UV irradiation on DNA is to induce cyclobutane pyrimidine dimers, while preserving the functional capacity of single-stranded oligonucleotide primers (Moore *et al.*, 1981). This method has the potential to eliminate contamination from PCR systems and would improve the use of PCR for routine diagnosis of HPV in cervical scrapes. However, before this technique

can be applied to PCR in this way it must be established that the UV irradiation of the oligonucleotide primers does not reduce the sensitivity of the final reaction.

An alternative method for elimination of contamination with amplified DNA carried-over from previous PCRs has been documented (Longo *et al.*,1990). The dTTP in the PCR nucleotide mix was substituted with dUTP, resulting in amplimers containing deoxyuradine, instead of deoxythymidine. Treatment of subsequent PCR samples with uracil DNA glycosylase, which removes uracil bases from DNA, prior to cycling prevents amplification of any contaminating DNA, but leaves template DNA or RNA intact. Uracil DNA glycosylase is inactivated by the high temperatures of the denaturation step and therefore new PCR products are unaffected. The method described assumes all contamination is from previously amplified material and would not eliminate contamination from recombinant plasmids containing target sequence DNA or cross-contamination between cervical scrape samples, which would both contain deoxythymidine.

The PCR system described in this chapter is capable of detecting and distinguishing the three most common papillomavirus types present on the uterine cervix. Each set of primers for the amplification of HPV 6, 11 and 16 was specific for the virus type for which it was designed and detected neither non-specific DNA nor the DNA from other HPV types. The PCR system was also highly sensitive, being capable of detecting five genome copies in 1μg of cellular DNA. This is comparable with several other PCR systems described in the literature. Simple measures have been adopted to reduce the possibility of contamination with HPV DNA and positive and negative controls implemented in order that erroneous results can be discarded, should contamination occur. Thus, the PCR

system has been developed and optimized to be capable of detecting HPV 6, 11 and 16 in DNA extracted from cervical scrapes and is suitable for routine analysis of these samples.

CHAPTER 4: STUDY A; ANALYSIS OF THE PREVALENCE OF HPV 16 IN WOMEN FROM A GENERAL PRACTICE.

4.1 INTRODUCTION.

It has been suggested that infection of the uterine cervix with human papillomavirus may be associated with the development of cervical carcinoma (zur Hausen, 1977, 1987; zur Hausen *et al.*, 1984; Peto, 1986). HPV is present in 40-90% of patients with cervical carcinoma and 30-60% of women with CIN (Durst *et al.*, 1983; McCance *et al.*, 1985b; Fuchs *et al.*, 1987; Meanwell *et al.*, 1987; Caussy *et al.*, 1988; Melchers *et al.*, 1988; Cornelissen *et al.*, 1989; van den Brule *et al.*, 1989, 1991; Mayelo *et al.*, 1990). Women with no cervical abnormalities demonstrate a lower prevalence of HPV DNA of 0-30% (Crum *et al.*, 1985; McCance *et al.*, 1985b; Wickenden *et al.*, 1985; Toon *et al.*, 1986; Caussy *et al.*, 1988; Melchers *et al.*, 1989a; van den Brule *et al.*, 1989, 1991; Mayelo *et al.*, 1990; Evander and Wadell, 1991; Kyo *et al.*, 1991). These results indicate that HPV infection is more common in women with CIN or carcinoma than in women without disease. However, the mere presence of HPV infection in patients with CIN or carcinoma is insufficient evidence to define the disease aetiology.

More informative data has been obtained by studies which have used hybridization detection methods under high stringency conditions in order to distinguish between the different HPV types. HPV 6 and 11 are more common in benign cervical lesions and normal tissue with prevalence values of 20-65% (Gissmann *et al.*, 1983; Crum *et al.*, 1985; McCance *et al.*, 1985a; Burk *et al.*, 1986; Toon *et al.*, 1986), but are less frequent in severe lesions and cancerous tissue

with detection rates of 0-28% (Gissmann *et al.*, 1983; Wagner *et al.*, 1984; McCance *et al.*, 1985a). The converse is true of HPV types 16 and 18 which are found in 50-90% of severe lesions and carcinoma samples (Durst *et al.*, 1983; McCance *et al.*, 1985b; Meanwell *et al.*, 1987; Webb *et al.*, 1987; Cornelissen *et al.*, 1989), but are less common in benign tissue (Cox *et al.*, 1986; Meanwell *et al.*, 1987). These observations correlate with the ability of HPV 16 and 18 to immortalize human keratinocytes and transform established cell lines *in vitro* (Yasumoto *et al.*, 1986; Storey *et al.*, 1988; Pecorado *et al.*, 1989; Woodworth *et al.*, 1989).

More recently, the prevalence of HPV in cervical tissue has been studied using PCR and the prevalence of all types has been found to be 84-100% in patients with cervical carcinoma and 31-100% in patients with CIN lesions (van den Brule *et al.*, 1989; Young *et al.*, 1989; Griffin *et al.*, 1990; Pao *et al.*, 1990; Riou *et al.*, 1990; Evander and Wadell, 1991). These results correlate well with those obtained by hybridization studies, but demonstrate a higher prevalence of HPV as expected from such a sensitive technique. However, very contradictory results have been obtained when PCR has been used to study women without cervical disease. The prevalence of all HPV types in normal tissue is 5-70% (Melchers *et al.*, 1989; van den Brule *et al.*, 1989; Young *et al.*, 1989; Griffin *et al.*, 1990; Pao *et al.*, 1990; Evander and Wadell, 1991) and the value for HPV 16 alo_w has been found to be 5.4-84% (Tidy *et al.*, 1989; Rakoczy *et al.*, 1990; Kyo *et al.*, 1991; Nishikawa *et al.*, 1991).

Cervical cytology is routinely used to detect cervical disease. However, there is a false negative rate of 32% associated with this technique (Giles *et al.*,

1988). It has been postulated that HPV 16 may be useful as an alternative screening method to identify women with cervical disease (Burk *et al.*, 1986, Campion *et al.*, 1986; McCance *et al.*, 1988; Cornelissen *et al.*, 1989; Melchers *et al.*, 1989) However, the very high prevalence of HPV 16 in normal women reported in some studies, must cast doubt on the use of PCR for HPV 16 in this manner. The prevalence of HPV 16 in women with and without disease requires further investigation to determine whether HPV 16 has a role to play in the development of cervical disease.

The aims of the study described in this chapter (Study A) were to determine the prevalence of HPV 16 in a group of women from a General Practice using the PCR system described in Chapter 3 and to correlate the results with the clinical diagnosis.

4.2 Patients and Clinical Analysis.

Between May and September 1987, a cervical screening service, combining cytology and colposcopy, was offered to all adult female patients of a General Practice on the borders of London and Hertfordshire, irrespective of the date of their last cervical smear. The Practice was in a predominantly middle-class area and 249 women volunteered for this service from a total of 2879 female patients over the age of 21.

Once a medical and cytological history had been obtained, all patients underwent a full cervical examination. Two scrapes were taken by rotating a wooden Ayre spatula through 360° over the transformation zone. One scrape was used for standard cytological analysis and the second scrape was placed in 10ml sterile PBS at 4°C for virological analysis. A colposcopic examination was then performed with solutions of normal saline (0.9% w/v), 5% acetic acid, and Lugol's iodine. Biopsy specimens were taken from any abnormal areas of the epithelium and fixed in formolsaline for histological examination.

4.3 Sample Collection and Preparation.

The cervical cells for virological analysis were collected by centrifugation at 1700g for ten minutes and washed twice with 5ml PBS. The resulting cell pellet was resuspended in 200µl of PBS and stored at -70°C until required for analysis.

The cellular DNA was extracted and the concentration and yield of each sample was estimated using the methods described in Chapter 2, section 2.4.

4.4 Polymerase Chain Reaction for the Detection of HPV 16 DNA.

PCR analysis was performed on two separate occasions using the PCR system described in Chapter 3 and 1μg of DNA from each sample for the target. Positive and negative controls of cloned HPV DNA and no target DNA respectively, were included in the PCR system with each separate analysis. Samples which gave discrepant results in the duplicate analyses were subjected to PCR a further two times. All of the laboratory experiments were conducted without knowledge of the patients' clinical status.

4.5 Statistical Analysis.

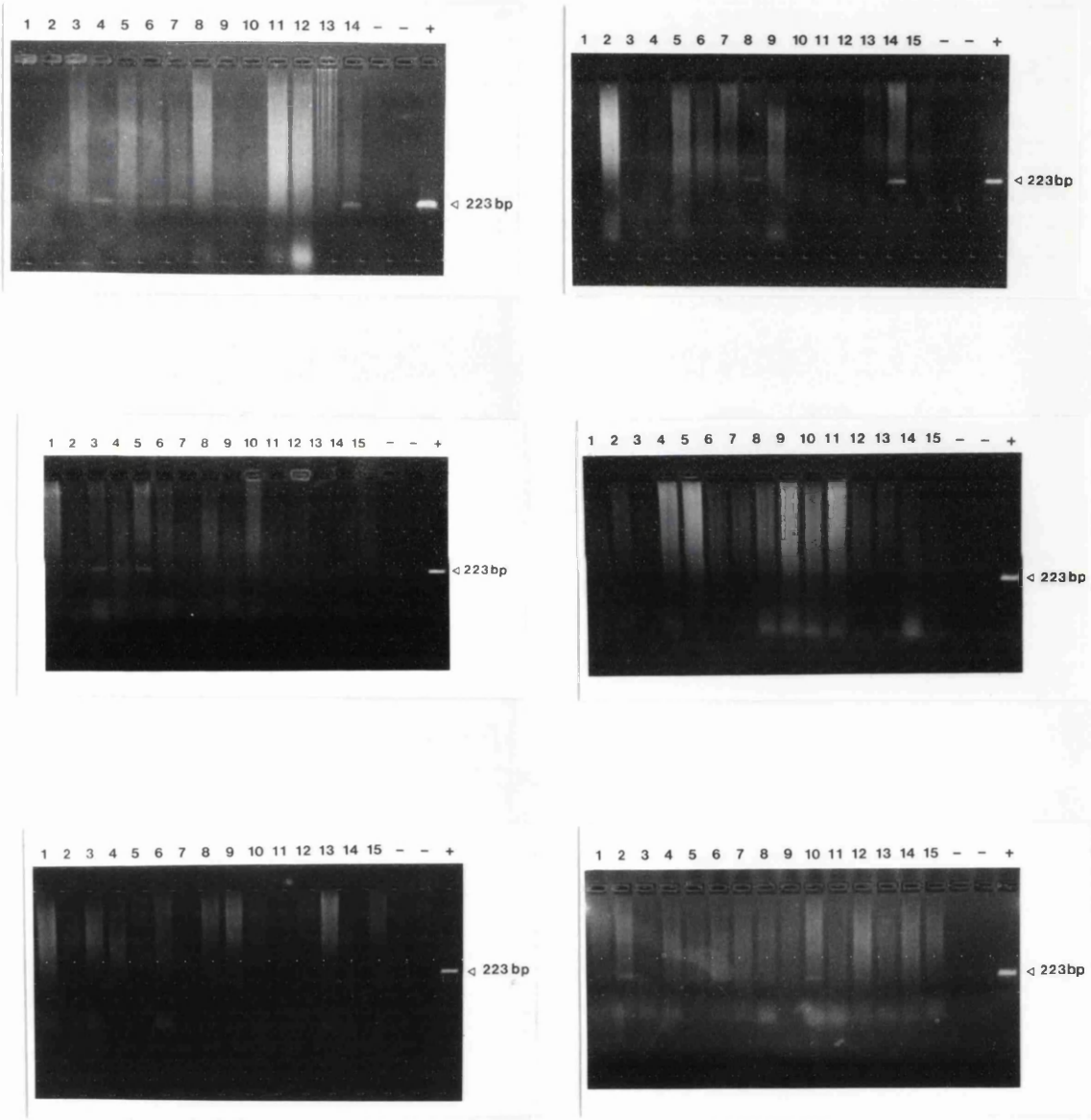
The statistical significance of the correlation of HPV 16 prevalence with cervical disease was determined by the Fishers exact test. The sensitivity was calculated by dividing the number of HPV 16 positive patients with cervical disease by the total number of patients with disease. The specificity was calculated by dividing the number of HPV 16 negative patients with no disease by the total number of patients without disease. The positive predictive value was calculated by dividing the number of HPV 16 positive patients with severe disease by the total number of HPV 16 positive women. The negative predictive value was calculated by dividing the number of HPV negative patients without disease by the total number of HPV negative women. The relative risk of having severe disease and HPV 16 was calculated by dividing the incidence rate of disease in the HPV 16 positive group by the incidence rate of disease in the HPV 16 negative group.

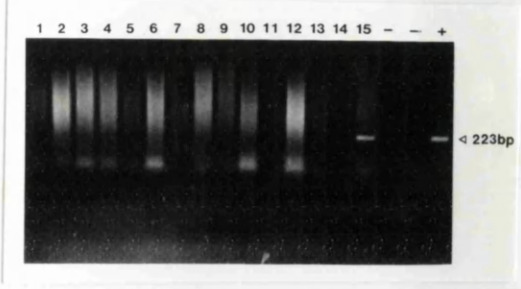
4.6 RESULTS.

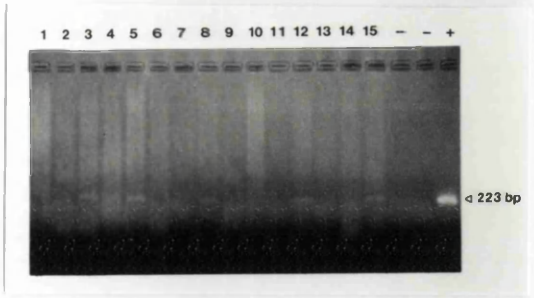
Women from a General Practice (n = 249) were examined by cytology, colposcopy and, where appropriate, histology for cervical abnormalities. Forty-nine women were excluded from this group due to a previous abnormal smear report, surgery for benign cervical conditions or abnormal vaginal bleeding. A final diagnosis could not be completed for a further 17 patients due to unsatisfactory smear samples, leaving 183 women in Study A. The mean age of the women in Study A was 39.4 years (range 18-76), the mean age of those with CIN was 29.5 and the mean age of those with no cervical abnormalities was 40.6 years. The mean time since the last cervical smear was two years (range 3 months-21 years). There were 10 women who had never had a cervical smear.

PCR analysis of cervical scrapes from the women in Study A showed that 33 of 183 (18%) were positive for HPV 16, whereas 160 (82%) had no detectable HPV 16 DNA. PCR analysis of cervical scrape samples from study A is shown in Figure 4.1. After clinical examination, the women in Study A were assigned to the following groups: normal, WVI, CIN 1, CIN 2, CIN 3. A diagnosis of histologically proven CIN 1, 2 or 3 was recorded for 22 patients. The prevalence of HPV 16 in these patients was 32% (7/22) whilst the prevalence in the colposcopically normal women was only 17% (26/151). None of the ten patients with cytological evidence of WVI were positive for HPV 16 DNA. The patients in Study A were stratified according to grade of disease and HPV 16 status and the results of this analysis are shown in Table 4.1.

Figure 4.1. PCR analysis of cervical scrape samples from Study A. PCR of HPV 16 positive samples resulted in a fragment of 223 base pairs in length. Negative (-) and positive (+) controls were included in each analysis.




1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 – – +
223bp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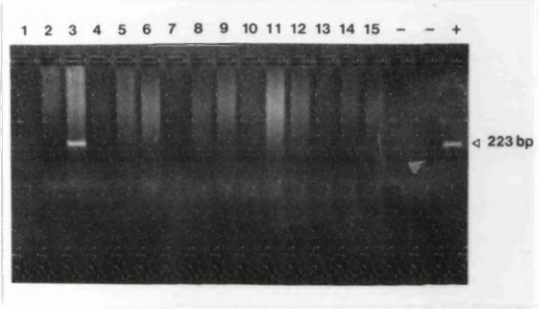
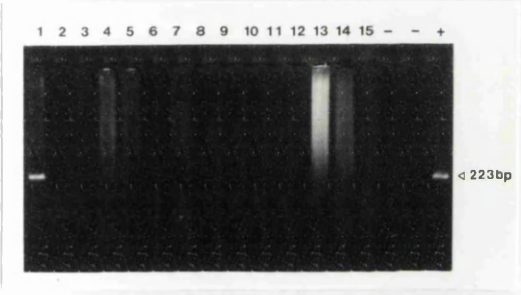
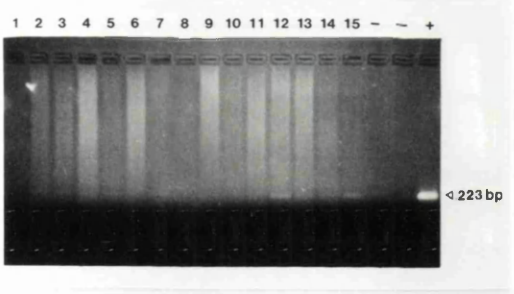
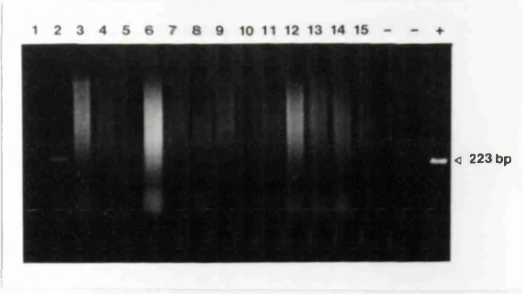
223 bp


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 – – +
223 bp


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 – – +
223bp


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 – – +
223 bp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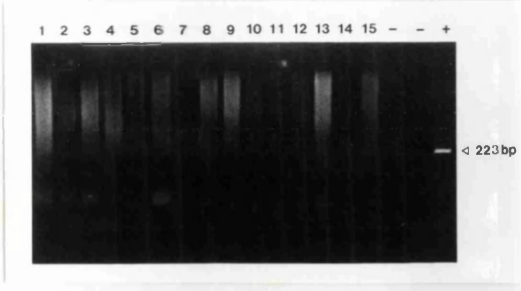

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 – – +
223 bp

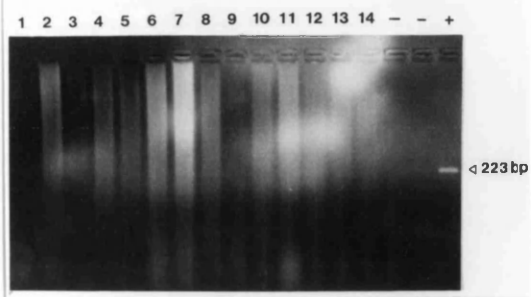

1 2 3 4 5 6 7 8 9 10 11 12 13 14 – – +
223 bp

Table 4.1. Correlation of the grade of cervical disease and prevalence of HPV 16 for women from a General Practice population.

Final Diagnosis	HPV 16 Positive	Percentage
Normal	26/151	17.2
WVI	0/10	0.0
CIN 1	2/13	15.4
CIN 2	2/5	40.0
CIN 3	3/4	75.0
Total	33/183	18.0

Table 4.2. Comparison of the prevalence of HPV 16 in women with severe disease (CIN 2/3) with that of women with mild disease (CIN 1/WVI) or no abnormalities in a General Practice population.

Clinical Diagnosis	HPV 16 Positive	HPV 16 Negative	Total Number in Group
Normal/WVI/CIN 1	28	146	174
CIN 2/3	5	4	9
Total	33	150	183

Fishers exact test: $p = 0.009$

Table 4.3. The sensitivity, specificity, positive predictive value, negative predictive value and relative risk associated with using HPV 16 as an indicator of significant cervical disease in a General Practice population.

Parameter	Value (%)
Sensitivity	5/9 (56)
Specificity	146/174 (84)
Positive predictive value	5/33 (15)
Negative predictive value	146/150 (97)
Relative risk	5.63
[95% confidence limits]	[1.61-20.02]

HPV 16 positivity increases with severity of disease from 15.4% in women with CIN 1, 40% in those with CIN 2 to 75% of women with CIN 3.

Correlation of CIN lesion with HPV 16 status within the HPV 16 positive and negative population is shown in Table 4.2. For analysis the women were separated into two groups; those with severe cervical disease (CIN 2 or 3) and those who were normal or who had mild disease (CIN 1/WVI). Five of the nine women (56%) with severe disease were positive for HPV 16 whilst 28 of the remaining 164 women, who were either normal, or had mild disease, were HPV 16 positive (17%). Analysis of these results by Fishers exact test yielded a p value of 0.009.

Table 4.3 shows the sensitivity, specificity, positive and negative predictive values and relative risk of HPV 16 with respect to severe cervical disease in this study group. Although the sensitivity and positive predictive values are low (56% and 15% respectively) the specificity and negative predictive value are high (84% and 97% respectively). The relative risk of 5.63 (95% confidence limits: 1.61-20.02) indicates a strong association between the presence of HPV 16 and severe cervical disease.

Correlation of the cytological analysis and HPV 16 positivity with the final diagnosis as determined by histological examination is shown in Table 4.4. These data were used to calculate the sensitivity of cytology and PCR for HPV 16 to identify women with cervical disease. The results are shown in Table 4.5 and demonstrate that although the sensitivity of the two techniques is comparable, cytology is more sensitive than PCR for HPV 16. However, when cytology and PCR for HPV 16 were combined, the number of women identified with cervical

disease increased.

The false negative and false positive rates for both cytology and PCR for HPV 16 are shown in Table 4.6. There was no significant difference between the false negative rate of 7.5% for cytology and 10.0% for PCR for HPV 16. However, there was a significantly higher probability of obtaining a false positive result in a healthy woman when using PCR for HPV 16 ($p < 0.05$).

Table 4.4. Correlation of the results of cytology and PCR for HPV 16 with the final diagnosis determined by histological analysis of biopsy samples.

Cytology Report (total number)	Final Diagnosis (total number)	PCR for HPV 16
Severe Dyskaryosis (3)	CIN 3 (3)	2/3
Moderate Dyskaryosis (4)	CIN 3 (1) CIN 2 (2) Normal (1)	1/1 1/2 0/1
Mild Dyskaryosis (15)	CIN 2 (1) CIN 1 (3) Normal (11)	0/1 1/3 1/11
Normal (161)	CIN 2 (2) CIN 1 (10) Normal (149)	1/2 1/10 25/149

Table 4.5. Comparison of the sensitivity of cytology and PCR for HPV 16 to identify all cervical disease and CIN 2 and 3.

Screening Method	Sensitivity for all Cervical Disease	Sensitivity for CIN 2 and 3
PCR for HPV 16	7/23 (30.4%)	5/9 (55.5%)
Cytology	10/23 (43.5%)	7/9 (77.7%)
Cytology and HPV 16	12/23 (52.2%)	8/9 (88.8%)

Table 4.6. False positive and false negative rates associated with the use of cytology and PCR for HPV 16 to identify women with cervical disease.

Screening Method	False Positive Rate	False Negative Rate
Cytology	12/22 (54.5%)*	12/161 (7.5%)**
PCR for HPV 16	26/33 (78.8%)*	5/150 (10.0%)**

* Chi squared $= 3.63, p < 0.05$
** Not significant

4.7 DISCUSSION.

The PCR system, described in Chapter 3, has been utilized for the detection of HPV 16 in cervical scrape samples from 183 women from a General Practice. HPV 16 was selected for the investigation because it has been implicated in cervical disease by a variety of epidemiological studies (McCance *et al.*, 1985b; Campion *et al.*, 1986; Meanwell *et al.*, 1987) and may be of use as an indicator of significant cervical disease (Crum *et al.*, 1985; Burk *et al.*, 1986; Campion *et al.*, 1986). In addition, *in vitro* studies have identified the transforming potential of HPV 16 (McCance *et al.*, 1988; Storey *et al.*, 1988; Woodworth *et al.*, 1989).

HPV 16 DNA was found to be present in 17% of normal women from a General Practice population, which is compatible with the data available from hybridization studies and some PCR data (McCance *et al.*, 1985b; Cox *et al.*, 1986; Meanwell *et al.*, 1987; Kyo *et al.*, 1991), but contrasts with data from certain studies using PCR to detect HPV 16 DNA (Tidy *et al.*, 1989, Rakoczy *et al.*, 1990). Thus, the results of Study A indicated that certain of the PCR studies, demonstrating a high prevalence of HPV 16 in 'normal' women, may be in error. This may be due to contamination of the PCR system (Tidy and Farrell, 1989), or incomplete diagnosis of the women when only cervical smear results are used to determine the diagnosis (Munoz *et al.*, 1988; Syrjanen *et al.*, 1990). Cytology has been shown to be associated with a 24% false-negative rate (Giles *et al.*, 1988) and therefore some women with cervical disease may be considered normal when examined by cytology only, without the additional sensitivity of both colposcopy and histology.

When the grade of lesion was correlated with HPV 16 positivity in those

women with cervical disease, HPV 16 infection was found to be present in 75% of women with CIN 3, 40% of women with CIN 2 and only 15.4% of women with CIN 1. The results demonstrate a trend of increasing prevalence of HPV 16 infection with greater severity of disease. Although the numbers of women with disease in this study were too small for the statistical significance to be accurately assessed, other studies have identified similar trends by both dot blot and PCR (McCance *et al.*, 1985b; Shirasawa *et al.*, 1986; Mayelo *et al.*, 1990; van den Brule *et al.*, 1991).

Analysis of the prevalence of HPV 16 in women with severe disease (CIN 2/3) versus women who were either normal, or who had mild disease (CIN 1/WVI) demonstrated that the presence of HPV 16 was significantly associated with severe cervical disease ($p = 0.009$). The relative risk indicated that women with either CIN 2 or 3 were 5.63 times more likely to have had an HPV 16 infection. The very high values obtained for the specificity and negative predictive value indicated that women who are not infected with HPV 16 are highly unlikely to have cervical disease. The low sensitivity and positive predictive value indicate that women with HPV 16 infection do not necessarily have current cervical disease.

There are problems associated with screening for cervical disease using cytological analysis. There is a high false negative rate associated with this technique (Giles *et al.*, 1988) and the smear result may be affected by the quality of the sample and number of cells obtained. The sensitivity of the PCR assay is such that the detection of HPV 16 DNA in cervical scrapes with low numbers of cells is feasible. The efficiency of cytology and PCR for HPV 16 for detecting

cervical disease was therefore assessed using the initial cytology report for each patient in Study A. PCR for HPV 16 compared favourably with cytology for identification of women with all grades of CIN and in certain cases complimented the results obtained with cytology. These results are compatible with those of Ritter *et al.* (1988) using conventional DNA hybridization for the detection of HPV 16 DNA. The false positive rate of PCR for HPV 16 was higher and the sensitivity was lower than that of cytology for detection of cervical disease. However, in combination the two techniques were more sensitive in detecting cervical disease.

Since the General Practice was in a predominantly middle class area and the mean age of the women was 39.4, ranging from 18 to 76, the prevalence of HPV 16 in the study group of 183 women volunteers should be representative of that in the screened population. Since HPV 16 DNA was present in 17% of women without cervical disease, the possible role of HPV 16 alone in the aetiology of cervical cancer remains an open question and additional agents may well play a role in the development of cervical disease. As expected for a sample from a General Practice, the number of patients with CIN 2 or 3 was small, however, three of the four women with CIN 3 and two of the five women with CIN 2 were successfully identified by the PCR system. The results demonstrate that HPV 16 is significantly associated with severe cervical disease and may be of use as an indicator of cervical disease when used in conjunction with standard cytological analysis.

CHAPTER 5: A COMPARISON OF SOUTHERN BLOT HYBRIDIZATION WITH PCR FOR THE DETECTION OF HPV 16 IN CERVICAL SCRAPE SAMPLES.

5.1 INTRODUCTION.

The results of Study A, described in Chapter 4, showed that HPV 16 is a useful indicator of severe cervical disease. Therefore, a rapid and simple diagnostic test for the detection of HPV 16 DNA may have the potential to highlight women at risk of cervical cancer. The purpose of this study was to compare the PCR system described in Chapter 3 with Southern blot hybridization in order to determine the system which would be most suitable for development as a possible diagnostic test for the analysis of HPV 16 DNA in cervical scrape samples.

