DEVELOPMENT OF CONSENSUS PRIMERS FOR AMPLIFICATION OF HUMAN PAPILLOMAVIRUS DNA

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by

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TABLE OF CONTENTS

ABSTRACT

The detection of human papillomavirus (HPV) sequences associated with anogenital tract infections is problematic due to the abundant diversity of HPV types found in the genital mucosa, the apparent geographic variation of HPV types, the prevalence of certain types in ethnic groups and the presence of yet unknown HPV types in anogenital samples.

The objective of this work was to develop a technique that would allow for the detection of all HPVs in a variety of clinical samples: diseased tissues, latent infections, and samples with limited amount of material. Because of its promising potential, the polymerase chain reaction (PCR) was selected as the method of choice. To fulfill the objective of amplifying all HPV sequences, primers were designed from regions of homology identified in the E1 ORF from a selected group of HPV types. The primers were assayed with a variety of HPV types and their utility evaluated in clinical samples. After amplification, Southern blot hybridization was performed for accuracy of typing as well as to increase the sensitivity of the assay.

PCR using E1 ORF consensus primers proved to be a successful tool in the detection of HPV DNA sequences in clinical samples. The primers are capable of amplifying known and unknown HPV types in diseased tissues and tissues latently infected with HPV. Their application allowed for the identification of potential new HPV sequences.

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Lastly, I would like to express my love to my daughter, Jessica. Her birth, just before writing began, was my inspiration to complete this work in a timely fashion. We were together for the writing of this dissertation. I will never forget those times.

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ATP	adenosine triphosphate
bp	base pair
BPV	bovine papillomavirus
BPV69T	bovine papillomavirus subgenomic fragment encompassing the noncoding and early region
BSA	bovine serum albumin
CIN	cervical intraepithelial neoplasia
CIP	calf intestinal phosphatase
CONDY	condyloma
CRV	cervicitis
Cys	cysteine
DB	dot blot hybridization
dNTP	deoxynucleotide triphosphate
E	early region
E2FL	full-length form of E2 protein
EGF	human epidermal growth factor
EGF-rec	human epidermal growth factor receptor
EM	electron microscopy
E2RE	E2 responsive element
E2sp	spliced form of E2 protein
Et-br	ethidium bromide
E2tr	truncated form of E2 protein
EV	epidermodysplasia verruciformis
fg	femtogram
GRE	glucocorticoid responsive element
DNA	deoxyribonucleic acid
H1	5′-ATTAGTGAGTATAGACATTA-3′
H2	5′-GGCTTTTGACAGTTAATACA-3′
H3	5′-GGTTTCTGGCACCGCAGGCA-3′
HG1	5′-GTGTTCTCAATATATTTTGGATG-3′
HG2	5′-AAAAACATCTGACTTGGTCTGGG-3′
HPV	human papillomavirus
IU	5′-TII(A/G)I(A/G)II(C/T)TAAAACGAAAGT-3′
IWDO	5′-(A/G)TC(A/G)(A/T)AIGCCCA(C/T)TGIACCAT-3′
Kd	kilodalton
L	late region
LCR	long control region
LG2	5′-AAAGTGATAAA(A/T)C(A/C)A(C/G)ITGT-3′
LG5	5′-(T/G)AAATGGTCT(A/T)ACTAA(A/T)TCI(A/G)IAAA-3′
LMA	low melting point agarose
LTR	long terminal repeat
M	modulation
mM	millimole
mRNA	messenger RNA
n	number
N	normal
NCR	noncoding region

nd	not done
neg	negative
NF1	nuclear factor 1
ng	nanogram
nt	nucleotide
ORF	open reading frame
P	promoter
Pap smear	Papanicolaou smear
PCR	polymerase chain reaction
PCR/SB	polymerase chain reaction followed by Southern blot hybridization
PDGF	platelet derived growth factor
PDGF-rec	platelet derived growth factor receptor
pg	picogram
P_L	late promoter
PMS	plasmid maintenance sequence
PV	papillomavirus
R	positive regulation
Rb	retinoblastoma gene product
RNA	ribonucleic acid
S	suppressor
SB	Southern blot hybridization
SIL	squamous intraepithelial neoplasia
SV40	simian virus 40
T AG	large T antigen
Taq	thermostable DNA polymerase from Thermus aquaticus
Td	temperature of dissociation
Tm	melting temperature
U	unit
µCi	microcurie
µg	microgram
µl	microliter
µM	micromole
UNI	unique sequence of dodecanucleotide string TAAAACGAAAGT
URR	upstream regulatory region
UV	ultra-violet

I. INTRODUCTION AND BACKGROUND

A. Physical Characteristics

Papillomaviruses (PV) represent one genus in the family of Papovaviridae, the other genus being the polyomaviruses (Melnick, 1962). The papillomavirus virion is a 55 nm diameter icosahedron, as first described for rabbit PV by electron microscopy (EM) (Finch and Klug, 1965). Empty virions band at a density of 1.29 g/ml in isopycnic cesium chloride gradients whereas DNA-containing particles migrate to a density of 1.34 g/ml (Breedis et al., 1962). No outer membrane or envelope has been identified in PV virions.

PVs infect a variety of species and are named according to the host they infect, eg: human PV (HPV), bovine PV (BPV), etc. PVs have a restricted natural host range; so far there is no report of naturally occurring cross-infection from species to species with the exception of BPV which is known to infect horses (Lancaster and Olson, 1982). In general, PVs have a tropism for cutaneous or mucosal epithelial tissue inducing papillomas; however, viruses from some species can infect both epithelial cells and fibroblasts to give rise to fibropapillomas.

B. Classification

Until 1979, it was believed that only a single PV type existed for each species. However, different restriction enzyme patterns for virus DNA isolated from individual lesions and lack of cross-hybridization revealed that for human and bovine PVs

multiple virus types existed (Coggin and zur Hausen, 1979).