Although Southern hybridization is less sensitive than PCR, the former technique has several advantages. The use of low stringency hybridization conditions allows the detection of related papillomaviruses, which may not be detectable by PCR if the DNA sequence is unknown (Williamson *et al.*, 1989; Brandsma *et al.*, 1989; Melchers *et al.*, 1989b). Integration of HPV DNA into the host cell genome, which is thought to be a significant event in the progression of CIN to cervical carcinoma, can be distinguished from episomal viral forms by Southern Blot hybridization (Durst *et al.*, 1983; Lehn *et al.*, 1988; Wagatsuma *et al.*, 1990). Southern blot hybridization requires high copy numbers of target DNA for detection and may preferentially identify women with severe cervical disease on the assumption that these women harbour greater quantities of viral DNA.

These higher levels of DNA may be a result of clonal expansion of cells containing integrated HPV sequences or duplication of the integrated HPV DNA, as has been demonstrated in CaSki cells which contain approximately 600 copies of HPV 16 per cell (Yee *et al.*, 1985). However, integration often results in a loss of episomal HPV DNA and prior to clonal expansion the quantity of HPV DNA within lesions containing integrated HPV DNA may be greatly reduced (Shirasawa *et al.*, 1986). Southern blot has therefore been used to analyse cervical scrape samples from women with and without disease which are known to be positive for HPV 16 DNA by PCR in order to determine whether Southern blot hybridization may be more selective in detecting the presence of HPV 16 in cervical scrapes from women with cervical disease.

5.2 Study Population.

The study samples for this analysis consisted of 48 cervical scrapes which had previously been shown to contain HPV 16 DNA by PCR as shown in Chapter 4. Thirty-six samples were from study group A and the remaining 12 were from patients who had been excluded from study A due to a history of cervical abnormalities or treatment for benign disorders as explained in Chapter 4, section 4.2. All patients had previously been analysed by cytology, colposcopy and where appropriate, histopathological analysis of biopsy specimens. Five control samples were randomly chosen from the 164 patients in study group A, who had no evidence of HPV 16 infection by PCR. The DNA had previously been extracted from the cervical scrapes and the DNA concentration of each sample measured as described in Chapter 2, section 2.4.

5.3 Southern Blot Hybridization.

Cervical scrape DNA (5μg) was digested with *Bam*H1 restriction enzyme and electrophoresed through 1% agarose gels. The DNA was transferred to nylon membranes and after hybridization with ^{32}P-labelled HPV 16 DNA under high stringency conditions the filters were exposed to X-ray film for between 16 and 48 hours. The methods are described in Chapter 2, sections 2.6-2.15.

The selection of samples and Southern blot hybridization were completed without knowledge of the clinical status of the patients and correlation of results with the clinical diagnosis was not effected until the analysis was complete.

5.4 Sensitivity of Southern Blot Hybridization and PCR.

The sensitivity of the Southern blot hybridization is shown in Chapter 2, Figure 2.4. The detection level of the assay was 10pg of cloned HPV 16 DNA, which corresponds with 500,000 copies of the viral genome. The sensitivity of the PCR is shown in Chapter 3, Figure 3.6 and the system was capable of detecting 0.1fg of cloned HPV 16 DNA which corresponds to approximately five genome equivalents. Thus, PCR is approximately 100,000 times more sensitive than Southern blot hybridization for the detection of HPV 16 DNA.

5.5 Southern Blot Hybridization.

Southern blot hybridization was carried out using cervical scrape samples which had previously been found to be HPV 16 positive by PCR. The results of these analyses are shown in Figure 5.1. Of the 48 PCR positive samples, only five samples resulted in positive signals by Southern blot hybridization, which were samples 20, 34, 37, 41 and 44. Sample 20 contained both episomal HPV 16 DNA of 7.9kbp in size and integrated HPV 16 of greater than 20kbp. The five control samples, which were HPV 16 negative by PCR, were also negative by Southern blot.

5.6 Correlation of the Southern Blot Analysis with Clinical Diagnosis.

The clinical status of each patient was correlated with the HPV 16 positivity, as analysed by Southern blot hybridization. Two of the five patients with detectable levels of HPV 16 had CIN 2 at the time of tissue sampling and the

remaining three patients were all cytologically normal. The Southern blot technique failed to detect HPV 16 DNA sequences in 43/48 samples which were known to be HPV 16 positive by PCR. Within this group there were 12 patients with cervical abnormalities, four of whom had CIN 3, three who had CIN 2 and the remaining seven patients had either CIN 1 or WVI. The results are summarized in Table 5.1.

Figure 5.1. Southern blot hybridization analysis of 48 PCR positive cervical scrape samples for the presence of HPV 16. Five PCR negative controls were included (-) and size markers (M) of cloned HPV 16 DNA.

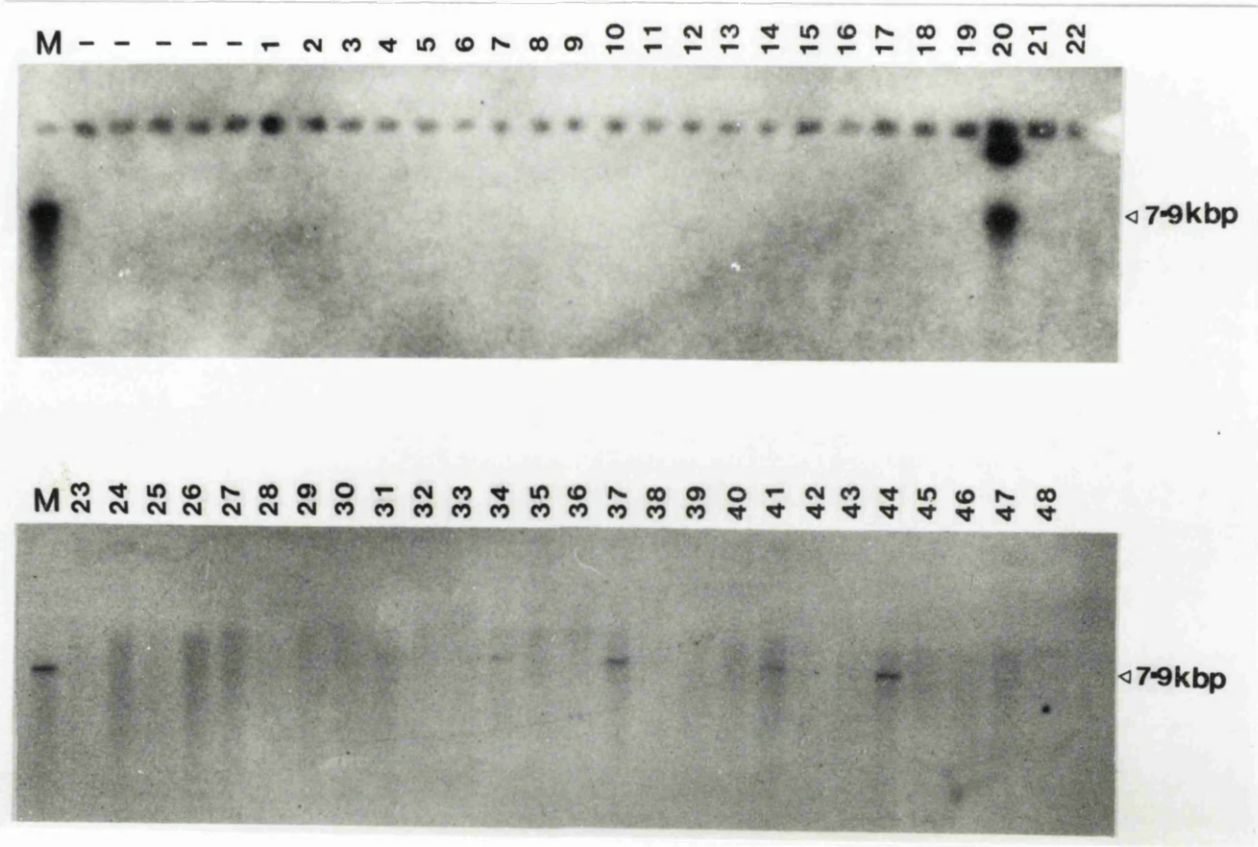


Table 5.1. Correlation of the results of Southern blot hybridization with grade of disease for patients who were positive for HPV 16 by PCR.

Clinical Status	Number of Patients	HPV 16 Positive by Southern Blot	HPV 16 Negative by Southern Blot
Normal	34	3 (9%)	31 (91%)
WVI	2	0	2 (100%)
CIN 1	3	0	3 (100%)
CIN 2	5	2 (40%)	3 (60%)
CIN 3	4	0	4 (100%)
Total	48	5 (10.4%)	43 (90%)

5.7 DISCUSSION.

The PCR system, described in Chapter 3, is approximately 100,000 times more sensitive than Southern blot hybridization in detecting cloned HPV 16 DNA and this result is in agreement with other studies (Tham et al., 1991). It would, therefore, seem to be the most accurate technique for screening women for the presence of HPV DNA. The results of Study A, described in Chapter 4, demonstrated a low prevalence of HPV 16 in normal women of 17%, however, several other studies have shown a very high prevalence of HPV 16 by PCR in women with no evidence of cytological abnormalities (Young *et al.*, 1989; Tidy *et al.*, 1989; Rakoczy *et al.*, 1990). It is possible that the increased sensitivity may render PCR unsuitable for the identification of women who are at risk of significant cervical disease.

Cervical scrapes from 48 women from a General Practice population, which were previously shown to contain HPV 16 DNA by PCR, were re-analysed by Southern blot hybridization to determine whether the latter technique may be more specific than PCR in identifying patients with severe cervical disease. Within this group there were 14 women who had CIN or WVI and the remaining 34 women had no cervical abnormalities. Five patients were found to be HPV 16 positive by Southern hybridization; two of these had CIN 2 and the other three women were all cytologically normal. Southern blot failed to detect HPV 16 sequences in 43 of the PCR positive cervical scrape samples, 12 of whom had cervical disease. As expected, none of the control samples, which had previously been shown to be negative by the PCR, were positive by Southern blot.

The results demonstrated that Southern blot hybridization was less

sensitive than PCR in detecting HPV 16 DNA in clinical specimens and are in agreement with the results of other studies (Melchers *et al.*, 1989b; Rakoczy *et al.*, 1990; Syrjanen *et al.*, 1990). The Southern blot system was unable to identify those women with cervical disease in preference to those who are cytologically normal, therefore PCR was the most accurate technique for screening women for the presence of HPV 16 in order to identify women who have cervical disease.

CHAPTER 6: STUDY B; ANALYSIS OF THE PREVALENCE OF HPV 16 IN WOMEN FROM A COLPOSCOPY CLINIC.

6.1 INTRODUCTION.

The results of Study A, described in Chapter 4, demonstrated that HPV 16 was significantly associated with cervical disease and detection for HPV 16 DNA was found to be useful as an indicator of cervical disease. The number of patients with CIN in Study A was small, but the results are comparable with those from other studies in which HPV 16 and 18 were found in 24-100% of women with CIN (Crum *et al.*, 1985; McCance *et al.*, 1985b; di Luca *et al.*, 1986; Bevan *et al.*, 1989; Cornelissen *et al.*, 1989; Mayelo *et al.*, 1990; Kyo *et al.*, 1991; van den Brule *et al.*, 1991). An increasing prevalence of HPV 16 with greater severity of disease was identified in Study A and this trend is identical to that of other studies in which HPV 16 and 18 which are present in 23-39% of CIN 1, 50-66% of CIN 2 and 55-71% of CIN 3 lesions (Wagner *et al.*, 1984; McCance *et al.*, 1985b; Campion *et al.*, 1986; Shirasawa *et al.*, 1986; Fuchs *et al.*, 1987; van den Brule *et al.*, 1991). The trend is reversed with HPV 6 and 11 which are present in 40-50% of CIN 1, 20% of CIN 2 and 15-23% of CIN 3 lesions (Wagner *et al.*, 1984; McCance *et al.*, 1985b; Campion *et al.*, 1986; Fuchs *et al.*, 1987).

CIN 2 and CIN 3 lesions are highly likely to progress to carcinoma (Harris and Peterson, 1956; Kinlen and Spriggs, 1978; McIndoe *et al.*, 1984) and therefore patients with these lesions are treated using local ablation therapy. However, CIN 1 lesions may regress, remain unchanged or progress to severe disease (Fox, 1967; Richart and Barron, 1969; Spriggs, 1984; Evans and Monaghan, 1985;

Weaver *et al.*, 1991). Women who are diagnosed as having CIN 1 are not usually treated immediately, but the state of the lesion is closely monitored by repeat cytology at regular intervals of 3-12 months. Morphological changes have not been found to be useful in predicting the prognosis of a particular lesion (Kottmeier, 1961; Fidler *et al.*, 1968; Spriggs, 1984), but several studies have suggested detection of HPV DNA as an alternative indicator which may be useful in determining whether low grade CIN lesions are likely to progress to more severe disease (Schneider *et al.*, 1985; Burk *et al.*, 1986; Campion *et al.*, 1986; McNicol *et al.*, 1989). The present study (Study B) was designed to examine whether HPV 16 positivity could be used as an indicator of severe disease in women who have been referred to a colposcopy clinic with a smear suggesting mild dyskaryosis.

6.2 Patients and Clinical Analysis.

Study group B was composed of 200 patients sequentially attending the Royal Free Hospital colposcopy clinic, having been referred with a cervical smear report suggesting mild dyskaryosis. Once a medical and cytological history had been obtained, each patient underwent a full cervical examination as outlined in Chapter 4, section 4.2. A full diagnosis could not be completed for 21 women in this group, due to unsatisfactory smear specimens, and these patients were excluded from the final analysis. Patients within the study group diagnosed as having CIN 2 or 3 were treated by local ablation therapy using a carbon dioxide laser. The remaining patients were monitored by cytological and colposcopic examination every four months.

6.3 Sample Collection and Preparation.

Cervical cell collection and preparation was carried out as detailed in Chapter 4, section 4.3.

6.4 Polymerase Chain Reaction for the Detection of HPV 16 DNA.

Duplicate PCR analyses were performed for each sample, on two separate occasions, using 1μg of DNA for the target. The PCR system described in Chapter 3 was used except that 0.05% polyoxyethylene sorbitan monolaurate (Tween-20) and 0.05% Nonidet P-40 were added to the 1 x Taq buffer. Positive and negative controls of cloned HPV DNA and no target DNA respectively were included in each PCR analysis. Samples which showed discrepant results in the duplicate

analyses were subjected to PCR on two additional occasions. All laboratory experiments were conducted without knowledge of the patients clinical status.

6.5 Statistical Analysis.

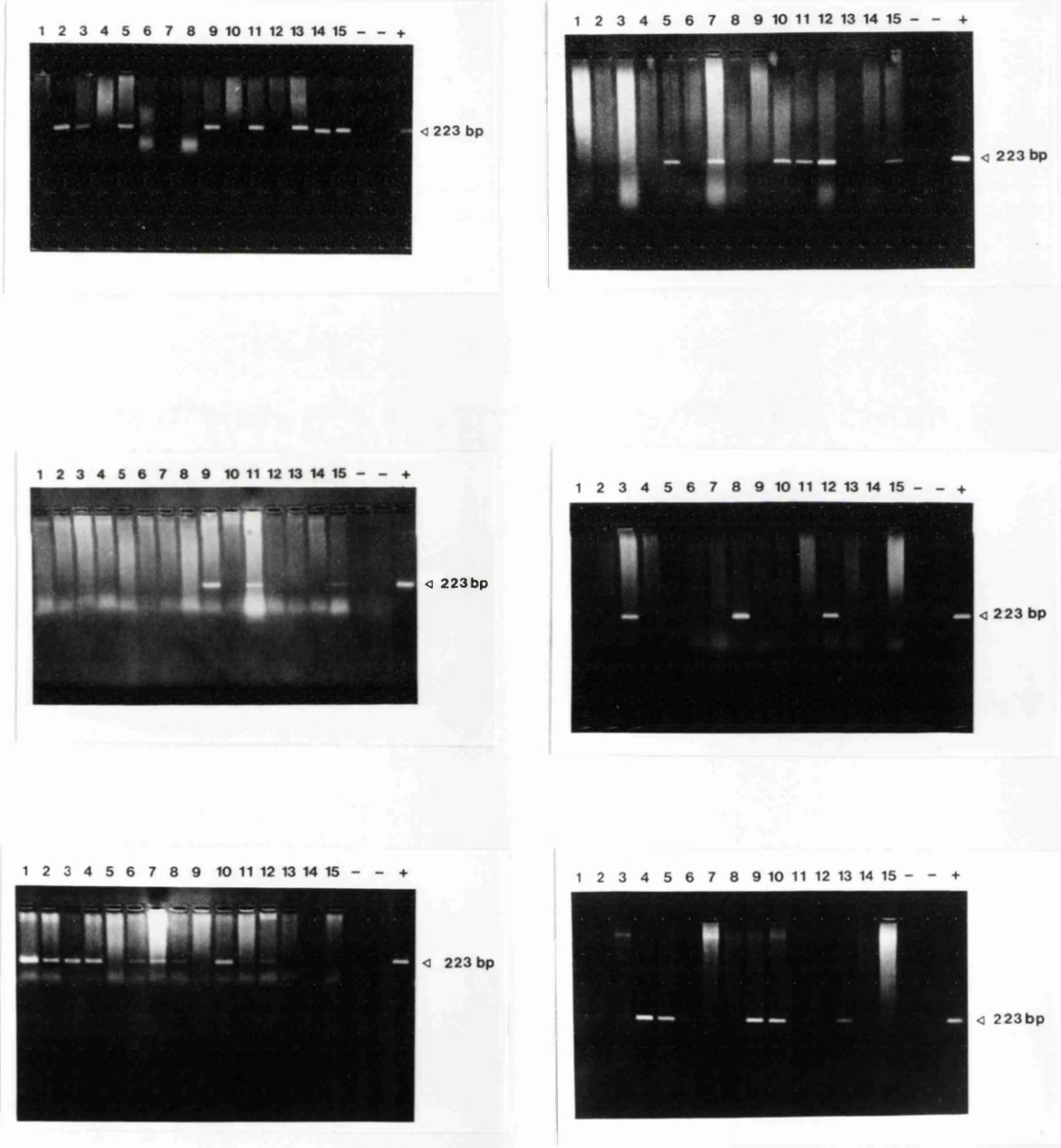
Statistical analysis of the distribution of results was determined using the Chi Squared test. The sensitivity, specificity, positive predictive value, negative predictive value and relative risk of severe cervical disease and HPV 16 were calculated as described in Chapter 4, section 4.5.

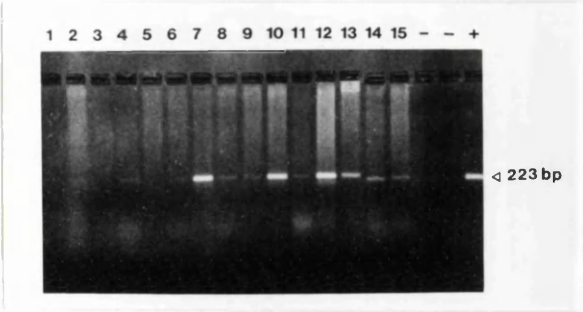
6.6 Study B.

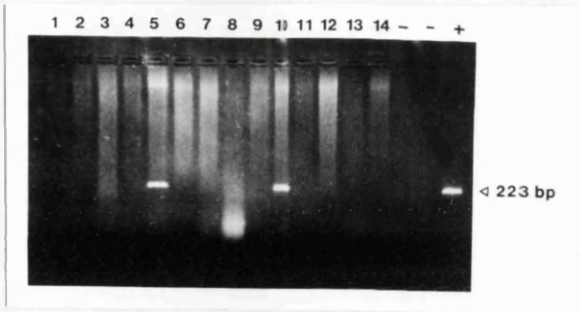
Two hundred women, referred to the Royal Free Hospital colposcopy clinic with a cervical smear report suggesting mild dyskaryosis, were examined by colposcopy, cytology and where appropriate histology. Each patient was assigned to the following groups according to the clinical examination: normal, WVI, CIN 1, CIN 2 or CIN 3. The results are summarized in Table 6.1. A final diagnosis could not be completed for 21 patients due to unsatisfactory cervical smears, leaving 179 women in study B. The mean age of these women was 29 years (range 17-61), the mean age of those with CIN was 26.9 years and the mean age of those with no cervical abnormalities was 33.5 years.

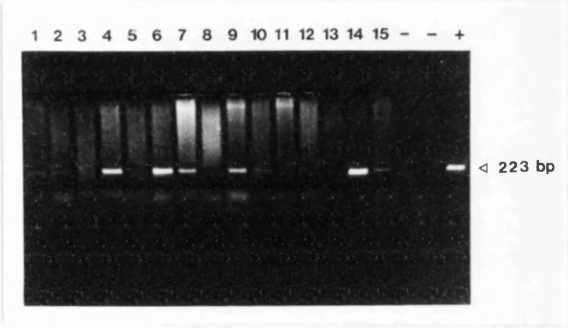
PCR analysis of cervical scrapes from the women in Study B showed that 109 of 179 (61%) were positive for HPV 16, whereas 70 (39%) had no detectable HPV 16 DNA. Results of the PCR analysis are shown in Figure 6.1. Clinical analysis of women in Study B resulted in a diagnosis of histologically proven CIN 1, 2 or 3 in 101 women. A further 14 women were diagnosed as having WVI while the remaining 54 women had no cervical abnormalities. The patients were stratified according to grade of disease and the prevalence of HPV 16 in these groups is shown in Table 6.1. HPV 16 positivity increased with greater severity of disease from 35.6% in women with WVI, 53% and 64% in women with CIN 1 and 2, to 74% of patients with CIN 3. The prevalence of HPV 16 in women who have had mild dyskaryosis, but who at the time of examination had normal cervical pathology was 63%.

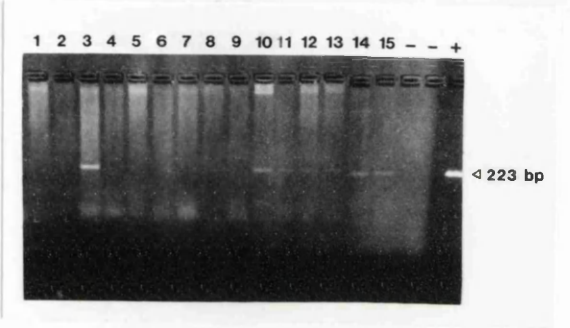
Figure 6.1. PCR analysis of cervical scrape samples from Study B. PCR amplification of HPV 16 DNA in positive samples resulted in a fragment of 223bp in length. Negative (-) and positive (+) controls were included in each analysis.

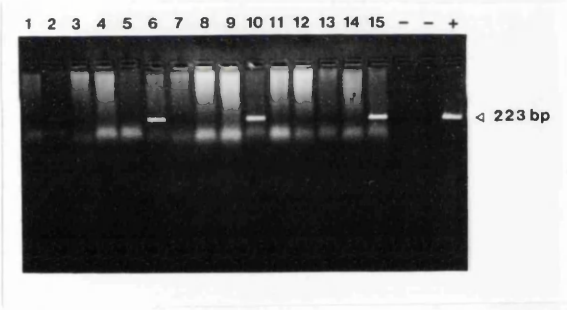


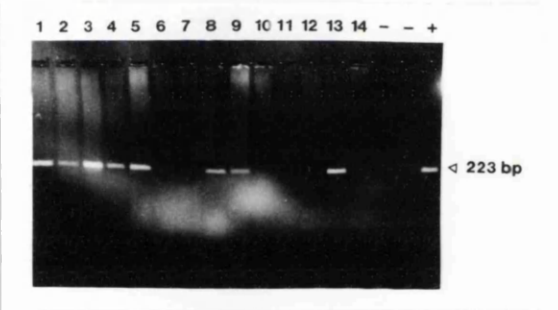

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 – – +
223 bp

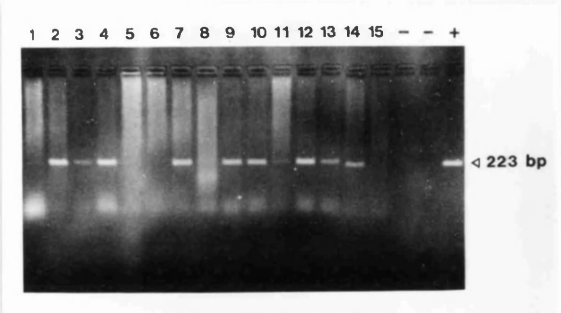

1 2 3 4 5 6 7 8 9 10 11 12 13 14 – – +
223 bp


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 – – +
223 bp


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 – – +
223 bp


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 – – +
223 bp


1 2 3 4 5 6 7 8 9 10 11 12 13 14 – – +
223 bp


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 – – +
223 bp

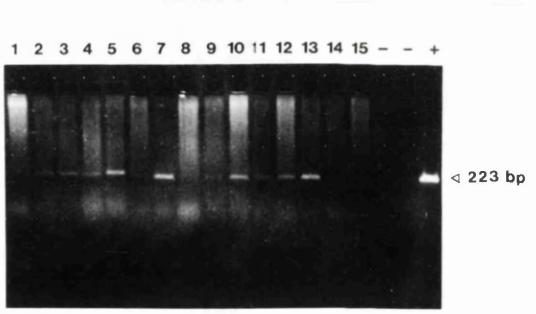

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 – – +
223 bp

Table 6.1. Correlation of grade of cervical disease and prevalence of HPV 16 DNA in patients referred to a colposcopy clinic with a smear suggesting mild dyskaryosis.

Final Diagnosis	HPV 16 Positive	Percentage
Normal	34/54	63.0
WVI	5/14	35.6
CIN 1	24/45	53.0
CIN 2	20/31	64.0
CIN 3	26/35	74.0
Total	109/179	61.0

Table 6.2. Comparison of the prevalence of HPV 16 in women with severe disease with that of women with mild disease or no abnormalities.

Clinical Diagnosis	HPV 16 Positive	HPV 16 Negative	TotalNumber in Group
Normal/WVI/CIN 1	63	50	113
CIN 2/CIN 3	46	20	66
Total	109	70	179

Chi squared $= 2.84; p < 0.0919$

Table 6.3. The sensitivity, specificity, positive predictive value, negative predictive value and the relative risk associated with using HPV 16 as an indicator of severe cervical disease in a colposcopy clinic population.

Parameter	Value (%)
Sensitivity	46/66 (69.7)
Specificity	50/113 (44.2)
Positive Predictive Value	46/109 (42.2)
Negative Predictive Value	50/70 (71.0)
Relative Risk [95% confidence limits]	1.48 [0.96-2.27]

A comparison of the prevalence of HPV 16 in women with severe cervical disease (CIN 2/3) with that of women with no abnormalities or mild disease (normal/WVI/CIN 1) is shown in Table 6.2. Of the 66 women who had severe disease, 46 were positive for HPV 16 (69.7%) whilst 63 of the remaining women who were either normal, or who had WVI/CIN 1, were HPV 16 positive (55.7%). There was no significant difference in the HPV 16 positivity between these two groups of women.

The sensitivity, specificity, positive and negative predictive values and relative risk of HPV 16 infection with respect to cervical disease in study group B are shown in Table 6.3. The sensitivity and negative predictive value were high (96.7% and 71% respectively), but the specificity and positive predictive value were low (44.2% and 42.2% respectively). The relative risk associated with having cervical disease and HPV 16 infection was 1.48.

6.7 Correlation of Study A with Study B.

The results of Study A, described in Chapter 4, were correlated with those of Study B. The overall prevalence of HPV 16 within these study groups is shown in Table 6.4. Thirty-three women from study A (18%) and 109 from study B (61%) had detectable levels of HPV 16 DNA. Statistical analysis of these results using the Chi squared test showed a significant difference in the prevalence of HPV 16 between the two populations ($p < 0.0001$).

Each woman from Study A and Study B was assigned to a clinical group according to the final diagnosis. The HPV 16 positivity for each clinical group in both studies is shown in Table 6.5. HPV 16 was most prevalent in patients with

Table 6.4. The prevalence of HPV 16 DNA in women from a General Practice population (Study A) and women referred to a colposcopy clinic with a smear suggesting mild dyskaryosis (Study B).

Study Population	Total	HPV 16 Positive (%)
A	183	33 (18)
B	179	109 (61)

$X^2 = 69.93; p < 0.0001$

Table 6.5. Comparison of the prevalence of HPV 16 DNA in women with a final diagnosis of normal cervical pathology, WVI, CIN 1, CIN 2 or CIN 3 within Study A and Study B. The statistical significance of differences between the two studies is shown (n.s. = not significant).

Final Diagnosis	Study A Positive/Total (%)	Study B Positive/Total (%)	Statistical Significance
Normal	26/51 (17.2)	34/54 (63.0)	< 0.0001
WVI	0/10 (0)	5/14 (35.7)	< 0.003
CIN 1	2/13 (15.4)	24/45 (53.3)	< 0.03
CIN 2	2/5 (40.0)	20/31 (64.5)	n. s.
CIN 3	3/5 (75.0)	26/35 (74.3)	n. s.

severe cervical disease (CIN 2 or 3) in both study A and Study B and there was no significant difference in the values for HPV 16. Women in Study B who were diagnosed as having WVI or CIN 1 were more likely to be HPV 16 positive than those in Study A who had the same diagnosis. These differences were statistically significant with p values of 0.03 and 0.003 for WVI and CIN 1 respectively.

There was a significant difference between the prevalence of HPV 16 in normal women who had never had cervical disease from Study A and that of women from Study B who had a history of cervical disease, but no abnormalities at the time of colposcopic examination ($p < 0.0001$).

After combining the results of the two studies a significant difference was found between women who have never had cervical disease and those who have current abnormalities ($p < 0.0001$). Results of this analysis are shown in Table 6.6.

The prevalence of HPV 16 infection in women with minor grade disease (WVI/CIN 1) versus those with major grade disease (CIN 2/CIN 3) was analysed and the results are shown in Table 6.7. Statistical analysis using the Chi Squared test gave a p value of < 0.0002, confirming that HPV 16 is significantly associated with major grade CIN.

Table 6.6. Comparison of the prevalence of HPV 16 DNA between women who have never had cervical disease and those who have current abnormalities.

Cervical Pathology	Number of Women in Study A and B	Number HPV 16 Positive (%)
Normal	151	26 (17.2)
Abnormal	157	82 (52.2)

Chi squared = 20.37; $p < 0.0001$

Table 6.7. Comparison of the prevalence of HPV 16 DNA between women with minor grade disease (CIN 1/WVI) and those who have severe cervical disease (CIN 2/3).

Cervical Pathology	Number of Women in Study A and B	Number HPV 16 Positive (%)
Minor Grade	82	31 (37.8)
Major Grade	75	51 (68.0)

Chi squared = 4.5, $p < 0.002$

6.8 Reproducibility of PCR for Detection of HPV 16 DNA.

In order to eliminate errant results, duplicate analyses of each cervical scrape sample were performed and the results are shown in Table 6.8.These data, obtained from multiple analyses of the 400 samples in Study A and Study B, allowed the false positive and false negative rates for the PCR assay to be calculated.

Discrepant results between the two analyses were obtained in 12% of samples. Upon further investigation, 8.8% of these were consistently positive in the subsequent analyses and were therefore scored as true positive results. The remaining 3.2% of discrepant samples were consistently negative on further examination and therefore the positive results in the initial analysis were presumed to be false positive signals. Thus, for a single PCR analysis 3.2% of positive signals and 8.8% of negative signals were presumed to be errant results. However, after duplicate analyses were performed the false positive rate was reduced to 0.1% ($100 \times [0.032]^2$) and the false negative rate reduced to 0.77% ($100 \times [0.088]^2$).

Table 6.8. Results of duplicate PCR analyses of the 400 cervical scrape samples from Study A and Study B.

First Run	Second Run	Total (%)	True Positive	True Negative
-ve	-ve	253 (63.2)	N/A	N/A
+ve	+ve	99 (24.8)	N/A	N/A
-ve	+ve	22 (5.5)	14	8
+ve	-ve	26 (6.5)	21	5
Cumulative Totals (%)		400	35 (8.8)	13 (3.2)

6.9 DISCUSSION.

Comparison of Southern blot hybridization with PCR, as described in Chapter 5, showed that PCR would be the most suitable method for routine screening of cervical scrape samples. However, the use of PCR for diagnostic use has not been fully assessed. Therefore, the reproducibility of the PCR system for detection of HPV 16 DNA was calculated using the PCR data from Study A and Study B. After PCR analysis of 400 samples it was found that on any one occasion 3.2% of signals may be due to false positive reactions, possibly caused by contamination with target DNA. The false negative rate of 8.8% may be attributable to DNA sampling error when assaying samples containing only a few copies of target sequence. The effect of performing duplicate analyses was to reduce the false positive rate to 0.1% and false negative rate to 0.77%. The adoption of duplicate testing could therefore achieve a substantial reduction in erroneous results generated by PCR and allow this technique to be used for clinical diagnosis.

The results of Study A, described in Chapter 4, demonstrated that detection of HPV 16 in cervical scrape samples was useful in identifying women with severe cervical disease from a General Practice population. A second population for whom detection of HPV 16 infection maybe a useful indicator of severe disease is that of women referred to colposcopy clinics with mild dyskaryosis. These women present a clinical dilemma since a proportion of low grade cervical disease may progress to severe CIN or carcinoma over a period of 10-40 years, whereas 26-60% of lesions may persist or regress (Fox, 1967; Hulka, 1968; Richart and Barron, 1969; McGregor and Lupen, 1978; Evans and

Monaghan, 1985; Campion *et al.*, 1986; Weaver *et al.*, 1990). Detection of HPV 16 DNA could be used to identify those women referred to colposcopy clinics with mild dyskaryosis who actually have more severe cervical disease and require urgent treatment.

Cervical scrapes from 200 women referred to the Royal Free Hospital colposcopy clinic with a smear report suggesting mild dyskaryosis were analysed by PCR for the presence of HPV 16 DNA and the results were correlated with the grade of lesion at the final diagnosis. In those women found to have current cervical disease there was a trend of higher prevalence of HPV 16 with increasing severity of CIN from 53% of those with CIN 1, to 64 and 74% of those with CIN 2 and 3. This trend confirms a close association of HPV 16 with high grade CIN and is in agreement with the results of Study A and those of other studies (McCance *et al.*, 1985b; Shirasawa *et al.*, 1986; Mayelo *et al.*, 1990; van den Brule *et al.*, 1991). There was no significant difference in the prevalence of HPV 16 in women with CIN 2 or 3 from Study A and B. However, patients in Study B with CIN 1 or WVI were more likely to have an HPV 16 infection than those from Study A. The prevalence of HPV 16 in women from Study B who had a previous abnormal smear, but who were normal at the time of examination was 63%. This is unexpectedly high when compared with the prevalence in Study A of 17%, but is in agreement with that found by other studies when investigating women from a similar population who have a history of cervical disease (Schneider *et al.*, 1987; Mayelo *et al.*, 1990; Borst *et al.*, 1991).

The values calculated for the sensitivity and positive predictive value were higher for Study B than those of Study A, because a greater proportion of women

with disease in Study B were positive for HPV 16. However, the specificity and negative predictive value were lower than those for Study A due to the high HPV 16 positivity in normal women from Study B. The relative risk indicates that women with CIN 2 or 3 in Study B were 1.48 (95% confidence limits: 0.96-2.27) times more likely to have an HPV 16 infection than those without current disease. Since the relative risk in Study B (1.48) is lower than that of Study A (5.63) the risk of having severe cervical disease and HPV 16 infection is greater in women from a General Practice than in those attending a colposcopy clinic with mild dyskaryosis.

The results of Study A and Study B show that HPV 16 is more prevalent in women with CIN than in those with no cervical abnormalities and demonstrate a close association of HPV 16 with severe cervical disease. Although HPV 16 was found to be useful as an indicator of severe cervical disease in patients from a General Practice population (Study A) HPV 16 was of little value in identifying women with cervical disease from a population referred to a colposcopy clinic with a smear suggesting mild dyskaryosis This result is due to the high prevalence of HPV 16 in the apparently normal women within this population.

CHAPTER 7: ANALYSIS OF THE LONG CONTROL REGION OF HUMAN PAPILLOMAVIRUS TYPE 16 AND POSSIBLE CO-FACTOR INVOLVEMENT IN THE DEVELOPMENT OF DISEASE.

7.1 INTRODUCTION.

Human papillomaviruses have been implicated in the development of cervical disease, with a high prevalence of certain types of HPV such as 16, 18, 31, 33 and 35 in cervical carcinoma biopsies (van den Brule *et al.*, 1989; Cornelissen *et al.*, 1989; Griffin *et al.*, 1990). The HPV genome is episomal in normal cervices and low grade CIN lesions, but is found to be integrated into the host DNA in cervical carcinomas, carcinoma derived cell lines and some high grade CIN lesions (Shirasawa *et al.*, 1986; Lehn *et al.*, 1988; Wagatsuma *et al.*, 1990; Cullen *et al.*, 1991). Regulation of viral gene expression in both integrated and non-integrated HPV is mediated by the LCR, which contains the constitutive enhancer, E2-dependent enhancer, tissue specific promoter and other regulatory sequences.

The interaction of proteins with sequences within the LCR is likely to be important in regulating viral gene expression as has been found for other viruses and cell systems (Serfling, *et al.*, 1985). Protein binding sites within the HPV LCR have been mapped by DNase I footprinting analysis and several of the binding proteins have been identified. There are four motifs to which the E2 protein binds, three motifs of 5'-TGACTCA, or related sequences, to which AP-1 binds and seven copies of the 5'-TTGGC motif with which NF-1 interacts (Garcia-Carranca *et al.*, 1988; Hawley-Nelson *et al.*, 1988; Gloss *et al.*, 1989; Li *et al.*,

1989). A glucocorticoid responsive element (GRE) has been mapped to the enhancer region of the LCR which interacts with both progesterone and dexamethasone (Chan *et al.*, 1989). An additional GRE is present in HPV 16 LCR which has some homology with the GRE consensus sequence, but has not been found to respond to steroid hormones and is therefore presumed to be non-functional (Sibbet and Campo, 1990). An Oct-1 motif of 5'-AATTGCAT is situated within the constitutive enhancer of the LCR and interacts with Oct-1 via a multi-protein complex (Hoppe-Seyler *et al.*, 1991). A keratinocyte-specific transcriptional activator, KRF-1, has recently been identified and found to compete with Oct-1 for binding to enhancer sequences (Mack and Laimins, 1991).

The LCR is a major determinant in the immortalization potential of the different papillomaviruses (Romanczuk *et al.*, 1991). This effect may be mediated by sequence variation within the LCR altering protein factor binding and hence influencing viral gene expression. However, sequence divergence within the HPV LCR has not been extensively investigated.

The aim of this study was to clone the HPV 16 LCR from cervical scrape samples from women with and without cervical disease, determine the nucleotide sequence for comparison with the prototype HPV 16 and identify any nucleotide changes, particularly those within defined protein binding domains. The functional significance of any variations could subsequently be assessed using a promoter assay system.

METHODS.

7.2 Oligonucleotide Primer Design and Synthesis.

Two oligonucleotide primers, of 20bp in length, were designed to amplify the entire LCR of HPV 16 between the L1 and E6 open reading frames. The primers were called LCR 16 A and LCR 16 B. Two additional primers, LCR 16 C and LCR 16 D, were synthesized to be complementary to sequences within the HPV 16 LCR for use in DNA sequencing procedures. The nucleotide sequence and position of these primers within the HPV genome are shown in Table 7.1. All primers were synthesized and purified using the methods described in Chapter 3, section 3.3.

7.3 PCR Amplification of LCR 16 DNA.

Prior to PCR amplification, 10μg of each PCR primer was phosphorylated by the addition of 5μl of 10 x kinase buffer (0.5M Tris.HCl pH 7.6, 0.1M $MgCl_2$, 50mM dithiothreitol, 1mM spermidine, 1mM EDTA), 2μl 10mM ATP and 1 unit of T4 polynucleotide kinase in a final volume of 50μl. The primers were incubated at 37°C for 30 minutes, after which 2μl of 0.5M EDTA were added. The primers were purified by phenol:chloroform extraction and centrifugation through a Sephadex G-25 column as described in Chapter 2, section 2.15.

The optimal magnesium ion concentration was determined for the LCR primers as described in Chapter 3, section 3.7 and the LCR region of HPV 16 was amplified using the PCR system described in Chapter 3. The cycling conditions were altered to include an extension step of two minutes at 72°C.

Table 7.1. Nucleotide sequence and position of oligonucleotide primers for PCR amplification and dideoxy sequencing of the HPV 16 long control region.

Name	Sequence	Position
LCR 16 A	5'-CGTAAGCTGTAAGTATTGTA	7143-7162
LCR 16 B	5'-CCTGTGGGTCCTGAAACATT	103-122
LCR 16 C	5'-GCTTCAACCGAATTCGG	7445-7461
LCR 16 D	5'-CCTTATGCCAAATATGC	7736-7752

HPV 16 LCR DNA was amplified from cervical scrape samples obtained from two patients with HPV 16 infection. One patient had histologically proven CIN 3 (C3) and the second had no evidence of cervical disease (C0). The PCR products were electrophoresed through 0.8% low melting point agarose gels for 1-2 hours at 40 volts as described in Chapter 2, section 2.4. The DNA was visualized by UV light and the fragment of amplified DNA excised from the gel. The gel slice was placed in a 1.5ml microcentrifuge tube and 4-5 gel volumes of elution buffer (20mM Tris.HCl pH 8.0, 1mM EDTA) were added and heated to 65°C for ten minutes. To this solution was added 0.5 volume of phenol mix and the temperature maintained at 65°C for one minute or until the agarose had completely melted. The mixture was extracted twice with phenol mix, once with phenol:chloroform and finally with chloroform. The methods and solutions for the extraction procedure are detailed in Chapter 2, section 2.4. The PCR amplified DNA was precipitated with 100% ethanol, washed with 70% ethanol, vacuum dried and the pellet resuspended in 20µl of SDW.

7.4 Filling in of Recessed 3' Termini of Double Stranded DNA.

The recessed termini of the PCR amplified DNA were filled by the addition of 2.5µl of 10 x medium salt buffer, 2µl of 2.5mM dNTPs and 1 unit of Klenow fragment of DNA polymerase I in a final volume of 25µl. The mixture was incubated at room temperature for 30 minutes and the DNA purified by phenol extraction and ethanol precipitation. The prepared insert DNA was resuspended in 20µl of SDW.

7.5 Preparation of pUC13 Vector DNA.

Vector pUC13 DNA (10μg) was digested with *Sma*I restriction endonuclease in the presence of 1 x medium salt buffer for 3-5 hours at 37°C. The DNA was purified by phenol:chloroform extraction and ethanol precipitation using the methodology described in Chapter 2, section 2.4. The DNA was collected by centrifugation in a microcentrifuge at 13 000 rpm for 20 minutes. The DNA pellet was washed with 70% ethanol and, after drying, was resuspended in 42μl of SDW. To this was added 5μl of 10 x CIP buffer (0.5M Tris pH 9.0, 10mM $MgCl_2$, 1mM $ZnCl_2$, 10mM spermadine) and 3 units of calf intestine alkaline phosphatase. The DNA mixture was incubated at 37°C for a minimum of two hours. To this was added 10μl of 10 x TNE (100mM Tris.HCl pH 8.0, 1M NaCl, 10mM EDTA), 5μl of 10% SDS and 40μl of SDW and incubated at 55°C for 15 minutes. The dephosphorylated DNA was phenol extracted, ethanol precipitated and, after drying, resuspended in 20μl of SDW.

7.6 Ligation of pUC13 DNA with HPV 16 LCR DNA.

The PCR amplified DNA (500ng) was ligated into the vector DNA (100-200ng) in 1 x ligation buffer (50mM Tris.HCl pH 7.6, 10mM $MgCl_2$, 10mM dithiothreitol, 500μg/ml bovine serum albumin), 1μl of 6mM ATP and 2 units of T4 DNA ligase in a final volume of 15μl. A control ligation of vector DNA and ligation reagents, but no insert DNA, was carried out with all ligation procedures. All ligations were incubated at 15°C for 24-48 hours.

7.7 Preparation of Transformation Competent E. coli Cells.

A single colony of *E. coli* cells (JM109) was inoculated into 10ml of LB without ampicillin and incubated at 37°C for 16-20 hours, with shaking. An aliquot (500µl) of the culture was inoculated into 400ml of LB and incubated at 37°C, with shaking, until the absorbance of the solution at 550nm reached 0.2. The cells were then harvested by centrifugation at 1700g for 15 minutes. The cells were resuspended in ice cold TFN buffer (10mM Tris.HCl pH 8.0,50mM $CaCl_2$) to give a total volume of 200ml and incubated on ice for 20 minutes. The cells were centrifuged as before, resuspended in 20ml of TFN buffer and retained on ice. The competent cells were dispensed into 0.4ml aliquots and immediately frozen in a dry ice and ethanol bath. The cells were stored at -70°C until required.

7.8 Transformation of Ligation products into E. coli cells.

Competent cells were thawed on ice, 100µl were added to each ligated DNA sample and incubated on ice for 15 minutes. The ligation-cell suspensions were transferred to 37°C and incubated for five minutes. To each sample was added 400µl of LB without ampicillin and incubation at 37°C continued for a further 45 minutes. An aliquot (80µl) of each transformation was plated onto agar containing 50µg/ml ampicillin and incubated at 37°C for 16-20 hours. A control of 0.1ng of pBR322 plasmid DNA was included in each transformation experiment.

7.9 Selection of Plasmids Containing LCRC0 and LCRC3.

E coli colonies containing pUC13-LCR were selected with 40µg/ml isopropyl-ß-D-thiogalactopyranoside (IPTG) and 40µg/ml 5-bromo 4-chloro 3-indolyl-ß-D-galactosidase (X-gal), which were added to the agar upon which the transformed cells were plated. White, insert-bearing, colonies were inoculated into 5ml of LB for mini-preparation of plasmid DNA.

7.10 Minipreparation of Plasmid DNA.

Each single white colony of *E. coli* transformed with ligated plasmid DNA was inoculated into 5ml of LB with 50µg ampicillin and incubated at 37°C for 16-20 hours. The same colonies were also inoculated onto an agar plate with ampicillin, incubated at 37°C for 16-20 hours, then sealed and stored at 4°C. Twelve colonies from each transformation experiment were analysed at any one time. The cells were collected by centrifugation at 13 000 rpm for five minutes in a microcentrifuge and resuspended in 200µl of solution I (50mM glucose, 10mM EDTA, 25mM Tris.HCl pH 8.0) containing 50µl of 5mg/ml lysozyme. The mixture was incubated at room temperature for five minutes. Solution II (500µl; 0.2M NaOH, 1% SDS) was added and the mixture incubated on ice. After five minutes, 375µl of solution III (60% 5M potassium acetate, 11.5% glacial acetic acid) were added, mixed vigorously and incubation on ice continued for a further five minutes. The cell debris was removed by centrifugation at 13 000 rpm for five minutes. The supernatant was retained, extracted with phenol and the DNA precipitated with 0.6 volumes of isopropanol at room temperature for 20 minutes. The pellet was collected by centrifugation at 13 000 rpm for 20 minutes, dried and

resuspended in 200µl of SDW. RNase A was added to a final concentration of 0.1mg/ml and incubated at 37°C for 30 minutes. DNase free-RNase A (10mg/ml) was prepared in 10mM Tris.HCl, 15mM NaCl, heated to 100°C for 15 minutes, cooled slowly and stored at -20°C. The DNA was phenol extracted, ethanol precipitated and, after drying, resuspended in 50µl of SDW.

7.11 Restriction Digest Analysis of pUC13-LCR DNA.

Plasmid DNA was analysed for the presence of LCRC0 and LCRC3 by restriction with *Eco*RI endonuclease. Plasmid DNA (2-5µl) was digested using two units of *Eco*R1 endonuclease in the presence of 1 x high salt buffer. The mixture was incubated at 37°C for 2-4 hours and the DNA analysed by electrophoresis through a 0.8% agarose gel using the methodology described in Chapter 2, section 2.6.

7.12 Large Scale Plasmid Preparation and CsCl gradient.

Large quantities of pUC13-LCRC0 and pUC13-LCRC3 plasmids were prepared according to the methods described in Chapter 2, section 2.8. Preparation of caesium chloride gradients was adapted to allow greater purification of supercoiled DNA and was carried out as follows:

A gradient was prepared by dissolving 8g CsCl in 8ml of TE buffer and adding 40µl of ethidium bromide (10mg/ml) in 14ml polycarbonate tubes. The plasmid DNA was resuspended in 1ml of SDW and gently layered onto the CsCl mixture. The remaining space in the tube was filled with paraffin oil and the sealed tube was centrifuged at 140 000g for 36 hours. The band of supercoiled

DNA was visualized under UV light (254 and 366nm) and collected with a hyperdermic needle (21G) and syringe. The ethidium bromide was extracted four times with an equal volume of water-saturated butan-1-ol and the DNA in the final aqueous phase was precipitated with 100% ethanol. The precipitated DNA was resuspended in 1ml of SDW, the concentration determined by measurement of the solution absorbance at 260nm as described in Chapter 2, section 2.4 and the plasmid DNA analysed by restriction digestion as described in section 7.11.

7.13 Dideoxy Sequencing of pUC13-LCRC0 and pUC13-LCRC3.

The pUC13-LCR plasmid DNA (3-5μg) was denatured by the addition of 5μl of 1M NaOH, 0.5mM EDTA in a final volume of 25μl and incubated at room temperature for 15 minutes. The denatured template was purified using a Sepharose CL-6B column made as follows: The base of a 0.5ml microcentrifuge tube was partially pierced, sealed with glass beads (450-600μm) and the tube filled with sepharose CL-6B equilibrated in TE buffer. The column was supported in a 1.5ml microcentrifuge tube, the base of which had been pierced, and centrifuged at 1700g for three minutes. The denatured plasmid DNA was applied to the column and collected in an intact microcentrifuge tube by centrifugation as before.

To 8μl of the template DNA was added 1μl of 10 x TM buffer (100mM $MgCl_2$, 100mM Tris.HCl pH 8.4) and 1μl of sequencing primer. The primers used to sequence the HPV 16 LCR were the M13 universal and reverse primers and the LCR 16 C and LCR 16 D primers described in section 7.3. The primers were annealed to the template DNA at 37°C for 15-20 minutes. Primed template

(2.5μl) and 2μl of Klenow mix (2μl deoxyadenosine [α-^{35}S] triphosphate, 1 unit of Klenow fragment of DNA polymerase I in 16μl of SDW) were added to 2μl of the four appropriate dideoxy/deoxynucleotide mixes (A mix: 0.125mM dCTP, dGTP, dTTP, 0.005mM ddATP; C mix: 0.125mM dGTP, dTTP, 0.006mM dCTP, 0.04mM ddCTP; G mix: 0.125mM dCTP, dTTP, 0.006mM dGTP, 0.08mM ddGTP; T mix: 0.125mM dCTP, dGTP, 0.006mM dTTP, 0.25mM ddTTP). The reactions were incubated at 42°C for ten minutes, after which 2μl of chase buffer (0.5mM dATP, dCTP, dGTP, dTTP) were added and incubation at 42°C continued for a further five minutes. The reaction was stopped by the addition of 4μl of formamide dye mix (98% deionized formamide, 10mM EDTA, 0.01% bromophenol blue, 0.001% xylene cyanol). The samples were heated to 95°C for three minutes and cooled on ice.

A 6% polyacrylamide sequencing gel (1 x TBE buffer, 50% urea, 1/7 volume of acrylamide mix [38% acrylamide, 2% N,N'-methylene bisacrylamide]) was solidified by the addition of 1/100 volume of 10% ammonium persulphate and 1/500 volume of N,N,N',N'-tetramethylethylenediamine (TEMED). The samples were electrophoresed through the sequencing gel at 38 watts for 2 hours in 1 x TBE buffer. The gel was fixed in 10% acetic acid for 30 minutes with occasional agitation and dried onto 3MM filter paper using a slab drier (Biorad 483) with vacuum pump at 80°C for 1 hour. The dried gel was exposed to X-ray film for 16-20 hours and the sequence of the DNA read manually from the developed autoradiograph.

7.14 Cloning of LCRC0 and LCRC3 into pCAT Basic Vector.

Preparation of pCAT vector DNA.

pCAT basic vector DNA (10μg) was digested with *Hind*III endonuclease in the presence of 1 x medium salt buffer for 3-5 hours at 37°C and the recessed termini filled in as described in section 7.4. The DNA was purified by phenol:chloroform extraction and ethanol precipitation. The 5' termini were dephosphorylated using the methodology described in 7.5.

Ligation and Transformation of pCAT-LCR Constructs.

The PCR amplified LCR DNA was ligated into *Hind*III restricted pCAT basic vector and the ligation products transformed into competent *E. coli* JM109 cells using the methods described in sections 7.6-7.8.

E. coli colonies transformed with pCAT-LCR were either inoculated directly into LB for minipreparation, or selected by colony hybridization. For the latter procedure ninety-six colonies were inoculated onto duplicate agar plates containing 50μg/ml ampicillin in a numbered grid formation. A positive control of *E. coli* transformed with pAT153-HPV 16 plasmid (see Chapter 2, section 2.8) was included on each plate, which were incubated at 37°C for 16-20 hours. One of the duplicate plates was sealed and stored at 4°C as a reference stock of colonies. The *E. coli* colonies from the second plate were transferred to 541 paper (Whatman) and incubated twice in 0.4M NaOH for three minutes at room temperature. The filter was incubated in the same manner in 0.5M Tris.HCl pH 7.5 and in 2 x SSC. The filter was washed in 100% ethanol, air dried and hybridized with a ^{32}P-labelled HPV 16 DNA probe using the methods described

in Chapter 2, section 2.15, except that hybridization of the filter was carried out in colony hybridization buffer (6 x SSC, 0.5% NP-40, 1mg/ml yeast RNA) at 65°C. Colonies which were positive by hybridization were inoculated into LB for minipreparation of plasmid DNA.

7.15 Plasmid Preparation of pCAT-LCR Constructs.

Plasmid preparation was carried out as described in section 7.10. The presence and orientation of LCRC0 and LCRC3 within the vector was determined by restriction digestion analysis with *Eco*RI endonuclease as described in section 7.11.

7.16 Partial Digestion of pUC13-LCRC3 with EcoRI Restriction Enzyme.

pUC13-LCRC3 DNA (10μg) was partially digested with 0.1 unit *Eco*RI endonuclease in the presence of 1 x high salt buffer. Three identical samples were prepared and incubated at 37°C for 5, 10 and 15 minutes. The products were electrophoresed through a 0.8% low melting point agarose gel, the LCRC3 fragment (883bp) excised from the gel and the DNA purified using methods described in section 7.3. The 3' recessed termini were converted to blunt ends and the DNA ligated into *Hind*III restricted pCAT basic using the methods described in sections 7.4 and 7.6.

7.17 Culture of Hela Cells.

HeLa cells were obtained from the American Type Culture Collection and cultured in Eagles' minimal essential medium (MEM) containing 1mM L-glutamine, 60μg/ml penicillin, 100μg/ml streptomycin and 5% foetal calf serum (FCS) at 37°C without carbon dioxide.

HeLa cells, grown to 75-95% confluence, were collected by trypsin digestion, centrifuged at 1700g for 5 minutes and resuspended in 4ml MEM with 10% FCS and antibiotics. HeLa cells (10^3-10^4) were applied to each well of a tissue culture plate (12 x 25mm diameter) with 2ml MEM containing 10% FCS and antibiotics. The cells were incubated at 37°C with 5% carbon dioxide for 24 hours.

7.18 Transfection of Hela Cells with pCAT-LCR DNA.

The HeLa cells were washed twice with 1ml of sterile PBS. The pCAT basic, pCAT promoter and the pCAT-HPV 16 LCR DNAs were diluted with PBS to a final volume of 85μl and total DNA content of 0.5μg. Diethylaminoethyl (DEAE)-dextran solution (85μl: 2mg/ml DEAE-dextran in 0.9% NaCl) was added and the mixture applied to a single well containing HeLa cells. The cells were incubated at room temperature for two hours after which the DEAE-dextran/DNA solution was removed and the cells washed with PBS. The cells were then covered with 2ml of MEM with 10% FCS and antibiotics and incubated at 37°C with 5% carbon dioxide for 48 hours.

7.19 Harvest of Transfected HeLa Cells.

Two methods for the collection of transfected cell lysate were utilized and compared.

a) Water Lysis

The MEM was removed from the HeLa cells which were washed twice with 1ml SDW. SDW (150µl) was added to each well and incubated at room temperature for 10-15 minutes. The lysate was collected with a plastic pastette and centrifuged at 13 000 rpm for five minutes in a microcentrifuge at 4°C.

b) Freeze-Thaw Lysis

The media was removed from the HeLa cells, which were washed twice with 1ml PBS. The cells were scraped from the well using a pastette, collected into 400µl of PBS and centrifuged for five minutes at 13 000 rpm in a microcentrifuge. The cell pellet was resuspended in 0.25M Tris.HCl, pH 7.8 and immersed in liquid nitrogen for three minutes. The samples were then immediately transferred to 37°C for three minutes. This process was repeated a further three times, after which the lysed cells were centrifuged at 13 000 rpm in a microcentrifuge at 4°C and the supernatant retained.

The absorbance of each cell lysate was measured at 260 and 280nm and the protein concentration calculated using the standard equation of:

$$\text{mg/ml protein} = 1.55 \times A_{280} - 0.77 \times A_{260}.$$

7.20 Detection and Quantification of CAT in Crude Cell Extracts.

The quantity of CAT protein in the cell extracts was analysed by enzyme-linked immunosorbant assay (ELISA) (5 Prime→3 Prime, Inc., Pennsylvania). Microwell plates, coated with antibody to CAT, were rehydrated by the addition of 400µl of PBS and incubated at room temperature for 15 minutes. Transfected cell lysates were diluted with dilution buffer (8mM Na_2HPO_4, 2mM KH_2PO_4, 150mM NaCl, 3mM KCl, 1% bovine serum albumin, 0.02% NaN_3, pH 7.4) to a concentration of 200µg/ml protein and then diluted further to 1:10, 1:20, 1:40 and 1:80 in a final volume of 200µl. Samples of CAT enzyme standards were prepared, containing 300pg, 200pg, 100pg and 50pg of protein. Negative controls consisted of a non-transformed cell extract at 200µg/ml and dilution buffer with no cell extract. The CAT standards, controls and cell extracts were applied to wells of the microtitre plate and incubated at room temperature for two hours. The wells were washed five times with wash buffer (8mM Na_2HPO_4, 150mM NaCl, 2mM KH_2PO_4, 3mM KCl, 0.02% NaN_3, 0.25% Tween-20, pH 7.4). To each well was added 200µl of a 1:5 000 dilution of biotinylated antibody to CAT and incubated at room temperature for one hour. Each well was washed as before and 200µl of a 1:2 500 dilution of streptavidin conjugated alkaline phosphatase was added to the microtitre plate and incubated at room temperature for 30 minutes. The wells were washed as before and 200µl of a 2mg/ml solution of p-nitrophenyl phosphate in diethanolamine buffer (10% diethanolamine, 1mM $MgCl_2$, 0.02% NaN_3, pH 9.8) was added to each well and incubated at room temperature for 30 minutes. The absorbance at 405nm of the solution in each well was measured using an ELISA plate reader (Wellcozyme MCC 340). A standard curve was

drawn from the results of the CAT protein standards and the quantity of CAT protein per well of cell lysate was extrapolated from the graph. The concentration of CAT protein in the cell lysates was calculated using the following equation:

Protein concentration = pg CAT protein/well x 5 x Y x Z

Y = dilution factor to reach 200μg/ml.

Z = dilution factor of Y for which A_{405} was within range of the standards.

7.21 PCR Amplification of HPV 16 LCR.

Two cervical scrape samples from Study A, described in Chapter 4, were used in this analysis, one from a woman with no cervical abnormalities (C0) and one from a patient with histologically proven CIN 3 (C3). PCR analysis had previously shown both samples to be positive for HPV 16. Southern blot analysis of C0 and C3 HPV DNA is shown in Figure 7.1. The HPV DNA in both samples was episomal as demonstrated by HPV specific fragments of 7.9kbp in size, in comparison with integrated HPV DNA which resulted in a fragment larger than 20kbp.

The LCR region of HPV 16 in both samples was amplified using the LCR 16 A and LCR 16 B primers and the PCR system described in Chapter 3. Optimal PCR amplification of HPV 16 LCR DNA was achieved with 8mM $MgCl_2$, as is shown in Figure 7.2, and the size of the resulting LCR fragment was 883bp as predicted from the prototype HPV 16 sequence.

7.22 Cloning of LCRC0 and LCRC3 into pUC13 Vector.

LCRC0 and LCRC3 amplified DNAs were ligated into *Sma*I digested pUC13 vector and putative clones analysed by digestion with *Eco*R1 endonuclease. A single *Eco*R1 site was present in each of the vector and LCR DNAs and therefore digestion of LCR-pUC13 with *Eco*R1 resulted in two fragments. The size of the fragments obtained was dependent on the orientation of the LCR within the vector as is shown in Figure 7.3.

Figure 7.1. Southern blot hybridization analysis of C0 and C3 HPV 16 DNA after restriction digest with *Bam*HI enzyme. Both samples are of 7.9kbp in size and therefore contain only episomal HPV 16. A sample containing integrated HPV 16 DNA (I) is shown for comparison.

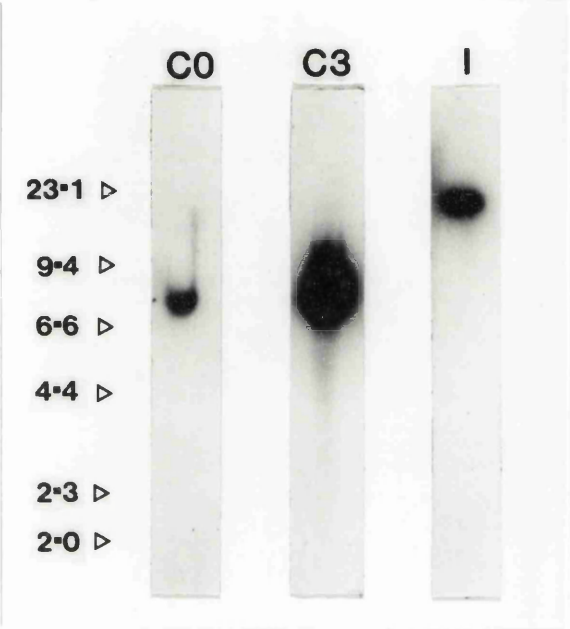


Figure 7.2. PCR amplification of HPV 16 LCR in 1, 3, 5, 8 and 10mM magnesium chloride to determine optimal amplification conditions. The resulting fragment is of 883bp.

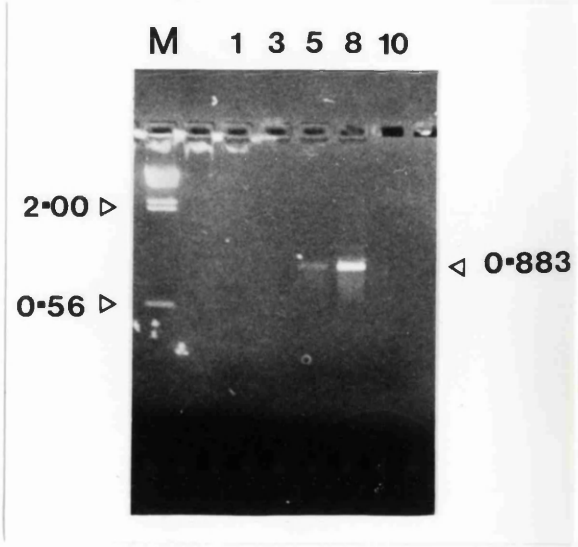


Figure 7.3. Cloning of HPV 16 LCR into *Sma*I cut pUC13 vector. The orientation of LCR DNA within the vector was determined by restriction digest analysis of putative clones with *Eco*RI endonuclease. Fragment sizes are shown in base pairs.

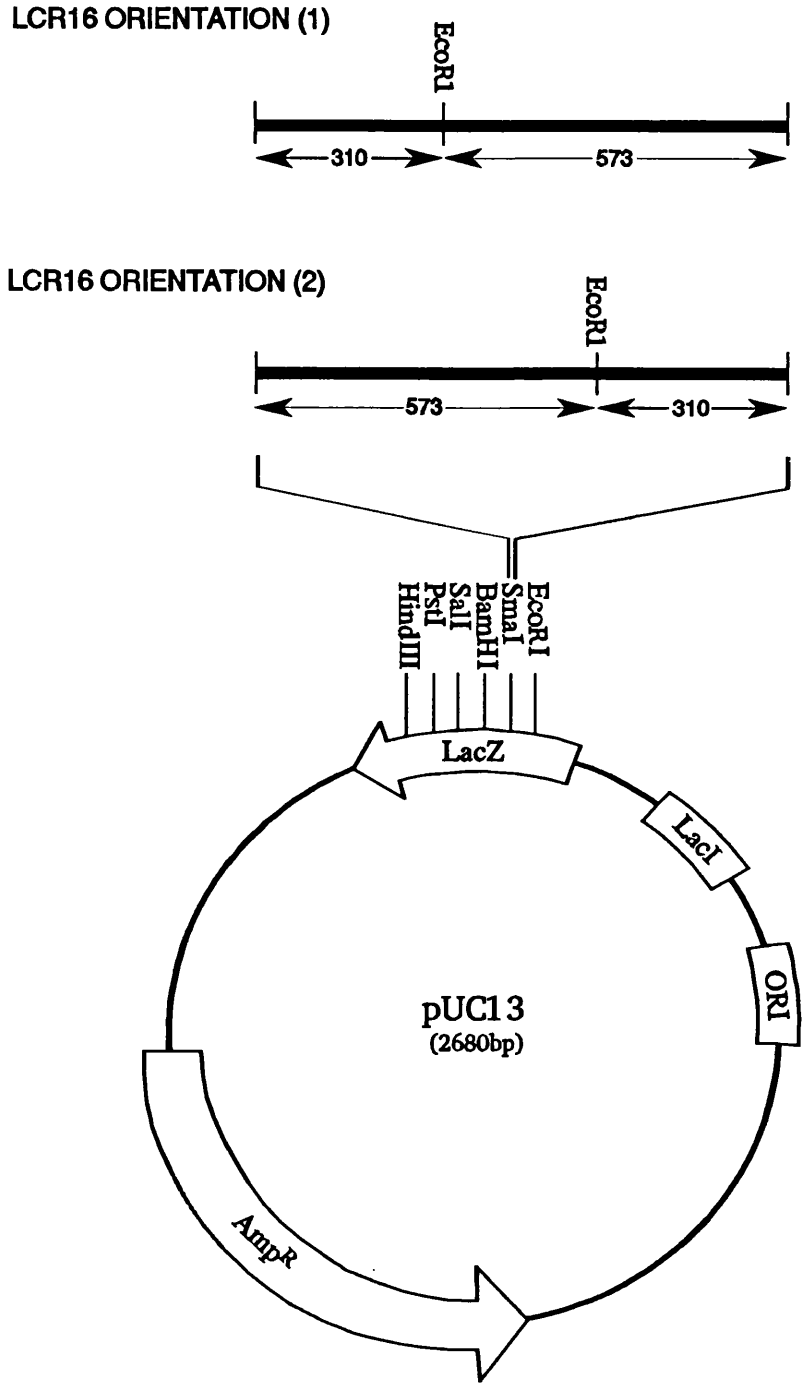
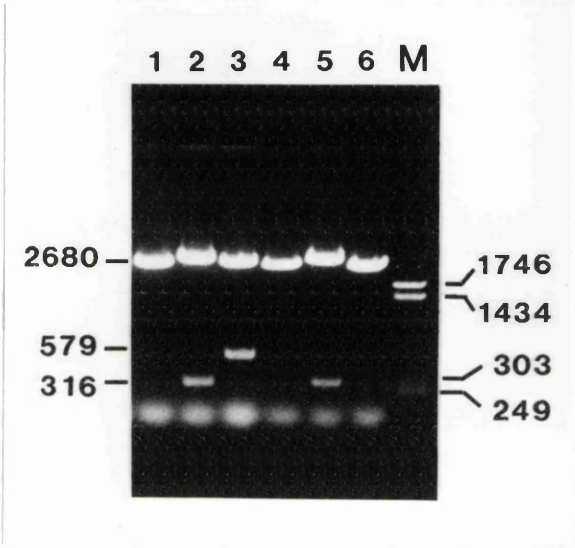


Figure 7.4. Restriction analysis of putative pUC13-LCRC3 clones with *Eco*RI. The LCR insert in clone 3 is in orientation (1) and clones 2 and 5 are in orientation (2). The remaining clones contain only pUC13 vector sequences. M = size marker of pBR322 digested with *Ava*II enzyme. Fragment sizes are shown in base pairs.



When the LCR fragment was cloned into pUC13 in orientation (1) the size of DNA fragments obtained on digestion with *Eco*R1 were 2984bp and 579bp. When in orientation (2) the fragments were 3247bp and 316bp. The results of *Eco*R1 restriction analysis of putative pUC13-LCRC3 clones are shown in Figure 7.4. Clone 3 was in orientation (1) and clones 2 and 5 were in orientation (2). The remaining clones contain pUC13 vector without insert DNA. A similar profile was obtained after *Eco*R1 restriction of putative pUC13-LCRC0 clones.

7.23 Sequence Analysis of pUC13-LCRC3 and pUC13-LCRC0.

Clones of pUC13-LCRC3 and pUC13-LCRC0 were sequenced using the dideoxy method and M13 universal, M13 reverse, LCR 16 C and LCR 16 D oligonucleotide primers. LCR 16 C could not be used to prime the sequencing reaction on the LCRC0 DNA template due to sequence divergence in the region to which this primer anneals.

The nucleotide sequence of LCRC0 and LCRC3 were compared with that of the prototype HPV 16 as is shown in Figure 7.5. LCRC0, which was derived from a woman with no cervical abnormalities, showed a total of three nucleotide deletions, one insertion, four transitions and three transversions. In contrast, LCRC3, derived from a patient with CIN 3, contained two nucleotide deletions, one insertion, eleven transitions and eight transversions. Details of the nucleotide changes between the clinical isolates and HPV 16 are shown in Table 7.2. The sequence variations were also analysed for disruption of known protein binding domains. Part of the GRE consensus sequence, positioned between nucleotides 7641-7655, was altered in LCR C3 from 5'-TGTACA to 5'-TGTACG.

Figure 7.5. Sequence analysis showing points of variation between LCRC0 and LCRC3 and the prototype HPV 16 sequence.

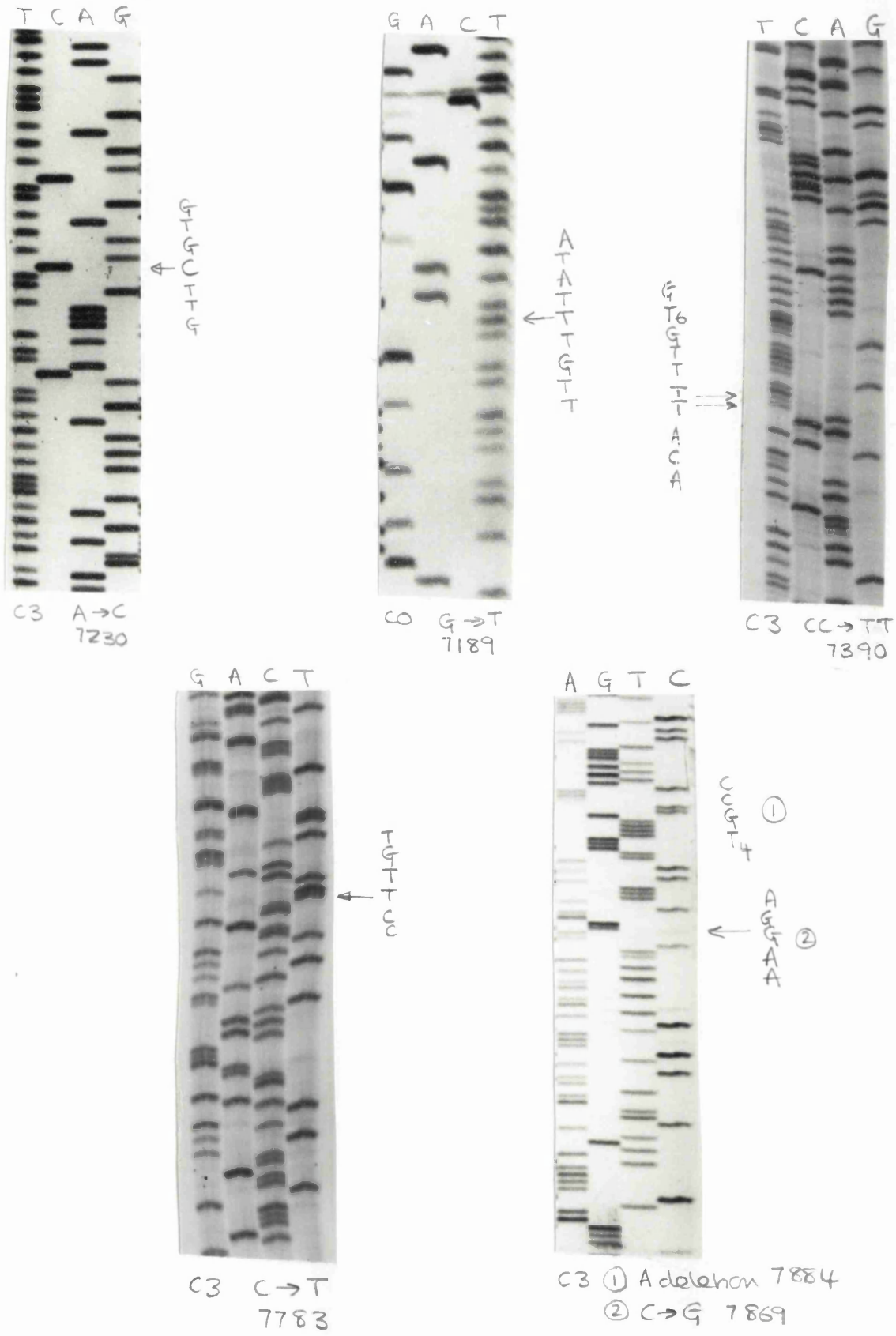


Figure 7.6. HPV 16 LCR showing sequence variations between LCRC0, LCRC3 and the prototype HPV 16. Defined protein binding regions are marked and labelled using nomenclature used in Gloss *et al.* (1989). The position of the sequencing primers, LCR 16 C and LCR 16 D, and the L1 and E6 ORFs are also shown.

```
          _L1 ORF__
          _______fp91_____________        ____fp81_
16        CGTAAGCTGTAAGTATTGTATGTATGTTGAATTAGTGTTGTTT
C3        *******************************************
C0        *******************************************
                                                7183

     _____fp71__      _fp61__   __fp51_
16   GTTGTGTATATGTTTGTATGTGCTTGTATGTGCTTGTAAATATTAAGTTG
C3   *****T*****************************************C****
C0   *****T************************************************
                                                7235

      __fp41___                  __fp31__   _____fp21__
16   TATGTGTGTTTGTATGTATGGTATAATAAACACGTGTGTATGTGTTTTTA
C3   ************************G************************
C0   ************************************************
                                                7285

     _____   ____________________fp11_____________________
16   AATGCTTGTGTAACTATTGTGTCATGCAACATAAATAAACTTATTGTTTC
C3   ************************************************
C0   ************************************************
                                                7335
```

```
                          fp1l
16   AACACCTACTAATTGTGTTGTGGTTATTCATTGTATATAAACTATATTTG
C3   *T**************************************************
C0   **************************************************
                                                    7385

                                          fp01
16   CTACACCTTGTTTTTGTTTTATATATACTATATTTTGTAGCCCGAGCGGCC
C3   *****TT*********************************G*AC-*****
C0   *****T*********************************G*C*-*****
                                                    7436

          LCR_16_C          fp1e
             E2                  GRE2
16   ATTTTGTAGCTTCAACCGAATTCGG-TTGCATGCTTTTTGGCACAAAATG
C3   ************************T*********************C**
C0   ***********C************T************************
                                                    7485

16   TGTTTTTTTAAATAGTTCTATGTCAGCAACTATGGTTTAAACTTGTACGT
C3   *A*********************************A***************
C0   ***********************************A**C*************
                                                    7535

                fp2e
16   TTCCTGCTTGCCATGCGTGCCAAATCCCTGTTTTCCTGACCTGCACTGCT
C3   **************************************************
C0   **************************************************
                                                    7585
```

```
     __fp3e_____                                          ___________
16   TGCCAACCATTCCATTGTTTTTTACACTGCACTATGTGCAACTACTGAAT
C3   **************************************************
C0   **************************************************
                                                        7635

         _______GRE1______

     _____fp4e_____________              ________fp5e_____
16   CACTATGTACATTGTGTCATATAAAATAAATCACTATGCGCCAACGCCTT
C3   **********G*******************T******************
C0   **************************************************
                                                        7685


     _                          _____fp6e_____
16   ACATACCGCTGTTAGGCACATATTTTTGGCTTGTTTTAACTAACCTAATT
C3   *A*************************************C********
C0   ****************************************_********
                                                        7735

     ____LCR_16_D____

     _______fp7e_________       _____fp8e______
16   GCATATTTGGCATAAGGTTTAAACTTCTAGGCCAACTAAATGTCACCCTA
C3   ****G*******************T********************T**
C0   **************************************************
                                                        7785


                          ______fp9e_______
16   GTTCATACATGAACTGTGTAAAGGTTAGTCATACATTGTTCATTTGTAAA
C3   **************************************************
C0   **************************************************
                                                        7835
```

```
                    ______fp3u______
16  ACTGCACATGGGTGTGTGCAAACCGATTTTGGGTTACACATTTACAAGCA
C3  ************************-**********************G*
C0  ************************-**********************
                                                  7885


                                          _tata_  __fp2u_
16  ACTTATATAATAATACTAAACTACAATAATTCATGTATAAAACTAAGGGC
C3  ************************************************
C0  ************************************************
                                                  7935
      ____E2______    _____E2_____   _tata_
    ______                  ___fp1u_____
16  GTAACCGAAATCGGTTGAACCGAAACCGGTTAGTATAAAAGCAGACATTT
C3  ************************************************
C0  ************************************************
                                                  7985


                        P97→_____ E6 ORF______
16  TATGCACCAAAAGAGAACTGCAATGTTTCAGGACCCACAGG
C3  ****************************************
C0  ****************************************
                                        1022
```

Table 7.2. Details of nucleotide changes and deletions between LCRC3 and LCRC0 and the prototype HPV 16 DNA sequence.

Nucleotide Position	Mutations in LCRC0	Mutations in LCRC3
7189	G→T	G→T
7231	-	A→C
7261	-	A→G
7391	C→T	C→T
7392	-	C→T
7427	C→G	C→G
7429	G→C	G→A
7430	-	A→C
7431	G deleted	G deleted
7448	T→C	-
7462	T insertion	T insertion
7483	-	A→C
7487	-	G→A
7519	G→A	G→A
7522	T→C	-
7646	-	A→G
7667	-	C→T
7687	-	C→A
7727	A deleted	A→C
7740	-	A→G
7762	-	C→T
7783	-	C→T
7861	A deleted	A deleted
8 7884	-	C→G

7.24 Cloning of LCRC0 and LCRC3 into pCAT basic Vector.

The PCR amplified LCRC0 DNA was ligated into *Hind*III restrictedpCAT basic vector and putative clones were analysed by digestion with *Eco*R1 endonuclease. The size of fragments obtained was dependent on the orientation of the LCR within pCAT basic as shown in Figure 7.7. It was necessary for the LCR insert to be in the correct orientation (1) for expression of CAT from the P_{97}promoter. When in this orientation, *Eco*R1 restrictionof the LCRC0 construct resulted in fragments of 2996bp, 1392bp and 859bp. When the insert was in orientation (2), the restriction fragments obtained by *Eco*R1 digestion were 3259bp, 1392bp and 596bp. The clones obtained with LCRC0 were all of the desired orientation as is shown in Figure 7.8.

Cloning of LCRC3 into PCAT basic could not be achieved by the above method, therefore an alternativestrategywas employed. LCRC3 was excised from pUC13-LCRC3 using *Eco*R1 restriction.The presence of an *Eco*R1 site within the LCR sequence necessitatedthe use of partial digestion to obtain an intactLCRC3 and the resulting fragments were 3.4, 3.17, 2.6, 0.8, 0.57 and 0.31kbp in size as shown in Figure 7.9. The 0.883kbp fragment, which corresponds with the full length LCRC3, was excised from the gel, purified and ligated into the pCAT basic vector.

Figure 7.7. Cloning of HPV 16 LCR into *Hind*III digested pCAT basic vector. The orientation of LCR DNA was determined by restriction analysis of putative clones with *Eco*RI endonuclease. Fragment sizes are shown in base pairs.

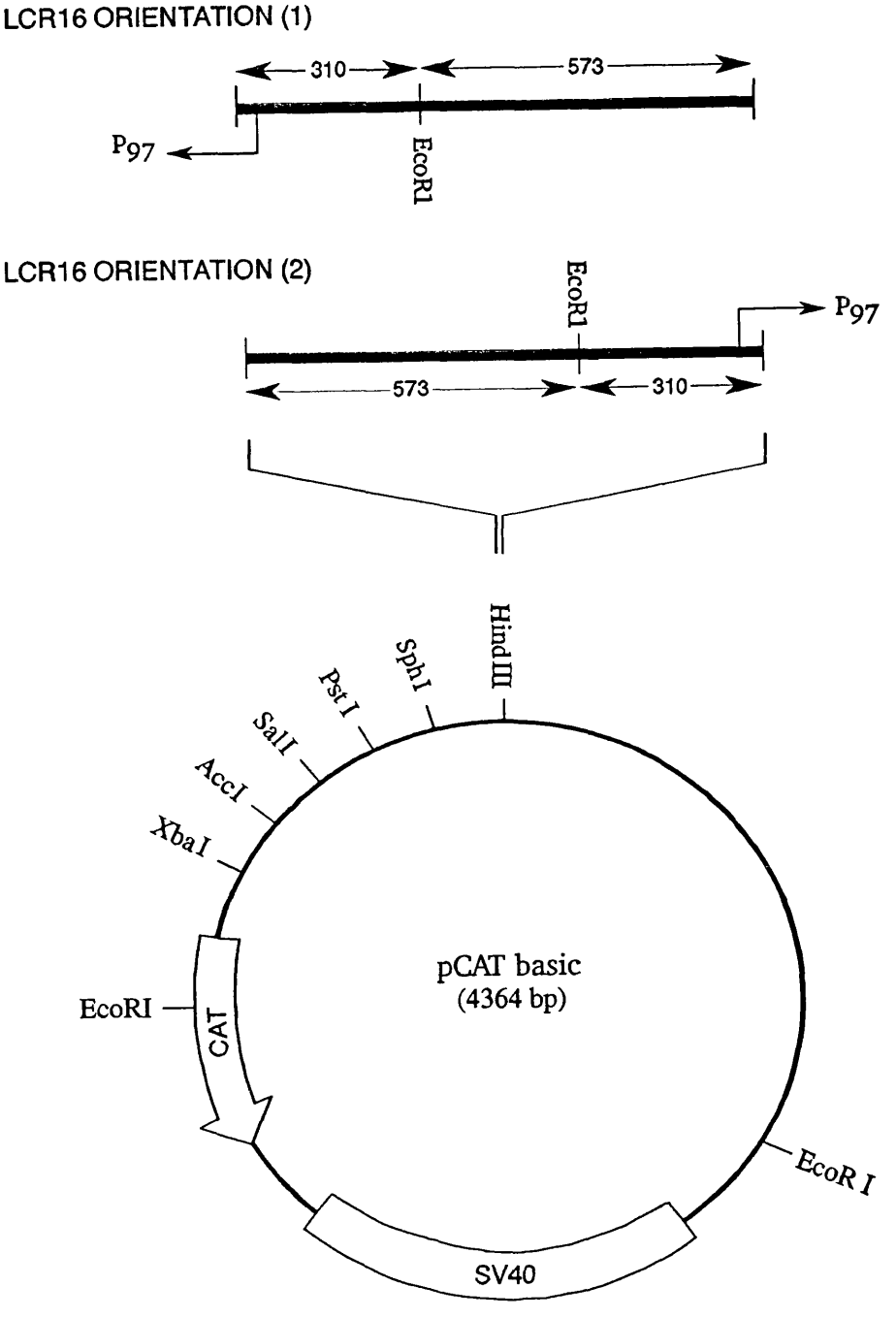


Figure 7.7b. Diagram of pCAT promoter vector, which contains the SV40 early promoter from which the CAT gene is expressed.

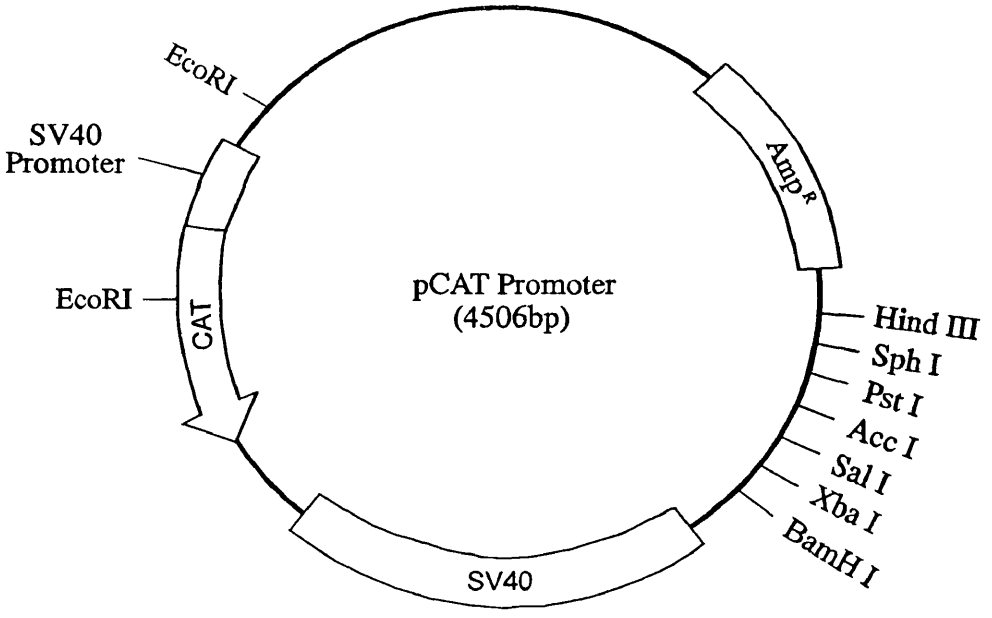


Figure 7.8. Restriction analysis of putative pCAT-LCRC0 clones with *Eco*RI endonuclease. The LCR DNA was located upstream of the CAT gene in all of the pCAT-LCRC0 clones.

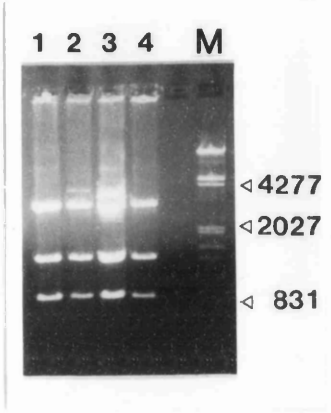
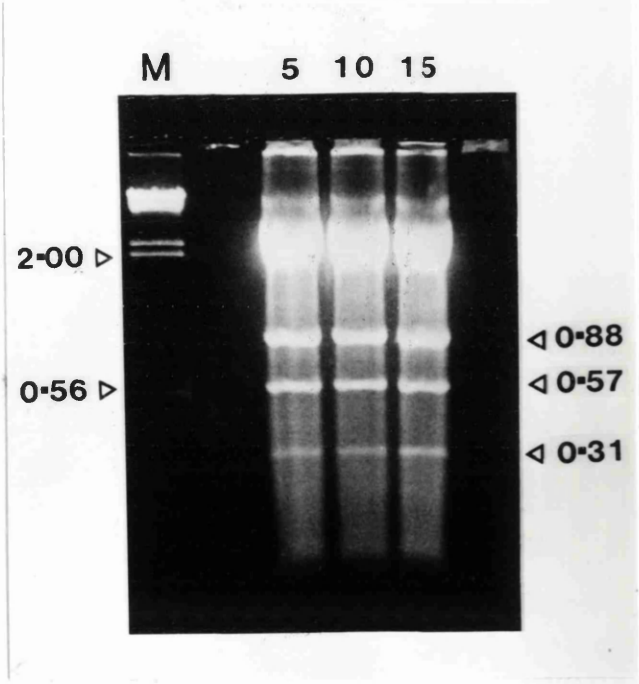


Figure 7.9. Partial digestion of pUC13-LCRC3 with *Eco*RI endonuclease at 5, 10 and 15 minutes. The 0.88kbp fragment contains the full length LCRC3. M = size marker of Lambda DNA digested with *Hind*III. Fragment sizes are shown in kilo base pairs.



7.25 Transfection of pCAT Constructs and Analysis of CAT Expression.

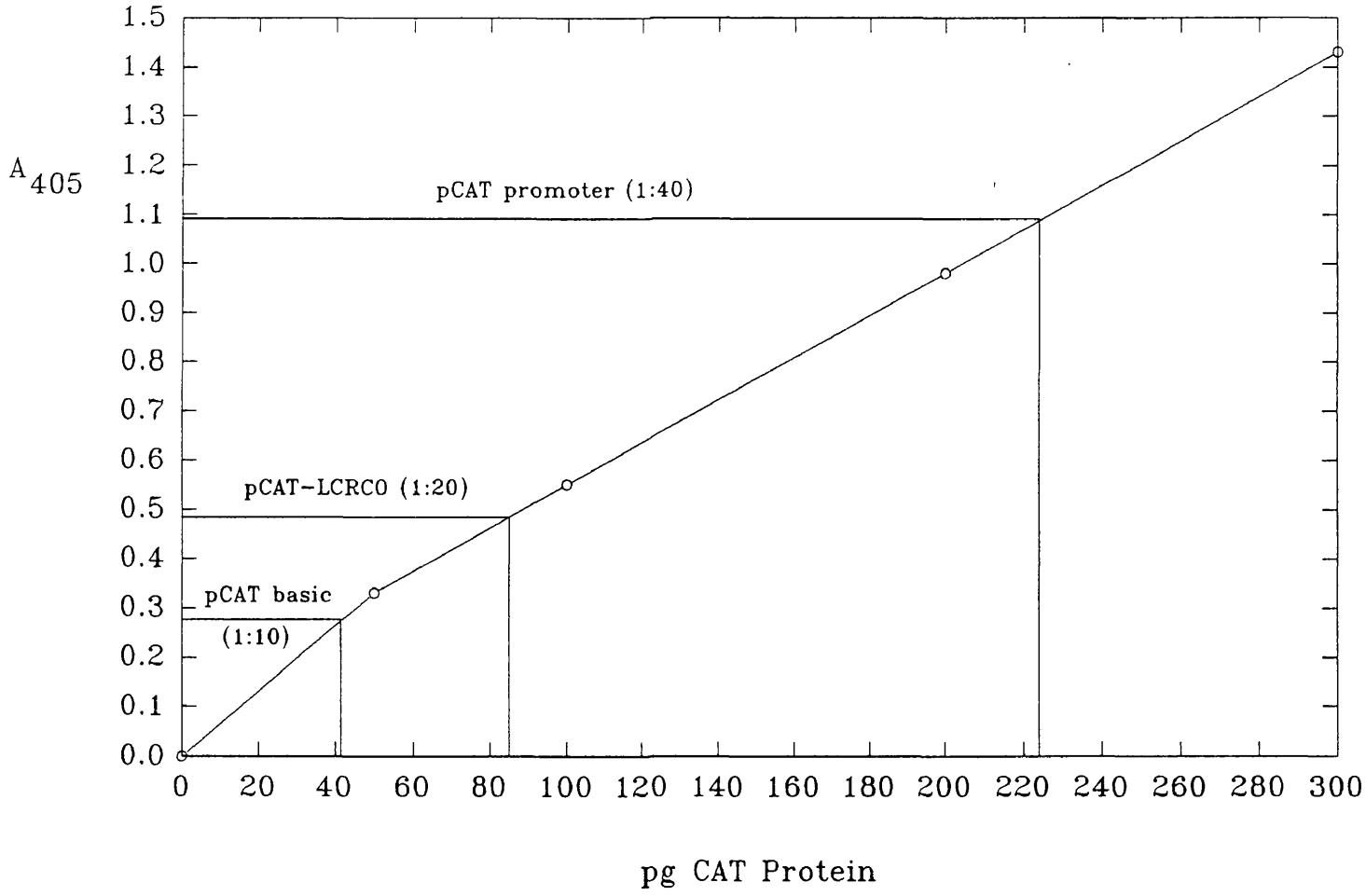
The pCAT-LCRC0 and pCAT-LCRC3 constructs were transfected into HeLa cells for CAT expression analysis. A pCAT promoter vector, containing the SV40 early promoter, was used as a positive control in these experiments. The cell lysates were obtained by two different methods, but since greater quantities of total cell protein were obtained with freeze-thaw lysis (150-300μg) than water lysis (50-120μg) the freeze-thaw method was used for all remaining experiments.

Expression levels from pCAT promoter, pCAT basic and pCAT-LCR constructs in HeLa cells were measured by an ELISA for CAT protein. The absorbance of the CAT protein standards and transfected cell lysates at 405nm is shown in Table 7.3 and the standard curve drawn from these values is shown in Figure 7.10. The concentration of CAT protein in each ELISA plate well was extrapolated from the standard curve. The level of CAT expression achieved from the pCAT promoter vector was 433ng/ml (223pg x 5 x 9.7 x 40) which corresponds to 2.3μg CAT/mg total protein, whereas the level achieved with pCAT basic, which lacks a promoter for transcription, was 15ng/ml (41pg x 5 x 7.5 x 10) which corresponds to 70pg CAT/mg total protein. The pCAT-LCRC0 construct resulted in expression of 94ng/ml of CAT protein (85pg x 5 x 11 x 20) which corresponds to 0.46μg CAT/ml total protein.

Table 7.3. Absorbance at 405nm of CAT protein standards and transfected cell lysates after ELISA for CAT protein.

Sample	A_{405}
50pg CAT protein	0.331
100pg CAT protein	0.550
200pg CAT protein	0.983
300pg CAT protein	1.433
pCAT basic	0.276
pCAT promoter	1.091
pCAT LCRC0	0.484

Figure 7.9. ELISA assay for CAT protein. CAT protein concentration versus the absorbance at 405nm. The protein concentration of pCAT promoter, pCAT basic and pCAT-LCRC0 transfected cell lysates were extrapolated from the graph.



7.26 DISCUSSION.

Human papillomaviruses type 16 and 18 are able to immortalize primary keratinocytes and transform established cell lines *in vitro* and the region of the viral genome to which these functions map is E6/E7/LCR (Yasumoto *et al.*, 1986; Bedell *et al.*, 1987; Laimins *et al.*, 1987; Watanabe *et al.*, 1989). The LCR of HPV contains the transcriptional regulatory sequences for control of viral gene expression and is a major determinant of the differential immortalization activities of HPV 16 and 18 (Romanczuk *et al.*, 1991). Protein binding sites within the LCR have been mapped by DNase I footprinting analysis and several of the binding proteins have been characterized (Garcia-Carranca *et al.*, 1988; Hawley-Nelson *et al.*, 1988; Gloss *et al.*, 1989). Sequence changes within the protein binding domains may alter HPV gene regulation and affect the oncogenic potential of HPV. In order to investigate nucleotide sequence variation within the LCR, PCR was used to amplify HPV 16 LCR from two cervical scrape samples; one from a woman with no cytological abnormalities (C0) and a second from a woman with CIN 3 (C3). DNA sequence analysis indicated that, although the majority of LCRC0 and LCRC3 sequence was identical to the prototype HPV 16 (> 98% homology), some nucleotide variation was apparent, with LCRC3 exhibiting greater sequence divergence than LCRC0. HPV 16 was originally isolated from an invasive carcinoma biopsy (Durst *et al.*, 1983), therefore it is interesting to note that, of the two samples, the LCR from a woman with no cervical abnormalities was most homologous to the prototype HPV 16. The two LCR isolates share an adenine deletion at position 7861, which results in the formation of a novel E2 binding site in this region. An identical deletion has recently been documented

and is considered to be a correction of the prototype sequence of HPV 16 (Krajinovic and Savic, 1991).

The LCR sequences were examined for nucleotide variations within defined protein binding domains. An A→G transition was identified within the GRE of LCRC3, which alters the GRE sequence from 5'-TGTAC<u>A</u>N_3TGTCAT to 5'TGTAC<u>G</u>N_3TGTCAT. A C→T mutation within the HPV 16 LCR has previously been documented (5'-TGTA<u>T</u>AN_3TGTCAT) which disrupts the palindrome of the GRE and leads to a reduction in the response of progesterone and glucocorticoid hormones (Chan *et al.*, 1989). The mutation identified in LCRC3 also disrupts the GRE palindrome and therefore LCRC3 is likely to have a reduced ability to be activated by steroid hormones. Since expression from the HPV 16 P_{97} promoter, which drives expression of E6 and E7, is dependent on the presence of glucocorticoid hormone (Pater *et al.*, 1988), mutations in the GRE may affect expression of the HPV transforming proteins and hence development of malignancy. An additional protein binding region in HPV 16 LCR shares some homology with the GRE consensus sequence (Sibbet and Campo, 1990), but has not been found to interact with glucocorticoid hormones (Chan *et al.*, 1989). A nucleotide transversion (A→C) is also present within the second GRE of LCRC3, but since the mutation is situated within the N_3 region of the consensus sequence the activity would not be affected should the second GRE be functional.

The levels of expression from the P_{97} promoter within the LCR were measured by transfection of HeLa cells with pCAT-LCR constructs and ELISA assay for CAT protein. Detection of CAT protein by ELISA confers several advantages over the conventional method of using ^{14}C-chloramphenicol

acetylation.These include detection of all forms of CAT, including denatured and enzymatically inactive forms, and safer and simpler procedures. The levels of CAT expressed from the HPV 16 LCR were 5-fold lower than those expressed from the pCAT promoter vector containing the SV40 promoter.

Analysis of the LCR of HPV 16 from two patients has shown that sequence variation occurs within this region and nucleotide mutations are present within defined protein binding motifs. Basal levels of transcription from the P_{97} promoter of the HPV 16 LCR are low and may require the presence of hormones for higher expression levels to be achieved. Detailed analysis of sequence divergence within the HPV 16 LCR has not previously been documented and therefore the isolates described in this chapter highlight differences between the LCRs of HPV 16 present in women with and without cervical disease and provide a unique resource for studying the role of LCR binding proteins in regulation of HPV transcription. The functional significance of any sequence variations and interaction of possible co-factors,such as glucocorticoid hormones or other viral *trans*activators,could be assessed using the promoter assay system.

CHAPTER 8: CONCLUSION.

There is a considerable quantity of epidemiological data suggesting that HPV is associated with cervical disease, however, there are flaws in the design of many of these studies. Firstly, study populations have included insufficient numbers of women and it is therefore very difficult to attach any statistical significance to the findings. Second, the problem may be compounded through poor definition of the populations being studied. Third, the clinical diagnosis is often based solely on cytological analysis which has a high false negative rate, therefore women who are classified as normal may have undetected cervical lesions. Fourth, there is enormous variation in the type of methods used and the sensitivity of these for the detection of HPV DNA. Detection methods which do not distinguish between the different viral types have also been utilised and do not give any clear data concerning the association of HPV with disease. It is important that any future investigations in this area should address all of these criticisms.

The major aim of this thesis was to examine the association of human papillomaviruses with cervical intraepithelial neoplasia. Assays were developed to detect HPV DNA to high specificity and sensitivity in cervical scrape samples. Initially, Southern blot and dot blot were compared to determine the most effective hybridization technique for detection of HPV DNA. Southern blot hybridization was twice as sensitive as dot blot in detecting HPV DNA and also more specific. However, polymerase chain reaction methodology became available during the initial phase of the project and, due to the extremely high potential of the method for specific and sensitive detection of nucleic acid sequences of

interest it was decided that a PCR assay offered several advantages for the detection of HPV DNA. Therefore, a PCR system was developed for the detection of HPV DNA in cervical scrape samples which was specific for the HPV types of interest and sufficiently sensitive to detect five copies of HPV target sequence in 1 μg of cellular DNA. PCR was shown to be approximately 100,000 times as sensitive as either Southern blot or dot blot hybridization and therefore considered to be the most sensitive and accurate technique for the detection of HPV DNA in cervical scrape samples from women with disease.

In the light of the criticisms outlined above, two studies were designed to assess the use of HPV 16 as an indicator of cervical disease in well-defined populations. All the women were examined by colposcopy, cytology, and histology when necessary and all virological analysis was carried out on cervical scrape samples obtained at the time of clinical examination. The PCR system had been optimised to give high specificity and sensitivity and each sample was analysed in duplicate to reduce errant results. I believe that only by performing studies which satisfy these criteria will meaningful analysis of the prevalence of HPV infection and the association with cervical disease be possible.

The PCR system was used to analyze cervical scrape samples from 200 women from a General Practice population (Study A) who had volunteered for a cervical screening programme. From this patient population, the prevalence of HPV 16 in women without cervical disease was found to be 17%. There has been considerable contention over the prevalence of HPV in normal women and the data from Study A contrast with that from certain studies showing very high levels of HPV in normal women. I believe that the value of 17% is more realistic,

because the other studies have not addressed the problems outlined above, particularly the absence of colposcopy for detection of cervical lesions. In addition, certain of these studies have acknowledged problems with PCR contamination. The prevalence of HPV 16 increased with greater severity of disease in those women found to have CIN within this population from 15.4% and 40% of those with CIN 1 and CIN 2, to 75% of women with CIN 3. HPV 16 was found to be of use as an indicator of severe cervicaldisease when used alone, but offered greatest benefit when used in conjunction with cytological analysis. Detection of HPV 16 could therefore be introduced as a primary screening method for cervicalabnormalities in low risk populations, or used to complement the existing screening procedure of cytology.

PCR for HPV 16 was used to analyze a further 200 women who had been referred to the Royal Free Hospital colposcopy clinic with a smear report suggesting mild dyskaryosis (Study B). In those women found to have cervical disease there was a trend of increasing prevalence of HPV 16 with greater severity of disease from 53% and 64% of those with CIN 1 and CIN 2, to 74% of those with CIN 3. The trend of increase of HPV 16 with disease was identical in both study groups and the prevalence of HPV 16 in women with CIN 2 and CIN 3 from Study A was similar to that of Study B, which included larger numbers of women with disease. Within Study B there were 54 women who had no evidence of current cervical abnormalities and the prevalence of HPV 16 within this group was 63%. The result is unexpectedly high when compared with the value of 17% for women without cervical disease in Study A and may reflect the fact that the former women have previously had cervical disease. This high

prevalence of HPV 16 in women with normal cervices from Study B who have had mild dyskaryosis in the past precludes the use of PCR for HPV 16 to identify women with cervical disease within this population.

A semi-quantitativePCR method has been described (Cuzick *et al.*, 1992) which may assist in the identificationof women who have severe cervicaldisease. Any signals obtained by PCR amplificationof HPV 16 DNA from cervicalscrape samples were designated high, intermediate or low according to the intensity of the DNA band on an agarose gel in comparison with HPV standards. The presence of high or intermediate levels of HPV 16 DNA correlated with severe cervical disease, whereas women with mild disease or no abnormalities were found to have low levels of HPV DNA or no signal at all. In the light of these data, it is possible that the 34 women with HPV 16 DNA but no cervical abnormalities in Study B may have low levels of HPV 16 DNA. In contrast,those with CIN 2 or CIN 3 are likely to have high or intermediate levels of viral DNA. Thus, semi-quantitativePCR may enable PCR for HPV 16 to be used to identify women who actually have severe grade CIN, although they have only been referred with mild dyskaryosis. It would be interesting to analyze the samples from Study B by semi-quantitative,or fully quantitative, PCR for HPV 16 to examine this possibility.

The samples from Study B are also an ideal source of material for investigating further the relationship between HPV 16 and p53 mutations. Loss of wild-typep53 function is believed to be important in the pathology of cervical cancer. In HPV 16 positive lesions, p53 binds to HPV 16 E6 protein and this association results in degradation of p53, whereas in HPV 16 negative lesions,

somatic mutations result in the loss of p53 function (Crook *et al.*, 1992; Kaebling *et al.*, 1992). It is possible that loss of wild-type p53 function is necessary for the development of cervical neoplasia and that in the absence of the HPV E6 protein, loss of function is mediated via somatic mutations. This hypothesis requires further investigation and since the HPV 16 status of the patients in Study B is known, direct comparison of p53 mutations with HPV 16 would enable this analysis to be carried out on a greater number of patients than has so far been documented.

Analysis of cervical scrape samples for HPV 16 DNA can only define the viral status at one particular time. Follow-up studies have shown that HPV infection may be transient, with frequent recurrence of infection (Kitchener *et al.*, 1991; Rosenfeld *et al*, 1992). However, other analyses have found HPV infections to persist, even after laser treatment for CIN (Ruge *et al.*, 1992). Like HPV detection, cytological analysis only samples cells from the cervix at any one time. It has been found that up to 80% of cervical lesions may progress to more severe disease, whereas the remainder persist or regress, and should they regress, may recur in the future (Carmichael and Maskens, 1989; Weaver *et al.*,1990). The fluctuation in HPV infection is of concern for screening procedures involving cytology and detection of HPV DNA since women found to be positive by either method may prove to be negative in a subsequent analysis. Several studies have demonstrated that detection of HPV 16 could be of use to identify lesions with a high risk of progression to more severe disease when left untreated (Kataja *et al.*, 1990, 1992; Harding *et al.*, 1991). However, other workers have not found such a clear correlation between HPV 16 infection and histological changes of the

cervix(Carmichael and Maskens, 1989; Kitchener *et al.*, 1991). Further, long term follow-up studies on women with and without HPV infection, but who have no cervical abnormalities or mild dyskaryosis are necessary to evaluate the use of HPV 16 as a prognostic indicator of severe CIN.

The extreme sensitivity of PCR generates problems of false positive signals resulting from contamination of samples with target sequences. Duplicate PCR analysis was performed on each sample from Study A and Study B and rigorous controls were included in each experiment. These data from Study A and Study B allowed the reproducibility of PCR for HPV 16 to be assessed. After a single PCR analysis of 400 cervical scrapes, 3.2% of samples resulted in false positive reactions and 8.8% resulted in false negative reactions. The effect of performing duplicate analyses was to reduce the false positive rate to 0.1% and the false negative rate to 0.77%. Duplicate testing of samples substantially reduced erroneous results and adoption of this procedure would further support the proposal that PCR could be used for routine screening of patients for HPV 16 DNA.

The LCR is a major determinant in the immortalization potential of the papillomaviruses (Romanczuk *et al.*, 1991) and this effect may be mediated by differences in the protein binding regions, particularly consensus sequences for transcription factors. Sequence divergence within the LCR have not been extensively investigated, therefore the LCR of HPV 16 was PCR amplified from cervical scrape samples from a woman with no cervical abnormalities (C0) and a woman with CIN 3 (C3) in order to analyze sequence divergence. Both samples contained only episomal HPV 16 sequences. Nucleotide variations between the

two isolates and the prototype HPV 16 sequence were identified by DNA sequence analysis, with LCRC3 exhibiting greater sequence divergence than LCRC0. The two LCR isolates share an adenine deletion at position 7861. An identical deletion has recently been documented and is considered to be a correction of the prototype sequence of HPV 16 (Krajinovic and Savic, 1991). The same mutation has also been documented (Cone *et al.*, 1992) as well as several changes within a short region of the protein binding region designated fp01. Nucleotide changes are present within this region (7427-7436) of both LCRC3 and LCRC0 and it is possible that fp01 could potentially be a hotspot for mutation in HPV 16. The number of nucleotide changes in the 883bp of LCRC3 was 22, and although this number appears to be high, another study has demonstrated between 1 and 10 mutations within a 364bp region of the LCR of HPV 16 isolated from 18 cervical lesions (Ho *et al.*, 1991).

The nucleotide sequences of LCRC0 and LCRC3 were investigated for changes in defined protein binding regions. A nucleotide transition was identified within the GRE of LCRC3, which disrupted the palindrome of the GRE consensus sequence. This mutation is likely to affect glucocorticoid receptor binding and may therefore affect HPV gene expression, which is mediated by glucocorticoid hormones. The levels of expression from the LCRC0 construct and pCAT promoter vector were measured using a CAT reporter assay system. Levels of expression from the SV40 early promoter were low when compared with those from other studies (Li *et al.*, 1992; Novak *et al.*, 1992) which may have been due to low efficiency of the transfection procedure. The efficiency could have been improved by the inclusion of a DMSO or glycerol shock treatment immediately

after removal of the DNA precipitate (Lopata *et al.*, 1984). The transfection assays were carried out using HeLa cells since previous studies had shown the HPV 16 LCR to be functional following transfection and these cells are easy to culture. However, HPV 18 sequences are integrated into the HeLa cell genome and their gene products may interact with the HPV 16 LCR and confuse the analysis. Consequently, similar transfection studies are necessary using established keratinocytes. In addition, levels of AP-1 are likely to influence the HPV-LCR. Variation in AP-1 levels between different cell lines could alter the relative promoter strength of the HPV LCR. The HPV 16 promoter was functional within the system, but the levels of CAT expressed from the HPV 16 LCR were 5-fold lower than those expressed from the SV40 early promoter. Glucocorticoid hormones are known to interact with the HPV 16 LCR to increase levels of expression from the P_{97} promoter. It would be interesting to determine the effects of hormones such as progesterone, dexamethasone and oestrogen on the expression levels from LCRC0 and LCRC3 promoters. Oestrogen has been demonstrated to increase expression of the E6 and E7 genes of HPV 16, although this report has not yet been verified by other studies (Mitrani-Rosenbaum *et al.*, 1989). Due to time constraints, the experiments were not performed, but they would have determined whether the mutation in GRE1 of LCRC3 would render this element non-functional. The ability of one virus to augment the expression of another virus has been documented, for example adenovirus, HSV, EBV and cytomegalovirus (CMV) can activate the expression of HTLV-1 or HIV (Ostrove and Berns, 1980; Davis *et al.*, 1987; Gendelman *et al.*, 1986; Kenney *et al.*, 1988). Certain studies have demonstrated an association between HSV and cervical

lesions and it is therefore possible that HPV expression may be *trans*activated by HSV-2 immediate early proteins. ICP4 and ICP0 of HSV-1 have both been shown to enhance transcription of HPV 16 by interaction with the LCR, but those of HSV-2 have yet to be analysed in a similar manner (McCusker and Bacchetti, 1988). Clones of HSV-2 immediately early genes were to have been used for co-transfection assays with the LCRC0 and LCRC3 constructs in order to examine the ability of HSV-2 to activate HPV 16 gene expression. Co-transfection assays with regions of the CMV genome had also been planned since CMV is known to be present in the genital tract and insertion of the human CMV enhancer upstream of the HPV 16 LCR enhances the transformation capability of HPV 16 in primary human cervical epithelial cells (Morgan *et al.*, 1992).

The results from Study A and Study B, as well as a considerable number of other clinical and epidemiological studies, have demonstrated a close association between cervical carcinoma and HPV infection. Transfection of epithelial tissue with HPV 16 leads to inhibition of differentiation and development of cellular morphology consistent with CIN (McCance *et al.*, 1988; Blanton *et al.*, 1991). Laboratory studies have shown that the E5, E6 and E7 ORFs code for transforming functions in some papillomaviruses and the protein products can act together, or with other oncogenes, to transform a variety of cell types *in vitro*. In addition, the E6 and E7 genes of HPV have been shown to interact with the tumour suppressor proteins p53 and Rb, respectively. These interactions result in a loss of function of the tumour suppressor proteins which may assist in the development of cervical malignancy. HPV 16 and 18 are often found integrated into the cellular genome within malignant tissue and carcinoma

derived cell lines. Integration usually disrupts the E2 ORF releasing expression of E6 and E7 genes from E2 regulation which may lead to increased levels of the oncogenic E6 and E7 gene products.

Malignant transformation is a multi-step process resulting from a variety of cellular abnormalities, action of carcinogenic agents or viral infections. It must therefore be recognised that infection of the uterine cervix with HPV alone may be insufficient to cause the development of cervical cancer. However, there is a wealth of data implicating HPV as a causal factor in the aetiology of cervical cancer in which interaction of HPV with other agents is necessary to alter cell growth to malignancy.

There are three important areas of papillomavirus research which remain to be elucidated. Firstly, the mechanism by which HPV induces a normal cell to become neoplastic and secondly, the co-factors which interact with HPV to bring about these changes. Thirdly, the clinical applicability of detection of HPV 16 for identifying women with severe cervical disease in specific patient groups. The investigations detailed in this thesis demonstrate that HPV 16 is of use as an indicator of severe disease in women from a General Practice. As expected from this population, only a small number of patients had CIN (22/183). Thus, the efficacy of using PCR for HPV 16 as a replacement or an additional screening procedure to cytology requires verification by a larger study of women attending a General Practice for cervical screening. Although detection of HPV 16 DNA was relatively poor in identifying women with severe CIN from a population referred with a mildly dyskaryotic smear report, the use of semi-quantitative PCR, or preferably truly quantitative PCR, may enable PCR for HPV 16 to be used as

an adjunct to cytology in the detection of cervical disease in this population. Finally, the HPV 16 LCR-CAT constructs should allow the interaction of co-factors with the HPV 16 LCR to be investigated.

In conclusion, I would argue that HPV 16 is likely to be involved in the development of cervical neoplasia. Qualitative PCR for HPV 16 DNA will be of use for the detection of high-grade cervical disease in the National cervical screening programme and future data will confirm whether quantitative PCR is of value when assessing women referred for colposcopy with a smear report suggesting mild dyskaryosis.

REFERENCES.

Ahola, H., Stenlund, A., Moreno-Lopez, J. and Pettersson, U. (1986). Promoters and processing sites within the transforming region of bovine papillomavirus type 1. J Virol **61**: 2240-2244.

Alexander, E. R. (1973). Possible etiologies of cancer of the cervix other than Herpesvirus. Cancer Res **33**: 1485-1496.

Ambinder, R. F., Charache, P., Staal, S., Wright, P., Forman, M., Hayward, D. and Hayward, G. S. (1986). The vector homology problem in diagnostic nucleic acid hybridization to clinical specimens. J Clin Microbiol **24**: 16-20.

Amtmann, E. and Sauer, G. (1982). Bovine papillomavirus transcription: polyadenylated RNA species and assessment of the direction of transcription. J Virol **43**: 59-66.

Androphy, E. J., Schiller, J. T. and Lowy, D. R. (1985). Identification of the protein encoded by the E6 transforming gene of bovine papillomavirus. Science **230**: 442-445.

Baker, C. C. and Howley P. M. (1987). Differential promoter utilization by the bovine papillomavirus in transformed cells and productively infected wart tissues. EMBO J **6**: 1027-1035.

Baker, C. C., Phelps, W. C., Lindgren, V., Braun, M. J., Gonda, M. A. and Howley, P. M. (1987). Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. J Virol **61**: 962-971.

Banks, L., Moreau, F., Vousden, K., Pim, D. and Matlashewski, G. (1991). Expression of the human papillomavirus E7 oncogene during cell transformation is sufficient to induce susceptibility to lysis by activated macrophages. J Immunol **146**: 2037-2042.

Barbosa, M. S., Lowy, D. R. and Schiller, J. T. (1989). Papillomavirus polypeptides E6 and E7 are zinc binding proteins. J Virol **63**: 1404-1407.

Barbosa , M. S., Edmonds, C., Fisher, C., Schiller, J. T., Lowy, D. R. and Vousden, K. H. (1990). The region of the HPV E7 oncoprotein homologous to adenovirus E1a and SV40 large T antigen contains separate domains for Rb binding and casein kinase II phosphorylation. EMBO J **9**: 153-160.

Barnes, W., Delgardo, G., Kurman, R. J., Petrilli, E. S., Smith, D. M., Ahmed, S., Lorincz, A. T., Temple, G. F., Jenson, A. B. and Lancaster, W. D. (1988). Possible prognostic significance of human papillomavirus type in cervical cancer. Gynecol Oncol **29**: 267-273.

Barton, S. E., Jenkins, D., Cuzick, J., Maddox, P. H., Edward, S. R. and Singer, A. (1988). Effect of cigarette smoking on cervical epithelial immunity: A mechanism for neoplastic change. Lancet ii: 632-634.

Bedell, M. A., Jones, K. H. and Laimins, L. A. (1987). The E6-E7 region of human papillomavirus 18 is sufficient for transformation of NIH 3T3 and Rat-1 cells. J Virol **61**: 3635-3640.

Bell, J. (1989). The polymerase chain reaction. Immunology Today **10**: 351-355.

Beral, V., Hannaford, P. and Kay, C. (1988). Oral contraceptives use and malignancies of the genital tract. Lancet ii: 1331-1334.

Berg, L., Singh, K. and Botchan, M. (1986). Complementation of a bovine papillomavirus low-copy-number mutant: evidence for a temporal requirement of the complementing gene. Mol Cell Biol **6**: 859-869.

Bertini, B. and Hornstein, M. (1971). The epidemiology of *Trichomonas* and the role of this infection in the development of carcinoma of the cervix. Acta Cytol **14**: 325-332.

Bevan, I. S., Blomfield, P. I., Johnson, M. A., Woodman, C. B. J. and Young, L. S. (1989). Oncogenic viruses and cervical cancer. Lancet i: 907-908.

Black, P. H., Hartley, J. W., Rowe, W. P. and Huebner, R. J. (1963). Transformation of bovine tissue culture cells by bovine papillomavirus. Nature **199**: 1016-1018.

Blanton, R. A., Perez-Reyes, N., Merrick, D. T. and McDougall, J. K. (1991) Epithelial cells immortalized by human papillomaviruses have premalignant characteristicsin organotype culture. Am J Pathol **138**: 673-683.

Borst, M., Butterworth, C. E., Baker, V., Kuykendall, K., Gore, H., Soong, S. J. and Hatch, K. D. (1991) Human papillomavirus screening for women with atypical Papanicolaou smears. J Reprod Med **36**: 95-99.

Boshart, M., Gissmann, L., Ikenberg, H., Kleinheinz, A., Scheurlen, W. and zur Hausen, H. (1984). 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.

Brandsma, J., Burk, R. D., Lancaster, W.D., Pfister, H. and Schiffman, M. H. (1989). Inter-laboratory variation as an explanation for varying prevalence estimates of human papillomavirus infection. Int J Cancer **43**: 260-262.

Brinton, L. A., Fraumeni, J. F., Juggins, G. R., Lehman, H. F., Mallin, K., Savitz, D. A., Trapido, E., Rosenthal, J. and Hoover, R. (1986). Long term use of oral contraceptives and risk of invasive cancer. Int J Cancer **38**: 339-344.

Broders, A. C. (1932). Carcinoma *in situ* contrasted with benign penetrating epithelium. JAMA **99**: 1670-1674.

Buckley, J. D., Harris, R. W., Doll, R., Vessey , M. P. and Williams, P. T. (1981). Case-control study of the husbands of women with dysplasia or carcinoma of the cervix uteri. Lancet ii: 1010-1015.

Buckley, C. H., Butler, E. B. and Fox, M. (1982). Cervical intraepithelial neoplasia. J Clin Pathol **35**: 1-13.

Burk, R.D., Kadish, K. S., Calderin, S. and Romney, S. L. (1986). Human papillomavirus infection of the cervix detected by cervicovaginal lavage and molecular hybridization: Correlation with biopsy results and Papanicolaou smear. Am J Obstet Gynecol **154**: 982-989.

Burns, J., Graham, A. K., Frank, C., Fleming, K. A., Evans, M. F. and McGee, J. O'D. (1987). Detection of low copy human papillomavirus DNA and mRNA in routine paraffin sections of cervix by non-isotopic *in situ* hybridization. J Clin Pathol **40**: 858-864.

Campion, M.J., McCance D.J., Cuzick, J. and Singer, A. (1986). Progressive potential of mild cervical atypia: Prospective cytological, colposcopic and virological study. Lancet ii: 237-240.

Campo, M. S., Moar, M. H., Sartirana, M. L., Kennedy, I. M. and Jarrett,W. G. F. (1985). The presence of BPV-4 DNA is not required for the progression to, or maintenance of, the malignant state in cancers of the alimentary tract in cattle. EMBO J **4**: 1819-1825.

Carmichael, J. A. and Maskens, P. D. (1989) Cervical dysplasia and human papillomavirus. Am J Obstet Gynecol **160**: 916-918.

Caussy, D., Orr, W., Daya, A.D., Roth, P., Reeves, W. and Rawls, W. (1988). Evaluation of methods for detecting human papillomavirus deoxyribonucleotide sequences in clinical specimens. J Clin Microbiol **26**: 236-243.

Chan, V. T. W., Fleming, K. A. and McGee, J. O'D. (1985). Detection of subpicogram quantities of specific DNA sequences on blot hybridization with biotinylated probes. Nucl Acid Res **13**: 8083-8091.

Chan, W.-K., Klock, G. and Bernard, H. (1989). Progesterone and glucocorticoid response elements occur in the long control regions of several human papillomaviruses involved in anogenital neoplasia. J Virol **63**: 3261-3269.

Chang, K.-W., Chang, C., Lai, K., Chou, M. and Choo, K. (1989). High prevalence of human papillomavirus infection and possible association with betal quid chewing and smoking in oral epidermoid carcinomas in Taiwan. J Med Virol **28**: 57-61.

Chen, E. Y., Howley, P. M., Levinson, A. D. and Seeburg, P. H. (1982). The primary structure and genetic organization of the bovine papillomavirus type 1 genome. Nature **299**: 529-534.

Chen, S-L. and Mounts, P. (1990) Transforming acivityof E5a protein of human papillomavirus type 6 in NIH 3T3 and C127 cells. J Virol **64**: 3226-3233.

Chin, M. T., Broker, T. R. and Chow, L. T. (1989). Identification of a novel constitutiveenhancer element and an associated binding protein: implications for human papillomavirus type 11 enhancer regulation. J Virol **63**: 2967-2976.

Choe, J., Vaillancourt, P., Stenlund, A. and Botchan, M. (1989). Bovine papillomavirus type 1 encodes two forms of a transcriptional repressor: structural and functional analysis of new viral cDNAs. J Virol **63**: 1743-1755.

Choo, K. B., Pan, C. C. and Han S. H. (1987). 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.

Chow, V. T. K., Tham, K. M. and Bernard, H. U. (1989). *Thermus aquaticus* DNA polymerase-catalysedchain reaction for the detection of human papillomaviruses. J Virol Methods **27**: 105-111.

Christopherson, W. M. and Parker, J. E. (1965). Relation of cervical cancer to early marriage and childbearing. N Engl J Med **27**: 235-239.

Clarke, E. A., Morgan, R. W. and Newman, A. M. (1982). Smoking as a risk factor in cancer of the cervix: additional evidence from a case-control study. Am J Epidemiol **115**: 59-66.

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

Coggin, J. R. and zur Hausen, H. (1979). Workshop on papillomaviruses and cancer. Cancer Res **39**:545-546.

Cole, S. T. and Streeck, R. E. (1986). Genome organization and nucleotide sequence of human papillomavirus type 33 which is associated with cervical cancer. J Virol **58**: 991-995.

Cole, S. T. and Danos, O. (1987). Nucleotide sequence and comparative analysis of the human papillomavirus type 18 genome. J Mol Biol **159**: 599-608.

Comerford, S. A., McCance, D. J., Dougan, G. and Tite, J. P. (1991). Identification of T- and B- cell epitopes of the E7 protein of human papillomavirus type 16. J Virol **65**: 4681-4690.

Cone, R. W., Minson, A. C., Smith, M. R. and McDougall, J. K. (1992) Conservation of HPV 16 E6/E7 ORF sequences in a cervical carcinoma. J Med Virol **37**: 99-107.

Cornelissen M. T. E., van der Velden, K. J., Walboomers, J. M. M., Briet, M. A., Smits, H. L., van der Noordaa, J. and ter Schegget, J. (1988). Evaluation of different DNA:DNA hybridization techniques in detection of HPV 16 DNA in cervical smears and biopsies. J Med Virol **25**: 105-114.

Cornelissen, M. T. E., van den Tweel, J. G., Struyk, A. P. H. B., Jebbink, M. F., Briet, M., van der Noordaa, J. and ter Schegget, J. (1989). Localization of human papillomavirus type 16 DNA using the polymerase chain reaction in the cervix uteri of women with cervical intraepithelial neoplasia. J Gen Virol **70**: 2555-2562.

Cox, M. F., Meanwell, C. A., Maitland, N. J., Blackledge, G., Scully, C. and Jordan, J. A. (1986). Human papillomavirus type-16 homologous DNA in normal human ectocervix. Lancet **i**: 157-158.

Cripe, T. P., Haugen, T. H., Turk, J. P., Tabatabai, F., Schmid, P. G., Durst, M., Gissmann, L., Roman, A. and Turek, L. P. (1987). Transcriptional regulation of the human papillomavirus-16 E6-E7 promoter by a keratinocyte-dependent enhancer, and by viral E2 trans-activator and repressor gene products: implications for cervical carcinogenesis. EMBO J **6**: 3745-3753.

Crook, T., Storey, A., Almond, N., Osborn, K. and Crawford, L. (1988). Papillomavirus type 16 cooperates with activated *ras* and *fos* oncogenes in bovine dependent transformation of primary mouse cells. Proc Natl Acad Sci. USA **85**: 8820-8824.

Crook, T., Almond, N., Murray, A., Stanley, M. and Crawford, L. (1989). Constitutive expression of c-*myc* oncogene confers hormone independence and enhanced growth-factor responsiveness on cells transformed by human papillomavirus type 16. Proc Natl Acad Sci. USA **86**: 5713-5717.

Crook, T., Wrede, D., Tidy, J., Scholerfield, J., Crawford, L., and Vousden, K. H. (1991). Status of c-*myc*, p53 and retinoblastoma genes in human papillomavirus positive and negative squamous cell carcinomas of the anus. Oncogene **6**: 1251-1257.

Crook, T., Wrede, D., Tidy, J. A., Mason, W. P., Evans, D. J. and Vousden, K. H. (1992) Lancet **339**: 1070-1073.

Crum, C. P., Mitao, M., Levine, R. U. and Silverstein, S. (1985). Cervical papillomaviruses segregate within morphologically distinct precancerous lesions. J Virol **54**: 675-681.

Crum, C. P., Nagai, N., Levine, R. U. and Silverstein, S. (1986). *In situ* hybridization analysis of HPV 16 DNA sequences in early cervical neoplasia. Am J Pathol **123**: 174-182.

Cullen, A. P., Reid, R., Campion, M. and Lorincz, A. T. (1991). Analysis of the physical state of different human papillomavirus DNAs in intraepithelial and invasive cervical neoplasia. J Virol **65**: 606-612.

Cuzick, J., Terry, G., Ho, L., Hollingworth, T. and Anderson, M. (1992) Human papillomavirus type 16 DNA in cervical smears as predictor of high-grade cervical cancer. Lancet **339**: 959-960.

Dallas, P. B., Flanagan, J. L., Nightingale, B. N. and Morris B. J. (1989). Polymerase chain reaction for fast, non-radioactive detection of high- and low-risk papillomavirus types in routine cervical specimens and biopsies. J Med Virol **27**: 105-111.

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

Danos, O., Giri, I., Thierry, F. and Yaniv, M. (1984). Papillomavirus genomes: sequences and consequences. J Invest Dermatol **83**: 7-11.

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

Davis, M., Kenney, S. C., Kamine, J., Pagano, J. S. and Huang, E. S. (1987) Immediate-early gene region of human cytomegalovirus trans-activates the promoter of human immunodeficiency virus. Proc Natl Acad Sci. USA **84**: 8642-8646.

de Caprio, J. A., Ludlow, J. W., Figge, J., Skew, J. Y., Huang, C. M., Lee, W. H., Marsilio, E., Paucha, E. and Livingston, D. M. (1988). SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. Cell **54**: 275-283.

de Martynoff, G., Brasseur, R., Vennin, P., Van Alphen, P., Burny, A. and Zeicher, M. (1989). Detection of antibodies specific to human papillomavirus type 16 in human sera: use of synthetic peptides and time resolved fluorescence. International papillomavirus workshop, Taos, New Mexico.

de Villiers, E.-M., Gissmann, L. and zur Hausen, H. (1981). Molecular cloning of viral DNA from human genital warts. J Virol **40**: 932-935.

de Villiers, E.-M., Schneider, A., Miklaw, H., Papendick, U., Wagner, D., Wesch,H., Wahrendorf, J. and zur Hausen, H. (1987). Human papillomavirus infections in women with and without abnormal cervical cytology. Lancet **ii**: 703-705.

Dillner, J., Dillner, L., Robb, J., Willems, J., Jones, I., Lancaster, W., Smith, R. and Lerner, R. (1989). A synthetic peptide defines a serological response to a human papillomavirus-encoding nuclear antigen expressed in virus carrying cervical neoplasia. Proc Natl Acad Sci. USA **86**: 3838-3841.

Dillner, J. (1990). Mapping of linear epitopes of human papillomavirus type 16: the E1, E2, E4, E5, E6 and E7 open reading frames. Int J Cancer **46**: 703-711.

di Luca, D., Pilotti,S., Stefanon, S., Rotola, A., Monini, P., Tognon, M., de Palo, G., Rilke, F. and Cassai, E. (1986). Human papillomavirus type 16 DNA in genital tumours: A pathological and molecular analysis. J Gen Virol **67**: 583-589.

di Maio, D., Guralski, D. and Schiller, J. T. (1986). Translation of open reading frame E5 of bovine papillomavirus is required for its transforming activity. Proc Natl Acad Sci. USA **83**: 1797-1801.

di Maio, D., Neary, K., Kaczmarek, L., Andrews, E., Horowitz, B. and Guralski, D. (1987). Mutational analysis of bovine papillomavirus type-1 transforming functions. In: Steinberg, B. M., Brandsma, J. L., Taichman, L. B. eds. Cancer cells vol 5: papillomaviruses. Cold Spring Harbor, New York: Cold spring Harbor Press: 187-193.

Dostatni, N., Thierry, F. and Yaniv, M. (1988). A dimer of BPV-1 E2 protein containing a protease resistant core interacts with its DNA target. EMBO J **7**:3807-3816.

Durst, M., Gissmann, L., Ikenberg, H. and zur Hausen, H. (1983). A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. Proc Natl Acad Sci. USA **80**: 3812-15.

Durst, M., Kleinheinz, A., Hotz, M. and Gissmann, L. (1985). The physical state of human papillomavirus type 16 DNA in benign and malignant genital tumours. J Gen Virol **66**: 1515-1522.

Durst, M., Dzarlieva-Petrskova,R. T., Boukamp, P., Fusenig, N. E. and Gissmann, L. (1987). Molecular and cytogenetic analysis of immortalized human primary keratinocytes obtained after transfection with human papillomavirus type 16 DNA. Oncogene **1**: 251-256.

Dvoretzky, I., Shober, R., Chattopadhyay, S. K. and Lowy, D. R. (1980). A quantitative *in vitro* focus assay for bovine papillomavirus. Virology **103**: 369-375.

Dyson, N., Howley, P. M., Munger, K. and Harlow, E. (1989). The human papillomavirus-16 E7 oncoprotein is able to bind the retinoblastoma gene product. Science **243**: 934-937.

Edmonds, C. and Vousden, K. H. (1989). A point mutational analysis of human papillomavirus type 16 E7 protein. J Virol **63**: 2650-2656.

Engel, L. W., Heilman, C. A. and Howley, P. M. (1983). Transcriptional organization of the bovine papillomavirus type 1. J Virol **47**:516-528.

Evander, M. and Wadell, G. (1991). A general primer pair for amplification and detection of genital human papillomavirus types. J Virol Methods **31**: 239-250.

Evans, A. S. and Monaghan, J. M. (1985). Spontaneous resolution of cervical wartydysplasia: the relevance of clinical and nuclear DNA features: a prospective study. Br J Obstet Gynaecol **92**: 165-169.

Evans, D. M. D., Hudson, E. A., Brown, C. L., Boddington, M. M., Hughes, H. C., Mackenzie, E. F. D. and Marshall, T. (1986). Terminology in gynaecological cytopathology: report of the working party of the British Society for Clinical Cytology. J Clin Pathol **39**: 933-944.

Feinberg, A. P. and Vogelstein, B. (1983). A technique for radiolabelling DNA restrictionendonuclease fragments to high specificactivity.Anal Biochem **132**: 6-13.

Feingold, A. R., Vermund, S. H., Burk, R. D., Kelley, K. F., Schrager, L. K., Schreiber, K., Munk, G., Friedland, G. H. and Klein, R. S. (1990). Cervical cytologic abnormalities and papillomavirus in women infected with human immunodeficiency virus. J Acquir Immune Defic Syndr **3**: 896-903.

Feitelson, M. A., Wettstein, F. O. and Stevens, J. G. (1985). Characterization of the structural polypeptides of the cottontail rabbit (Shope) papillomavirus by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and tryptic peptide mapping. In: Howley, P. M. and Broker, T. R. (eds) Papillomavirus: molecular and clinical aspects. Alan R. Liss, Inc. New York. 327-340.

Ferenczy, A., Braun, L. and Shah, K. (1981). Human papillomavirus in condylomatous lesions of cervix: a comparative ultrastructural and immunohistochemical study. Am J Surg Pathol **5**: 661-670.

Ferenczy, A. (1982). Cervical intraepithelial neoplasia. In: Pathology of the Female Genital Tract (ed A. Blaustein) 2nd edn. Springer-Verlag, New York, Heidelberg, Berlin, 156-177.

Fidler, H. K., Boyes, D. A. and Worth, A. J. (1968). Cervical cancer detection in British Columbia. J Obstet Gynaecol Br Commonwlth **75**: 392-404.

Fox, C. H. (1967). Biological behaviour of cervical dysplasia and carcinoma-in-situ. Am J Obstet Gynaecol **99**: 960-974.

Friedman, J. C., Levy, J. P., Lasneret, J., Thomas, M., Boiron, M. and Bernard, J. (1963). Induction de fibromes sous-cutanes chez le hamster dore par inoculation d'extraitsacellulaires de papillomes bovins. C R Acad Sci **257**: 2328-2331.

Fuchs, P. G., Girardi, F. and Pfister,H. (1987). Human papillomavirus DNA in normal, metaplastic,preneoplastic and neoplastic epithelia of the cervixuteri. Int J Cancer **40**: 41-45.

Fujinaga, Y., Shimada, M., Okazawa, K., Fukushima, M., Kato, I. and Fujinaga, K. (1991). Simultaneous detection and typing of genital human papillomavirus DNA using the polymerase chain reaction. J Gen Virol **72**: 1039-1044.

Furuta, Y. (1990). Molecular pathologic study of human papillomavirus infection in inverted papilloma and sqamous cell carcinoma of the nasal cavities and paranasal sinuses. Hokkaido Igaku Zasshi **65**: 568-582.

Gage, J. R., Meyers, C. and Wettstein, F. O. (1990). The E7 proteins of the nononcogenic human papillomavirus type 6b (HPV6b) and of the oncogenic HPV 16 differ in retinoblastoma protein binding and other properties. J Virol **64**: 723-730.

Galloway, T. C., Soper, G. R. and Elsen, G. (1960). Carcinoma of the larynx after irradiation for papilloma. Arch Otolaryngol **72**: 289-294.

Garcia-Carranca, A., Thierry, F. and Yaniv, M. (1988). Interplay of viral and cellular proteins along the long control region of human papillomavirus type 18. J Virol **62**: 4321-4330.

Gassenmaier, A., Lammel, M. and Pfister,H. (1984). Molecular cloning of the DNAs of human papillomaviruses 19, 20 and 25 from a patient with epidermodysplasia verruciformis. J Virol **52**: 1019-1023.

Gendelman, H. E., Phelps, W., Feigenbaum, L. et al. (1986) Transactivation ot the human immunodeficiency virus long terminal repeat sequence by DNA viruses. Proc Natl Acad Sci. USA **83**: 9759-9763.

Gibbs, R. A. and Chamberlain, J. S. (1989). The polymerase chain reaction: a meeting report. Genes Develop **3**: 1095-1098.

Giles, J. A., Hudson, E., Crow, J., Williams, D. and Walker, P. (1988). Colposcopic assessment of the accuracy of cervical cytology screening. Br Med J **296**: 1099-1102.

Giniger, G. M. and Ptashne, M. (1987). Transcription in yeast activated by a putative amphipathic alpha helix linked to a DNA binding unit. Nature **330**: 670-672.

Giri, I., Danos, O. and Yaniv, M. (1985). Genomic structure of the cottontail rabbit (Shope) papillomavirus. Proc Natl Acad Sci. USA **82**: 1580-1584.

Giri, I. and Yaniv, M. (1988). Study of the E2 gene product of the cottontail rabbit papillomavirus reveals a common mechanism of transactivation among the papillomaviruses. J Virol **62**: 1573-1581.

Gissmann, L., Diehl, V., Schulz-Coulon, H. J. and zur Hausen, H. (1982). Molecular cloning and characterization of human papillomavirus DNA derived from a laryngeal papilloma. J Virol **44**: 393-400.

Gissmann, L., Wolnik, L., Ikenberg, H., Koldovsky, V., Schnurch, H. and zur Hausen, H. (1983). Human papillomavirus type 6 and 11 DNA sequences in genital and laryngeal papillomas and in some cervical cancers. Proc Natl Acad Sci USA **80**: 560-3.

Gius, D., Grossman, S., Bedell, M. A. and Laimons, L. A. (1988). Inducible and constitutive enhancer domains in the noncoding region of human papillomavirus type 18. J Virol **62**: 665-672.

Gius, D. and Laimins, L. A. (1989). Activation of human papillomavirus type 18 gene expression by herpes simplex virus type-1 viral transactivators and a phorbolester. J Virol **63**: 555-563.

Gloss, B., Bernard, H. U., Seedorf, K. and Klock, G. (1987). The upstream regulatory region of the human papillomavirus 16 contains an E2 protein-independent enhancer which is specific for cervical carcinoma cells and regulated by glucocorticoid hormones. EMBO J **6**: 3735-3743.

Gloss, B., Chong, T. and Bernard, H. (1989). Numerous nuclear proteins bind the long control region of human papillomavirus type 16: a subset of 6 of 23 DNase I-protected segments coincides with the location of the cell-type specific enhancer. J Virol **63**: 1142-1152.

Greenspan, D., de Villiers, E. M., Greenspan, J. S., de Souza, Y. G. and zur Hausen, H. (1988). Unusual HPV types in oral warts in association with HIV infection. J Oral Pathol **17**: 482-487.

Gregoire, L., Arella, M., Campione-Piccardo, J. and Lancaster, W. D. (1989). Amplification of human papillomavirus DNA sequences by using conserved primers. J Clin Microbiol **27**: 2660-2665.

Griffin, N. R., Bevan, I. S., Lewis, F. A., Wells, M. and Young, L. S. (1990). Demonstration of multiple HPV types in normal cervix and in cervical squamous cell carcinoma using the polymerase chain reaction on paraffin wax embedded material. J Clin Pathol **43**: 52-56.

Groff, D. E. and Lancaster, W. D. (1985). Molecular cloning and nucleotide sequence of deer papillomavirus. J Virol **56**: 85-91.

Hallam, N., Gibson, P., Green, J. and Charnock, M. (1989). Detection and typing of human papillomavirus infection of the uterine cervix by dot blot hybridization: Comparison of scrapes and biopsies. J Med Virol **27**: 317-321.

Harris, J. H. and Peterson, P. (1956). Cold-knife conization and residual pre-invasive carcinoma of the cervix. Am J Obstet Gynecol **70**: 1092-1099.

Harrison, S. M., Gearing, K. L., Kim, S. Y., Kingsman, A. J. and Kingsman S. M. (1987). Multiple *cis* active elements in the long control region of bovine papillomavirus type 1. Nucleic Acids Res **15**: 10267-10284.

Haugen T. H., Cripe, T. P., Ginder, G. D., Karin, M. and Turek, L. P. (1987). *Trans*activation of an upstream early gene promoter of bovine papillomavirus-1 by a product of the viral E2 gene. EMBO J **6**: 145-152.

Haugen, T. H., Turek, L. P., Mercurio, F. M., Cripe, T. P., Olsen, B. J., Anderson, R. D., Seidl, D., Karin, M. and Schiller, J. (1989). Sequence specific and general transcriptional transactivation by the BPV-1 E2 transactivator require an N-terminal amphipathic helix-containing E2 domain. EMBO J **7**: 4245-4253.

Hawley-Nelson, P., Androphy, E. J., Lowy, D. R. and Schiller J. T. (1988). The specific DNA recognition sequences of the bovine papillomavirus E2 protein is an E2-dependent enhancer. EMBO J **7**: 525-531.

Heike, T., Miyatake, S., Yoshida, M. Arai, K. and Arai, N. (1989). Bovine papillomavirus encoded E2 protein activates lymphokine genes through DNA elements, distinct from the consensus motif, in the long control region of its own genome. EMBO J **8**: 1411-1417.

Heilman, C. A., Law, M. F., Israel, M. A. and Howley, P. M. (1980). Cloning of human papillomavirus genomic DNAs and analysis of homologous polynucleotide sequences. J Virol **36**: 395-407.

Hermonat, P. L. and Howley, P. M. (1987). Mutational analysis of the 3' open reading frames and the splice junction at nucleotide 3225 of bovine papillomavirus type 1. J Virol **61**: 3889-3895.

Hermonat, P. L., Spalholz, B. A. and Howley, P. M. (1988). The bovine papillomavirus P_{2443} promoter is E2 *trans*-responsive: evidence for E2 autoregulation. EMBO J **7**: 2815-2822.

Herrero, R., Brinton, L. A., Reeves, W. C., Brenes, M. M., Tenorio, F., de Britton, R. C., Gaitan, E., Montalvan, P., Garcia, M. and Rawls, W. E. (1990). Risk factors for invasive carcinoma of the uterine cervix in Latin America. Bull Pan Am Health Organ **24**: 263-283.

Hildesheim, A., Mann, V., Brinton, L. A., Szklo, M., Reeves, W. C. and Rawls, W. E. (1991). Herpes simplex virus type 2: a possible interaction with human papillomavirus types 16/18 in the development of invasive cervical cancer. Int J Cancer **49**: 335-340.

Hirochika, H., Hirochika, R., Broker, T. R. and Chow, L. T. (1988). Functional mapping of the human papillomavirus type 11 transcriptional enhancer and its interaction with the trans-actingE2 protein. Genes Dev **2**: 54-67.

Ho, L., Chan, S. Y., Chow, V., Chong, T., Tay, S. K., Villa, L.L. and Bernard, H. U. (1991) Sequence variants of human papillomavirus type 16 in clinical samples permit verification and extension of epidermological studies and construction of a phylogenetic tree. J Clin Microbiol **29**: 1765-1772.

Hoppe-Seyler, F., Butz, K. and zur Hausen, H. (1991). Repression of the human papillomavirus type 18 enhancer by the cellular transcription factor Oct-1. J Virol **65**: 5613-5618.

Hording, U., Daugaard, S., Bock, J. E., Sebbelov, A. M. and Norrild, B. (1991) Eur J Obstet Gynaecol **40**: 43-48.

Hubbert, N. L., Schiller, J. T., Lowy, D. R. and Androphy E. J. (1988). Bovine papillomavirus transformed cells contain multiple E2 proteins. Proc Natl Acad Sci. USA **85**: 5864-5868.

Hulka, B. S. (1968). Cytologic and histologic outcome following an atypical cervical smear. Am J Obstet Gynaecol **101**: 190-199.

Jarrett, W. F. (1980). Bracken fern and papillomavirus in bovine alimentary cancer. Br Med Bull **36**: 79-81.

Jarrett, W. F. (1985). The natural history of bovine papillomavirus infection. In: Klein, G. (ed) Advances in Viral Oncology. Raven Press, New York vol 5: 83-101.

Jarrett, W. F., Smith, K. T., O'Neil, B. W., Gaukroger, J. M., Chandrachud, L. M., Grindlay, G. J., McGarvie, G. M. and Campo, M. S. (1991). Studies on vaccination against papillomaviruses: prophylactic and therapeutic vaccination with recombinant structural proteins. Virology **184**: 33-42.

Jenison, S. A., Firzlaff, J. M., Langenberg, A. and Galloway, D. A. (1988). Identification of immunoreactive antigens of human papillomavirus type 6b using *Escherichia coli*-expressed fusion proteins. J Virol **62**: 2115-2123.

Jenson, A., Rosenthal, J., Olsen, C., Pass, F., Lancaster, W. and Shah, K. (1980) Immunological relatedness of papillomaviruses from different species. J Natl Cancer Inst **64**: 495-500.

Jochmus-Kudielka, I., Schneider, A., Braun, R., Kimming, R., Koldovsky, V., Schneeweis, K. E., Seedorf, K. and Gissmann, L. (1989). Antibodies against the human papillomavirus type 16 early proteins in human sera: correlation of anti-E7 reactivity with cervical cancer. J Natl Cancer Inst **82**: 1698-1703.

Kaelbling, M., Burk, R. D., Atkin, N. B., Johnson, A. B. and Klinger, H. P. (1992) Loss of heterozygosity on chromosome 17p and mutant p53 in HPV-negative cervical carcinomas. Lancet **340**:140-142.

Kataja, V., Syrjanen, K., Syrjanen, S., Mantyjarvi, R., Yliskoski, M., Saarikoski, S. and Salonen, J. T. (1990) Prospective follow-up of genital HPV infections: survival analysis of the HPV typing data. Eur J Epidemiol **6**: 9-14.

Kataja, V., Syrjanen, S., Mantyjarvi, R., Yliskoski, M., Saarikoski, S. and Syrjanen, K. (1992) Prognostic factors in cervical HPV infections. Sexually transmitted diseases **19**: 154-160.

Kenney, S., Kamine, J., Markovitz, D., Fenrick, R. and Pagano, J. (1988) An Epstein-Barr virus immediate early gene product transactivates gene expression from the human immunodeficiency virus long terminal repeat. Proc Natl Acad Sci. USA **85**: 1652-1656.

Kessler, I. I. (1976). Human cervical cancer as a venereal disease. Cancer Res **36**: 783-791.

Kinlen, L. J. and Spriggs, A. I. (1978). Women with positive cervical smears, but without surgical intervention: a follow-up study. Lancet **ii**: 463-465.

Kirii, Y., Iwamoto, S. and Matsukura, T. (1991). Human papillomavirus type 58 DNA sequence. Virology **185**: 424-427.

Kitchener, H. C., Neilson, L., Burnett, R. A., Young, L. and Macnab, J. C. (1991) Prospective serial study of viral change in the cervix and correlation with human papillomavirus genome status. Br J Ostet Gynaecol **98**: 1042-1048.

Kitchin, P. A., Szotyori, Z., Fromholc, C. and Almond, N. (1990). Avoidance of false positives. Nature **344**: 201.

Kiviat, N. B., Koutsky, L. A., Paavonen, J. A., Galloway, D. A., Critchlow, C. W., Beckmann, A. M. McDougall, J. K., Peterson, M. L. Stevens, C. E. and Lipinski, C. M. (1989). Prevalence of genital papillomavirus infection among women attending a college student health clinic or a sexually transmitted disease clinic. J Inf Dis **159**: 293-302.

Kloster, B. E., Manias, D. A., Ostrow, R. S., Shaver, M. K., Mc Pherson, S. W., Rangen, S. R., Uno, H. and Faras, A. J. (1988). Molecular cloning and characterizationof the DNA of two papillomaviruses from monkeys. Virology **166**: 30-40.

Kochel, H. G., Monazahian, M., Sievert, K., Hohne, M., Thomssen, C., Teichmann, A., Arendt, P. and Thomssen, R. (1991a) Occurrence of antibodies to L1, L2, E4 and E7 gene products of human papillomavirus types 6b, 16 and 18 among cervical cancer patients and controls. Int J Cancer **48**: 682-688.

Kochel, H. G., Sievert, K., Monozahian, M., Mittelstadt-Deterding, A., Teichmann, A. and Thomssen, R. (1991b). Antibodies to human papillomavirus type 16 in human sera as revealed by the use of prokaryotically expressed viral gene products. Virology **182**: 644-654.

Kogan, S. C., Doherty, M. and Gitschier, J. (1987). An improved method for prenatal diagnosis of genetic disease by analysis of amplified DNA sequences. N Engl J Med **317**: 985-990.

Komly, K. A., Breitburd, F., Croissant, O. and Streeck R. E. (1986). The L2 open reading frame of human papillomavirus type 1a encodes a minor structural protein carrying type specific antigens. J Virol **60**: 813-816.

Kondoh, G., Murata, Y., Aozasa, K., Yutsudo, M. and Hakura, A. (1991). Very high incidence of germ cell tumorigenesis (seminomagenesis) in human papillomavirus type 16 transgenic mice. J Virol **65**: 3335-3339.

Kottmeier, H. L. (1961). Evolution et traitement des epitheliomas. Rev Franc Gynecolet Obstet **56**: 821-825.

Krajinovic, M. and Savic, A. (1991). Sequencing data on the long control region of human papillomavirus type 16. J Gen Virol **72**: 2573-2576.

Kreider, J. W., Howett, M. K., Stoler, M. H., Zaino, R. J. and Welsh, P. (1987). Susceptability of various human tissues to transformation *in vivo* with human papillomavirus type 11. Int J Cancer **39**: 459-465.

Kremsdorf, D., Jablonska, S., Favre, M. and Orth, G. (1983). Human papillomaviruses associated with epidermodysplasia verruciformis. Molecular cloning and biochemical characterizationof human papillomavirus 3a, 8, 10 and 12 genomes. J Virol **48**: 340-351.

Kremsdorf, D., Favre, M., Jablonska, S., Obalek, S., Rueda, L. A., Lutzner, M. A., Blanchet-Bardon, C., Van Voost Vader, P. C. and Orth, G. (1984). Molecular cloning and characterization of the genomes of nine newly recognized human papillomavirus types associated with epidermodysplasia verruciformis. J Virol **52**: 1013-1018.

Kurman, R. J., Schiffman, M. H., Lancaster, W. D., Reid, R., Jenson, A. B., Temple, G. F. and Lorincz, A. T. (1988). Analysis of individual human papillomavirus types in cervical neoplasia. A possible role for type 18 in rapid progression. Am J Obstet Gynecol **159**: 293-296.

Kwok, S. and Higuchi, R. (1989). Avoiding false positives with PCR. Nature **339**: 237-238.

Kyo, S., Inoue, M., Nakazawa, A., Ogawa, H., Ueda, G. and Tanizawa, O. (1991). Detection of HPV DNA in the uterine cervical lesions by polymerase chain reaction and *in situ* hybridization. Nippon Sanka Fujinka Gakkai Zasshi **43**: 541-548.

Laimins, L. A., Bedell, M. A., Jones, K. H. and Long, J. A. (1987). Transformation of NIH 3T3 and primary rat embryo fibroblasts by human papillomavirus type 16 and type 18. In: Steinberg, B. M., Brandsma, J. L., Taichman, L. B. Eds. Cancer cells, vol 5: papillomaviruses. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory, 201-207.

Lambert, P. F., Spalholz, B. A. and Howley, P. M. (1987). A transcriptional repressor encoded by BPV-1 shares a common carboxy terminal domain with the E2 transactivator. Cell **50**: 68-78.

Lambert, P. F., Baker, C. C. and Howley, P. M. (1989a). The genetics of bovine papillomavirus type 1. Annu Rev Genet **22**: 235-258.

Lambert, P. F., Hubbert, N. L., Howley, P. M. and Schiller, J. T. (1989b). Genetic assignment of the multiple E2 gene products in bovine papillomavirus transformed cells. J Virol **63**: 3151-3154.

Lancaster, W. D., Olsen, C. and Meinke, W. (1977). Bovine papillomavirus: presence of virus-specific DNA sequences in naturally occuring equine tumors. Proc Natl Acad Sci. USA **74**: 524-528.

Lancaster, W. D. and Olsen, C. (1982). Animal papillomaviruses. Microbiol Rev **46**: 191-207.

Lancaster, W. D. and Sundberg, J. P. (1982). Characterization of papillomaviruses isolated from cutaneous fibromas of white tailed deer and mule deer. Virology **123**: 212-216.

Landschultz, W. H., Johnson, P. F. and McKnight, S. L. (1988). The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science **240**: 1759-1764.

Laverty,C. R., Russell, P., Hills, E. and Booth, N. (1978). The significance of non condylomatous wart virus infection of the cervical transformation zone. A review with discussion of two illustrative cases. Acta Cytol **22**: 195-201.

Lehn, H., Krieg, P. and Sauer, G. (1985). Papillomavirus genomes in human cervical tumours: Analysis of their transcriptional activity.Proc Natl Acad Sci. USA **82**: 5540-5544.

Lehn, H., Villa, L. L., Marziona, F., Hilgarth, M., Hillemans, H. and Sauer, G. (1988). Physical state and biological activity of human papillomavirus genomes in precancerous lesions of the female genital tract. J Gen Virol **69**:187-196.

Leptak, C., Ramon y Cajal, S., Kulke, R., Horwitz, B. H., Riese, D. J., Dotto, G. P. and diMaio, D. (1991) Tumorigenic transformation of murine keratinocytes by the E5 genes of bovine papillomavirus type 1 and human papillomavirus type 16. J Virol **65**: 7078-7083.

Lewin, B. (1990). Genes IV, Oxford University Press.

Ley, C., Bauer, H. M., Reingold, A., Schiffman, M. H., Chambers, J. C., Tashiro,C. J. and Manos, M. M. (1991). Determinants of genital human papillomavirus infection in young women. J Natl Cancer Inst **83**: 978-979.

Li, A. P., Myers, C. A. and Kaminski, D. L. (1992) Gene transfer in primary cultures of human hepatocytes. In Vitro cell develop biology **28**: 373-375.

Li, R., Knight, J., Bream, G., Stenlund, A. and Botchan, M. (1989). Specific recognition nucleotides and their DNA context determine the affinity of E2 protein for 17 binding sites in the BPV-1 genome. Genes Dev **3**: 510-526.

Linz, U. and Baker, C. C. (1988). Promoters of bovine papillomavirus type 1: *In vitro* activity and utilization. J Virol **62**: 2537-2543.

Lorincz, A. T., Lancaster, W. D. and Temple, G. F. (1986). 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.

Longo, M. C., Berninger, M. S. and Hartley, J. L. (1990). Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. Gene **93**: 125-128.

Lopata, M. A., Cleveland, D. W. and Solher-Webb, B. (1984) High level expression of a CAT gene by DEAE-dextran mediated DNA transfection coupled with a DMSO or glycerol shock treatment. Nucl Acids Res **12**: 5707.

Lowy, D. R., Dvoretsky, I., Shober, R., Law, M. F., Engel, L. and Howley, P. M. (1980). *In vitro* tumorigenic transformation by a defined sub-genomic fragment of bovine papillomavirus DNA. Nature **287**: 72-74.

Lusky, M. and Botchan, M. R. (1984). Characterization of the bovine papillomavirus plasmid maintenance sequences. Cell **36**: 391-401.

Lusky, M. and Botchan, M. R. (1985). Genetic analysis of bovine papillomavirus type 1 trans-acting replication factors. J Virol **53**: 955-965.

Lusky, M. and Botchan, M. R. (1986a). Transient replication of bovine papillomavirus type 1 plasmids: cis and trans requirements. Proc Natl Acad Sci. USA **83**: 3609-3613.

Lusky, M. and Botchan, M. R. (1986b). A bovine papillomavirus type 1 encoded modulator function is dispensable for transient viral replication, but is required for establishment of the stable plasmid state. J Virol **60**: 729-742.

Mack, D. H. and Laimins, L. A. (1991) A keratinocyte-specific transcription factor, KRF-1, interacts with AP-1 to activate expression of human papillomavirus type 18 in squamous epithelial cells. Proc Natl Acad Sci. USA **88** 9102-9106.

Marich, J. E., Ponster, A. V., Rice, S. A., McGraw, K. A. and Dubensky, T. W. (1992). The phylogenetic relationship and complete nucleotide sequence of human papillomavirus type 35. Virology **186**: 770-776.

Matlashewski, G. Schneider, J., Banks, L., Jones, N., Murray, A. and Crawford, L. (1987). Human papillomavirus type 16 DNA cooperates with activated *ras* in transforming primary cells. EMBO J **6**: 1741-1746.

Matorras, R., Ariceta, . M., Rementeria, A., Corral, J., Gutierrez-de-Teran, G., Diez, J., Montoya, F. and Rodriguez-Escudero, F. J. (1991). Human immunodeficiency virus-induced immunosupression: a risk factor for human papillomavirus infection. Am J Obstet Gynecol **164**: 42-44.

Mayelo, V., Coursaget, P., Fignon, A., Lhuintre, Y., Lansac, J. and Anthonioz, P. (1990). Detection of human type 16 papillomavirus in epithelial cells of the uterine cervix. Presse Med **19**: 1978-1980.

Mc Bride, A. A., Schlegel, R. and Howley, P. M. (1988). The carboxy-terminal domain shared by the bovine papillomavirus E2 transactivator and repressor proteins contains a specific DNA binding activity. EMBO J **7**: 533-539.

Mc Bride, A. A., Byrne, J. C. and Howley, P. M. (1989). E2 polypeptides encoded by bovine papillomavirus 1 form dimers through the carboxyl terminal DNA binding domain: transactivation is mediated through the conserved amino-terminal domain. Proc Natl Acad Sci. USA **86**: 510-514.

McCance, D. J., Walker, P. G., Dyson, J. L., Coleman, D. C. and Singer, A. (1983). Presence of human papillomavirus DNA sequences in cervical intraepithelial neoplasia. Br Med J **287**: 784-788.

McCance, D. J., Clarkson, P. K., Dyson, J. L., Walker, P. G. and Singer, A. (1985a). Human papillomavirus types 6 and 16 in multifocal intraepithelial neoplasias of the female lower genital tract.Br J Obstet Gynaecol **92**: 1093-1100.

McCance, D. J.,Campion, M. J.,Clarkson, P. K., Chesters,P. M., Jenkins, D. and Singer, A. (1985b). Prevalence of human papillomavirus type 16 DNA sequences in cervical intraepithelial neoplasia and invasive carcinoma of the cervix. Br J Obstet Gynaecol **92**: 1101-1105.

McCance, D. J.,Campion, M. J. and Singer, A. (1986). Non-invasive detection of cervical papillomavirus DNA. Lancet **i**: 558-559.

McCance , D. J., Kopan, R., Fuchs, E. and Laimins, L. A. (1988). Human papillomavirus type 16 alters human epithelial cell differentiation *in vitro*.Proc Natl Acad Sci. USA **85**: 7169-7173.

McDonnell, J. M., Mayr, A. J. and Martin, W. J. (1989). DNA of human papillomavirus type 16 in dysplastic and malignant lesions of the conjunctiva and cornea. N Engl J Med **320**: 1442-1446.

McGregor, J. E. and Lupen, S. (1978). Uterine cervical cytology and young women. Lancet **i**: 1029-1031.

McIndoe, W. A., McClean, M. R., Jones, R. N. and Mullins, P. R. (1984). The invasive potential of carcinoma-in-situ of the cervix. Obstet Gynaecol **64**: 451.

McNicol, P. J., Guijon, F. B., Paraskevas, M. and Brunham, R. C. (1989). Comparison of filter *in situ* deoxyribonucleic acid hybridization with cytologic, colposcopic and histopathologic examination for detection of human papillomavirus infection in women with cervical intraepithelial neoplasia. Am J Obstet Gynaecol **160**: 265-270.

Meanwell, C. A., Cox, M. F., Blackledge, G. and Maitland N. J. (1987). HPV 16 DNA in normal and malignant cervical epithelium: implications for the etiology and behaviour of cervical neoplasia. Lancet **i**: 703-707.

Meinke, W. and Meinke, G. C. (1981). Isolation and characterization of the major capsid protein of bovine papillomavirus type 1. J Gen Virol **52**: 15-24.

Meischke, H. R. C. (1979). *In vitro* transformation by bovine papillomavirus. J Gen Virol **43**: 473-487.

Meisels, A. and Fortin, R. (1976). Cytological lesions of the cervix and vagina I. Cytological patterns. Acta Cytologica **20**: 505-509.

Meisels, A., Fortin, R. and Roy, M. (1977). Condylomatous lesions of the cervix II. Cytologic, colposcopic and histopathologic study. Acta Cytol **21**: 379-390.

Meisels, A., Morin, C. and Casas Cordero, M. (1982). Human papillomavirus infection of the uterine cervix. Int J Guynecol Pathol **1**: 75-94.

Melchers, W., Herbrink, P., Quint, W., Walboomers, J., Meijer, C. and Lindeman J. (1988). Prevalence of genital HPV infections in a regularly screened population in the Netherlands in relation to cervical cytology. J Med Virol **25**: 11-16.

Melchers, W. J. G., Herbink, P., Walboomers, J. M. M., Meyer, C. J. L. M., Drift, H., Lindeman, J. and Quint, W. G. V. (1989a). Optimization of HPV genotype detection in cervical scrapes by a modified filter *in situ* hybridization test. J Clin Microbiol **27**: 106-110.

Melchers, W., van den Brule, A., Walboomers, J., de Bruin, M., Herbrink, P., Meijer, C., Lindeman, J. and Quint, W. (1989b). Increased detection rate of human papillomavirus in cervical scrapes by the polymerase chain reaction as compared to modified FISH and Southern blot analysis. J Med Virol **27**: 329-335.

Meyers, C., Harry, J., Lin, Y. and Wettstein, F. O. (1992) Identification of three transforming proteins encoded by cotton-til rabbit papillomavirus. J Virol **66**: 1655-1664.

Mitrani-Rosenbaum, S., Tsvieli, R. and Tur-Kaspa, R. (1989). Oestrogen stimulates differential transcription of human papillomavirus type 16 in SiHa cervical carcinoma cells. J Gen Virol **70**: 2227-2232.

Moore, P. D., Bose, K. K., Rabkin, S. D. and Strauss B. S. (1981). Sites of termination of *in vitro* DNA synthesis on ultraviolet-and N-acetylaminofluorine-treated ϕ X179 templates by prokayotic and eukaryotic DNA polymerases. Proc Natl Acad Sci. USA **78**: 110-114.

Moreno-Lopez, J., Pettersson, U., Dinter, Z. and Philipson, L. (1981). Characterization of a papillomavirus from the European elk (EEPV). Virology **112**: 589-595.

Moreno-Lopez, J., Morner, T. and Pettersson, U. (1986). Papillomavirus DNA associated with pulmonary fibromatosis in European elks. J Virol **57**: 1173-1176.

Morgan, D. M., Peorado, G., Rosenberg, I. and Defendi, V. (1992) Transformation by human papillomavirus type 16 DNA but not HPV 6b DNA is enhanced by addition of the human cytomegalovirus enhancer. Virology **189**: 687-694.

Morris, B. J., Rose, B. R., Flanagan, J. L., McKinnon, K. J., Loo, C. Y., Thompson, C. H., Flampoulidou, M., Ford, R. M., Hunter, J. C., Nightingale, B. N. and Cossart, Y. E. (1990). Automated polymerase chain reaction for papillomavirus screening of cervicovaginal lavages: Comparison with dot blot hybridization in a sexually transmitted diseases clinic group. J Med Virol **32**: 22-30.

Munger, K., Werness, B., Dyson, N., Phelps, W. C., Harlow, E. and Howley, P. M. (1989). Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumour supressor gene product. EMBO J **8**: 4099-4105.

Munoz, N., Bosch, X. and Kaldor, J.M. (1988). Does human papillomavirus cause cervical cancer? The state of the epidemiological evidence. Br J Cancer **57**: 1-5.

Nagai, N., Nuovo, G., Friedman, D. and Crum, C. (1987). Detection of papillomavirus nucleic acids in genital precancers with the in situ hybridization technique. Int J Gynecol Pathol **6**: 366-379.

Nakai, Y., Lancaster, W. D., Lim, Y. and Jenson, B. (1986). Monoclonal antibodies to genus- and type-specific papillomavirus structural antigens. Intervirology **25**: 30-37.

Neary, K., Horwitz, B. H. and DiMaio, D. (1987). Mutational analysis of open reading frame E4 of bovine papillomavirus type 1. J Virol **61**: 1248-1252.

Neary, K. and di Maio, D. (1989). Open reading frames E6 and E7 of bovine papillomavirus type 1 are both required for full transformation of mouse C127 cells. J Virol **63**: 259-266.

Nishikawa, A., Fukushima, M., Shimada, M., Yamakawa, Y., Shimano, S., Kato, I. and Fujinaga, K. (1991). Relatively low prevalence of human papillomavirus 16, 18 and 33 DNA in the normal cervices of Japanese women shown by polymerase chain reaction. Jpn J Cancer Res **82**: 532-538.

Novak, T. J., Yoshimura, F. K. and Rothenberg, E. V. (1992) In vitro transfection of fresh thymocytes and T cells shows subset specific expression of viral promoters. Molecular and cellular biology **12**:1515-1527.

Ocadiz, R., Sauceda, R., Cruz, M., Graef, A. and Gariglio, P. (1987). High correlation between molecular alterations of the c-*myc* oncogene and carcinoma of the cervix. Cancer Res **47**: 4173-4177.

Oltersdorf, T., Campo, M. S., Favre, M., Dartmann, K. and Gissmann, L. (1986). Molecular cloning and characterization of human papillomavirus type 7 DNA. Virology **149**: 247-250.

Orth, G., Favre, M. and Croissant, O. (1977). Characterization of a new type of human papillomavirus that causes skin warts. J Virol **24**: 108-120.

Orth, G., Jablonska, S., Favre, M., Croissant, O., Jarzabek-Chorzelska, M. and Rzesa, G. (1978). Characterization of two types of human papillomaviruses in lesions of epidermodysplasia verruciformis. Proc Natl Acad Sci. USA **75**: 1537-1541.

Orth, G., Jablonska, S., Favre, M., Croissant, O., Obalek, S., Jarzabek-Chorzelska, M. and Jibard, N. (1981). Identification of papillomavirus in butchers' warts. J Invest Dermatol **70**: 97-102.

Orth, G. (1987). Epidermodysplasia verruciformis. In: Salzman N. P., Howley, P. M. (eds) The papovaviridae, vol 2. New York: Plenum Press, 199-243.

O'Shea, E. K., Rutkowski, R. and Kim, P. S. (1989). Evidence that the leucine zipper is a coiled coil. Science **243**: 538-542.

Ostrow, R. S., Bender, M., Niimura, M., Seki, T., Kawashima, M., Pass, F. and Faras, A. J. (1982). Human papillomavirus DNA in cutaneous primary and metastasized squamous cell carcinomas from patients with epidermodysplasia verruciformis. Proc Natl Acad Sci. USA **79**: 1634-1638.

Palefsky, J. M., Winkler, B., Rabanus, J. P., Clark, C., Chan, S., Nizet, V. and Schoolnik, G. K. (1991a). Characterization of *in vivo* expression of the human papillomavirus type 16 E4 protein in cervical biopsy tissues. J Clin Invest **87**: 2132-2141.

Palefsky, J. (1991b). Human papillomavirus infection among HIV-infected individuals. Implications for development of malignant tumours. Hematol Oncol Clin North Am **5**: 357-70.

Pao, C. C., Lin, C., Maa, J., Lai, C., Wu, S. and Soong, Y. (1990). Detection of human papillomaviruses in cevicovaginal cells using the polymerase chain reaction. J Infectious Diseases **161**: 113-115.

Papanicolaou, G. N. (1949). A survey of the actualities and potentialities of exfoliative cytology in cancer diagnosis. Am Int Med **31**: 661-674.

Parkkinen, S., Mantyjarvi, R., Syrjanen, K. and Ranki, M. (1986). Detection of human papillomavirus DNA by the nucleic acid sandwich hybridization method from cervical scraping. J Med Virol **20**: 279-288.

Pater, M. M., Hughes, G. A., Hyslop, D. E., Nakshatri, H. and Pater, A. (1988). Glucocorticoid-dependent oncogenic transformation by type 16 but not type 11 human papillomavirus DNA. Nature **335**: 832-835.

Pater, M. M. and Pater, A. (1991). RU486 inhibits glucocorticoid hormone-dependent oncogenesis by human papillomavirus type 16 DNA. Virology **183**: 799-802.

Pecorado, G., Morgan, D. and Defendi V. (1989). Differential effectsof human papillomavirus type 6, 16 and 18 DNAs on immortalization and transformation of human cervical epithelial cells. Proc Natl Acad Sci. USA **86**:563-567.

Peto, R. (1986). Introduction: geographic patterns and trends. In: Peto, R., zur Hausen (eds) Viral etiology of cervical cancer. Banbury report 21. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory, 3-15.

Petti,L., Nilson, L. A. and DiMaio, D. (1991). Activation of the platelet-derived growth factor receptor by the bovine papillomavirus E5 transforming protein. EMBO J **10**: 845-855.

Pfister,H., Gissmann, L. and zur Hausen, H. (1977). Partial characterizationof proteins of human papillomaviruses (HPV) 1-3. Virology **83**: 131-137.

Pfister, H. (1984). Biology and biochemistry of papillomaviruses. Rev Physiol Biochem Pharmacol **99**: 111-181.

Phelps, W. C., Yee, C. L., Munger, K. and Howley, P. M. (1988). The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to adenovirus E1a. Cell **53**: 539-547.

Pietenpol, J. A., Stein, R. W., Moran, E., Yaciuk, P., Schlegel, R., Lyons, R. M., Pihelkow, M. R., Munger, K., Howley , P. M. and Moses, H. L. (1990). TGF-ß1 inhibition of c-*myc*transcription and growth in keratinocytesis abrogated by viral transforming proteins with p-Rb binding domains. Cell **61**: 777-785.

Pinion, S. B., Kennedy, J. H., Miller, R. W. and MacLean, A. B. (1991). Oncogene expression in cervical neoplasia and invasive cancer of cervix. Lancet **337**: 819-820.

Pirisi, L., Yasumoto, S., Feller, M., Doninger, J. and DiPaolo, J. A. (1987). Transformation of human fibroblasts and keratinocyteswith human papillomavirus type 16 DNA. J Virol **61**: 1061-1066.

Poulsen, M. E., Taylor, C. W. and Sobin, L. M. (1975). Histological typing of female genital tract tumours. World Health Organization, Geneva, 15-18.

Prakesh, S. S., Reeves, W. C., Sisson, G. R., Brenes, M., Godoly, I., Bachetti, S., de Britton, R. and Rawls, W. E. (1985). HSV-2 and HPV-16 in cervicitis,dysplasia and invasive cevical cancer. Int J Cancer **35**: 51-57.

Ptashne, M. (1986). Gene regulation by proteins acting nearby and at a distance. Nature **322**: 697-701.

Purola, E. and Savia, E. (1977). Cytology of gynecologic condyloma acuminatum. Acta Cytol **23**: 26-31.

Rabson, M. S., Yee, C., Yang, Y. C. and Howley, P. M. (1986). Bovine papillomavirus type 1 3' early region transformation and plasmid maintenance functions. J Virol **60**: 626-634.

Rakoczy, P., Sterrett,G., Kulski, J., Whitaker, D., Hutchinson, L., MacKenzie, J. and Pixley, E. (1990). Time trends in the prevalence of human papillomavirus infections in archival Papanicolaou smears: Analysis by cytology, DNA hybridization and polymerase chain reaction. J Med Virol **32**: 10-17.

Rando, R. F., Groff, D. E., Chirikjian, J. G. and Lancaster, W. D. (1986). Isolation and characterizationof a novel human papillomavirus type 6 DNA from an invasive vulvar carcinoma. J Virol **57**: 353-356.

Rawls, W. E., Garfield, C. H. and Seth, P. (1976). Serological and epidemiologic considerations of the role of herpes simplex virus type 2 in cervicalcancer. Cancer Res **36**: 829-835.

Rawls, W. E., Clarke, A., Smith, K. O., Docherty, J. J., Gilman, S. C. and Grahams, S. (1980). Specific antibodies to herpes simplex virus type 2 among women with cervical cancer. In: Essex, M., Todaro, G., zur Hausen, H. Eds. Viruses in naturally occurring cancers. Vol A, Cold Spring Harbor: Cold Spring Harbor Lab Press, 117-33.

Reagan, J. W., Seidemand, I. L. and Saracusa, Y. (1953). The cellular morphology of carcinoma *in situ* and dysplasia or atypical hyperplasia of the uterine cervix. Cancer **6**: 224-235.

Reeves, W. C., Brinton, L. A., Garcia, M., Brenes, M. M., Herrero, R., Gaitan, E., Tenorio, F., de Britton, R. C. and Rawls, W.E. (1989). Human papillomavirus infection and cervical cancer in Latin America. N Engl J Med **320**: 1437-1441.

Reid, R., Stanhope, C. R., Herschman, B. R., Booth, E., Phibbs, D. G. and Smith, P. J. (1982). Genital warts and cervical cancer I: evidence of an association between subclinical papillomavirus infection and cervicalmalignancy. Cancer **50**: 377-387.

Reszka, A. A., Sundberg, J. P. and Reichmann, M. E. (1991). *In vitro* transformation and molecular characterization of Colobus monkey venereal papillomavirus DNA. Virology **181**: 787-92.

Richart, R. M., Lerch, V. and Barron, B. A. (1967). A time lapse cinematographic study *in vitro* of mitosis in normal human cervical epithelium, dysplasia and carcinoma *in situ*. J Natl Cancer Inst **39**: 571-577.

Richart, R. M. and Barron, B. A. (1969). A follow-up study of patients with cervical neoplasia. Am J Obstet Gynecol **105**: 386-393.

Riou, G., Favre, M., Jeannel, D., Bourhis, J., le Doussel, V. and Orth, G. (1990). Association between poor prognosis in early-stage invasive cervical carcinomas and non-detection of HPV DNA. Lancet **335**: 1171-1174.

Romanczuk, H., Villa, L. L., Schlegel, R. and Howley, P. M. (1991). The viral transcriptional regulatory region upsteam of the E6 and E7 genes is a major determinant of the differential immortalization activities of human papillomavirus types 16 and 18. J Virol **65**: 2739-44.

Roseto, A., Pothier, P., Guillemin, M. C., Peries, J., Breitburd, F., Bonneaud, N. and Orth, G. (1984). Monoclonal antibodies to the major capsid protein of human papillomavirus type 1. J Gen Virol **65**: 1319-1324.

Rotkin, I. D. (1973). A comparison review of key epidemiological studies in cervical cancer related to current searches for transmissible agents. Cancer Res **33**: 1353-1367.

Rous, P. and Beard, J. W. (1935). Carcinomatous changes in virus-induced papillomas of rabbits. Proc Soc Exp Biol Med **32**: 578-580.

Rous, P. and Kidd, J. G. (1936). The carcinogenic effect of a virus upon tarred skin. Science **83**: 468-469.

Rudlinger, R., Smith, I. W., Bunney, M. H. and Hunter, J. A. (1986). Human papillomavirus infections in a group of renal transplant recipients. Br J Dermatol **115**: 681-692.

Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, T., Erlich, H. and Arnheim, N. (1985). Enzymatic amplification of ß-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science **230**: 1350-1354.

Saiki. R. K., Bugawan, T. L., Horn, G. T., Mullis, K. B. and Erlich, H. A. (1986). Analysis of enzymatically amplified ß-globin and HLA-DQ α DNA with allele specific oligonucleotide probes. Nature **324**: 163-166.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. and Erlich, H.A. (1988). Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**: 487-491.

Sarkar, G. and Sommer, S.S. (1990). Shedding light on PCR contamination. Nature **343**: 27.

Sarver, N., Rabson, M. S., Yang, Y. C., Byrne, J. C. and Howley, P. M. (1984). Localization and analysis of bovine papillomavirus type 1 transforming functions. J Virol **52**: 377-388.

Scheffner, M. Werness, B. A., Holbregtse, J. M., Levine, A. J. and Howley, P. M. (1990). The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell **63**: 1129-1136.

Scheffner, M., Munger, K., Byrne, J. C. and Howley, P. M. (1991). The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. Proc Natl Acad Sci. USA **88**: 5523-5527.

Schiller, J. T., Vass, W. C., Vousden, K. H., Lowy, D. R. and Pfister, H. (1986). The E5 open reading frame of bovine papillomavirus type 1 encodes a transforming gene. J Virol **57**: 1-6.

Schiller, J. T., Kleiner, E., Androphy, E. J., Lowy, D. R. and Pfister, H. (1989). Identification of bovine papillomavirus E1 mutants with increased transforming and transcriptional activity. J Virol **63**: 1775-1782.

Schlegel, R., Wade-Glass, M., Rabson, M. and Yang, Y. C. (1986). The E5 transforming gene of bovine papillomavirus encodes a small, hydrophobic polypeptide. Science **233**: 464-467.

Schlegel, R., Phelps, W. C., Zhang, Y. L. and Barbosa, M. (1988). Quantitative keratinocyte assay detects two biological activities of human papillomavirus DNA and identifies viral types associated with cervical carcinoma. EMBO J **7**: 3181-3187.

Schneider, A., Kraus, H., Schumann, R. and Gissmann, L. (1985). Papillomavirus infection of the lower genital tract: detection of viral DNA in gynaecological swabs. Int J Cancer **35**: 443-448.

Schneider, A. and Schwarz, E. (1986). Different human cervical carcinoma cell lines show similar transcription patterns of human papillomavirus type 18 early genes. EMBO J **5**: 2285-2292.

Schneider, A. (1987). Methods of identification of human papillomavirus. In: Syrjanen, K. J., Gissmann, L., Koss L. G. eds. Papillomavirus and human disease. Berlin, Springer-Verlag **7**: 19-39.

Schneider, A., Sawada, E., Gissmann, L. and Shah, K. (1987). Human papillomaviruses in women with a history of abnormal Papanicolaou smears and in their male partners. Obstet Gynecol **69**: 554-562.

Schneider-Gadicke, A. and Schwarz, E. (1986). Different human cervical carcinoma cell lines show similar transcription patterns of human papillomavirus type 18 early genes. EMBO J **5**: 2285-2292.

Schneider-Maunoury, S., Croissant, O. and Orth, G. (1987). Integration of human papillomavirus type 16 DNA sequences: a possible early event in the progression of genital tumors. J Virol **61**: 3295-3298.

Schwarz, E., Durst, M., Demankowski, G., Latterman, O., Zech, D., Wolfsberger, E., Suhai, S. and zur Hausen, H. (1983). DNA sequence and genome organization of human genital papillomavirus type 6b. EMBO J **2**: 2341-2348.

Schwarz, E., Freese, U. K., Gissmann, L., Mayer, W., Roggenbuck, B., Stremlau, A. and zur Hausen, H. (1985). Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature **314**: 111-114.

Sedman, S. A., Barbosa, M. S., Vass, W. C., Hubbert, N. L. Haas, J., Lowy, D. R. and Schiller, J. T. (1991). The full length E6 protein of human papillomavirus type 16 has transforming and transactivating activities and co-operates with E7 to immortalize keratinocytes in culture. J Virol **65**: 4860-4866.

Seedorf, K., Krammer, G., Durst, M., Suhai, S. and Rowekamp, W. G. (1985). Human papillomavirus type 16 DNA sequence. Virology **145**: 181-185.

Seedorf, K., Oltersdorf, T., Krammer, G. and Rowekamp, G. (1987). Identification of early proteins of the human papillomavirus type 16 and type 18 in cervical carcinoma cells. EMBO J **6**: 139-144.

Serfling, E., Jasin, M. and Scheffner, W. (1985). Enhancers and eukaryotic gene transcription. Trends Genet **1**: 224-230.

Shirasawa, H., Tomita, Y., Kubota K., Kasail, T., Sekiyas, S., Takamizawa, H., and Simizu, B. (1986). Detection of human papillomavirus type 16 DNA and evidence for integration into the cell DNA in cervical dysplasia. J Gen Virol **67**: 2011-5.

Shope, R. E. and Hurst, E. W. (1933). Infectious papillomatosis of rabbits; with a note on the histopathology. J Exp Med **58**: 607-624.

Sibbet, G. J. and Campo, M. S. (1990). Multiple interactions between cellular factors and the non-coding region of human papillomavirus type 16. J Gen Virol **71**: 2699-2707.

Smith, K. T. and Campo, M. S. (1985). The biology of papillomaviruses and their role in oncogenesis. Anticancer Res **5**: 31-47.

Smotkin, D. and Wettstein, F. O. (1986). Transcription of human papillomavirus type 16 early genes in cervical cancer and a cervical cancer derived cell line and identification of the E7 protein. Proc Natl Acad Sci. USA **83**: 4680-4684.

Smotkin, D., Prokoph, H. and Wettstein, F. O. (1989). Oncogenic and non oncogenic human genital papillomaviruses generate the E7 mRNA by different mechanisms. J Virol **63**: 1441-1447.

Snijders, P. J. F., van den Brule, A. J. C., Schrijnemakers, H. F. J., Snow, G., Meijer, C. J. L. M. and Walboomers, J. M. M. (1990). The use of general primers in the polymerase chain reaction permits the detection of a broad spectrum of human papillomavirus genotypes. J Gen Virol **71**: 173-181.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol **98**: 503.

Spalholz, B. A., Lambert, P. F., Yee, C. L. and Howley, P. M. (1987). Bovine papillomavirus transcriptional regulation: localization of the E2-responsive elements of the long control region. J Virol **61**: 2128-2137.

Spalholz, B. A., Byrne, J. C. and Howley, P. M. (1988). Evidence for cooperativity between E2 binding sites in E2 trans-regulation of bovine papillomavirus type 1. J Virol **62**: 3143-3140.

Spalholz, B. A., Vande-Pol, S. B. and Howley, P. M. (1991). Characterization of the *cis* elements involved in basal and E2-transactivated expression of the bovine papillomavirus P2443 promoter. J Virol **65**: 743-53.

Spriggs, A. I. (1984). Natural history of cervical dysplasia. Clin Obstet Gynecol **8**: 65-69.

Stanbridge, C. M., Mather, J., Curry, A. and Butler, E. B. (1981). Condylomata of the uterine cervix and koilocytosis of cervical intraepithelial neoplasia. J Clin Pathol **34**: 524-531.

Stanley, M. A., Browne, H. M., Appleby, M. and Minson, A. C. (1989). Properties of a non-tumorigenic human cervical keratinocyte cell line. Int J Cancer **15**: 672-676.

Stenlund, A., Bream, G. L. and Botchan, M. R. (1987). A promoter with an internal regulatory domain is part of the origin of replication in BPV-1. Science **236**: 1666-1671.

Sterling, J., Stanley, M., Gatward, G. and Minson, T. (1990). Production of human papillomavirus type 16 virions in a keratinocyte cell line. J Virol **64**: 6305-6307.

Stoler, M. H., Rhodes, C. R., Whitbeck, A., Chow, L. T. and Broker, T. R. (1990). Gene expression of HPV types 16 and 18 in cervical neoplasia. In: Papillomaviruses 1-11. Edited by P. M. Howley. New York: Wiley-Liss.

Storey, A., Pim, D., Murray, A., Osborn, K., Banks, L. and Crawford, L. (1988). Comparison of the *in vitro* transforming activities of human papillomavirus types. EMBO J **7**: 1815-1820.

Storey, A., Osborn, K. and Crawford, L. (1990). Co-transformation by human papillomavirus types 6 and 11. J Gen Virol **71**: 165-171.

Suchankova, A., Ritterova, L., Kromar, V., Vagner, J., Jochmus, I., Gissmann, L., Kanka, J. and Vonka, V. (1991). Comparison of ELISA and western blotting for human papillomavirus type 16 E7 antibody determination. J Gen Virol **72**: 2577-2581.

Sussman, D. J. and Milman, G. (1984) Short-term, high efficiencyexpression of transfectedDNA. Mol Cell Biol **4**: 1641.

Syrjanen, S., Saastamoinen, J., Chang, F., Ji, H. and Syrjanen, K. (1990). Colposcopy, punch biopsy, in situ DNA hybridization and the polymerase chain reaction in searching for genital human papillomavirus (HPV) infections in women with normal Pap smears. J Med Virol **31**: 259-266.

Tham, K. M., Chow, V. T., Singh, P., Tock, E. P., Ching, K. C., Lim-Tan, S. K., Sng, I. T. and Bernard, H. U. (1991). Diagnostic sensitivityof polymerase chain reaction and Southern blot hybridization for the detection of human papillomavirus DNA in biopsy specimens from cervicallesions. Am J Clin Pathol **95**: 638-646.

Thierry, F., Garcia-Carranca, A. and Yaniv, M. (1987). Elements that control the transcription of genital papillomavirus type 18. Cancer Cells **5**: 23-32.

Thierry , F. and Howley, P., M. (1991). Functional analysis of E2-mediated repression of the HPV 18 P105 promoter. New Biol **3**: 90-100.

Thomas, M., Boiron, M., Tanzer, J., Levy, J. P. and Bernard, J. (1964). *In vitro* transformation of mouse cells by bovine papillomavirus. Nature **202**: 709-710.

Thorner, L., Bucay, N., Choe, J. and Botchan, M. (1988). The product of the bovine papillomavirus type 1 modulator gene (M) is a phosphoprotein. J Virol **62**: 2474-2482.

Tidy, J. A., Parry, G. C. N., Ward, P., Coleman, D. V., Peto, J., Malcolm, A. D. B. and Farrell, P.J. (1989). High rate of human papillomavirus type 16 infection in cytologicallynormal cervices. Lancet **i**, 434.

Tidy, J. A. and Farrell, P. J. (1989). Retraction: Human papillomavirus subtype 16b. Lancet **ii**, 1535.

Tindle, R. W., Smith, J. A., Geysen, H. M., Selvey, L. A. and Frazer, I. H. (1990). Identification of B epitopes in human papillomavirus type 16 E7 open reading frame protein. J Gen Virol **71**: 1347-1354.

Tomita, Y., Shirasawa, H., Sekine, H. and Simizu, B. (1987a). Expression of human papillomavirus type 6b L2 open reading frame in *Escherichia coli*: L2-ß-galactosidase fusion proteins and their antigenic properties. Virology **158**: 8-14.

Tomita, Y., Shirasawa, H. and Simizu, B. (1987b). Expression of huamn papillomavirus types 6b and 16 L1 open reading frames in *Escherichia coli*: detection of a 56,000 dalton polypetide containing genus-specific (common) antigens. J Virol **61**: 2389-2394.

Toon, P. G., Arrand, J. R., Wilson, L. P. and Sharp, D. S. (1986). Human papillomavirus infection of the uterine cervix of women without cytological signs of neoplasia. Br Med J **293**, 1261-4.

Trevathan, E., Layde, P., Webster, L. A., Adams, J. B., Benigno, M. D. and Ory, H. (1983). Cigarette smoking and dysplasia and carcinoma in situ of the uterine cervix. JAMA **250**: 499-502.

van den Brule, A. J. C., Claas, E. J. C., du Maine, M., Melchers, W. J. G., Helmerhorst, T., Quint, W. G. V., Lindeman, J., Meijer, C. J. L. M. and Walboomers, J. M. M. (1989). Use of anticontamination primers in the polymerase chain reaction for the detection of human papillomavirus genotypes in cervical scrapes and biopsies. J Med Virol **29**: 20-27.

van den Brule, A. J., Walboomers, J. M., du Maine, M., Kenemans, P. and Meijer, C. J. (1991). Difference in prevalence of human papillomavirus genotypes in cytomorphologically normal cervical smears is associated with a history of cervical intraepithelial neoplasia. Int J Cancer **30**: 404-408.

Vermund, S. H., Schiffman, M. H., Goldberg, G. L., Ritter, D. B., Weltman, A. and Burk, R. D. (1989). Molecular diagnosis of genital human papillomavirus infection: Comparison of two methods used to collect exfoliated cervical cells. Am J Obstet Gynecol **160**: 304-308.

Vessey, M. P., Lawless, M., McPherson, K. and Yates, D. (1983). Neoplasia of the cervix uteri and contraception: a possible adverse effect of the pill. Lancet **ii**: 930-934.

Vousden, K. H., Androphy, E. J., Schiller, J. T. and Lowy, D. R. (1989). Mutational analysis of bovine papillomavirus E6 gene. J Virol **63**: 2340-2342.

Wagatsuma, M., Hashimoto, K. and Matsukura, T. (1990) Analysis of integrated human papillomavirus type 16 DNA in cervical cancers: Amplification of viral sequences together with cellular flanking sequences. J Virol **64**: 813-821.

Wagner, D., Ikenberg, H., Boehm, N. and Gissmann, L. (1984). Identification of human papillomavirus in cervical swabs by deoxyribonucleic acid *in situ* hybridization. Obstet Gynecol **64**: 767-772.

Waldeck, W., Rosl, F. and Zentgraf, H. (1984). Origin of replication in episomal bovine papillomavirus type 1 DNA isolated from transformed cells. EMBO J **3**: 2173-2178.

Walker, J., Bloss, J. D., Liao, S., Berman, M., Bergen, S. and Wilczynski, S. P. (1989). Human papillomavirus genotype as a prognostic indicator in carcinoma of the uterine cervix. Obstet Gynecol **74**: 781-785.

Walker, P. G., Singer, A., Dyson, J. L., Shah, K. V., To, H. and Coleman, D. V. (1983a). The prevalence of human papillomavirus antigen in patients with cervical intraepithelial neoplasia. Br J Cancer **48**: 99-101.

Walker, P. G., Singer, A., Dyson, J. L., Shah, K. V., Wilters, J. and Coleman, D. V. (1983b). Colposcopy in the diagnosis of papillomavirus infection of the uterine cervix. Br J Obstet Gynaecol **90**: 1082-1086.

Wank, R. and Thomssen, C. (1991). High risk of squamous cell carcinoma of the cervix for women with HLA-DQw3. Nature **352**: 723-725.

Watanabe, S. and Yoshiike, K. (1988). Transformation of rat 3Y1 cells by human papillomavirus type 18 DNA. Int J Cancer **41**: 896-900.

Watanabe, S., Kanda, T. and Yoshiike, K. (1989). Human papillomavirus type 16 transformation of primary human embryonic fibroblasts requires expression of open reading frames E6 and E7. J Virol **63**: 965-969.

Weaver, M. G., Abdul-Karim, F. W., Dale, G., Sorensen, K. and Huang, Y. T. (1990). Outcome in mild and moderate cervical dysplasias related to the presence of specific human papillomavirus types. Mod Pathol **3**: 679-683.

Webb, D.H., Rogers, R.E. and Fife, K. H. (1987). A one-step method for detecting and typing human papillomavirus DNA in cervical scrape specimens from women with cervical dysplasia. J Infect Diseases **156**: 912-919.

Whyte, P., Buchkovich, K. J., Horowitz, J. M., Friend, S. H., Raybuck, M., Weinberg, R. A. and Harlow, E. (1988). Association between an oncogene and an anti-oncogene: the adenovirus E1a proteins bind to the retinoblastoma gene product. Nature **334**: 124-129.

Wickenden, C., Steele, A., Malcom, A. and Coleman, D. (1985). Screening for wart virus infection in normal and abnormal cervices by DNA hybridization of cervical scrapes. Lancet **i**, 65-7.

Williams, J. F. (1989). Optimization strategies for the polymerase chain reaction. Biotechniques **7**: 762-769.

Williamson, A., Dehaeck, C. M. C. and Soeters, R. (1989). Typing of human papillomaviruses in cervical intraepithelial neoplasia grade 3 biopsies from Cape Town. J Med Virol **28**: 146-149.

Winkelstein, W. (1977). Smoking and cancer of the uterine cervix: Hypothesis. Am J Epidemiol **106**: 257-259.

Woods, P. L., Hobson, D. and Rees, E. (1984). Genital infections with *Chlamydia trachomatis* in women attending an antenatal clinic. Br J Obstet Gynaecol **91**: 1171-1176.

Woodworth, C. D., Doniger, J. and di Paolo, J. A. (1989). Immortalization of human foreskin keratinocytes by various human papillomavirus DNAs corresponds to their association with cervical carcinoma. J Virol **63**, 159-164.

Woodworth, C. D., Waggoner, S., Barnes, W., Stoler, M. H. and DiPaolo, J. A. (1990a). Human cervical and foreskin epithelial cells immortalized by human papillomavirus DNAs exhibit dysplastic differentiation *in vivo*. Cancer Res **50**: 3709-3715.

Woodworth, C. D., Notario, V. and DiPaolo, J. A. (1990b). Transforming growth factors ß-1 and 2 transcriptionally regulate human papillomavirus type 16 early gene expression in HPV-immortalized human genital epithelial cells. J Virol **64**: 4767-4775.

Yasumoto, S., Burkhardt, A. L., Doniger, J. and di Paolo, J. A. (1986). Human papillomavirus type 16 DNA-induced malignant transformation of NIH 3T3 cells. J Virol **57**, 572-577.

Yasumoto, S., Taniguchi, A. and Sohma, K. (1991). Epidermal Growth Factor (EGF) elicits down-regulation of human papillomavirus type 16 (HPV 16) E6/E7 mRNA at the transcriptional level in an EGF-stimulated human keratinocyte cell line: functional role of EGF-responsive silencer in the HPV 16 long control region. J Virol **65**: 2000-9.

Yee, C., Krishnan-Hewlett, I., Baker, C. C., Schlegel, R. and Howley, P. M. (1985). Presence and expression of human papillomavirus sequences in human cervical carcinoma cell lines. Am J Pathol **119**: 361-366.

Young, L. S., Bevan, I. S., Johnson, M. A., Blomfield, P. I., Bromidge, T., Maitland, N. J. and Woodman, C. B. J. (1989). The polymerase chain reaction: a new epidemiological tool for investigating cervical human papillomavirus infection. Br Med J **298**, 14-18.

Zachow, K. R., Ostrow, R. S. and Faras, A. J. (1987). Nucleotide sequence and genome organization of human papillomavirus type 5. Virology **158**: 251-254.

zur Hausen, H. (1977). Human papillomaviruses: a possible role in squamous cell carcinoma. Current Top Microbiol Immunol **78**: 1-3.

zur Hausen, H. (1982). Human genital cancer: Synergism between two virus infections or synergism between a virus infection and initiating events. Lancet **ii**: 1370-1372.

zur Hausen, H., Gissmann, L. and Schlehofer, J. R. (1984). Viruses in the aetiology of human genital cancer. Prog Med Virol **30**: 170-186.

zur Hausen, H. (1991). Human papillomaviruses in the pathogenesis of anogenital cancer. Virology **184**: 9-13.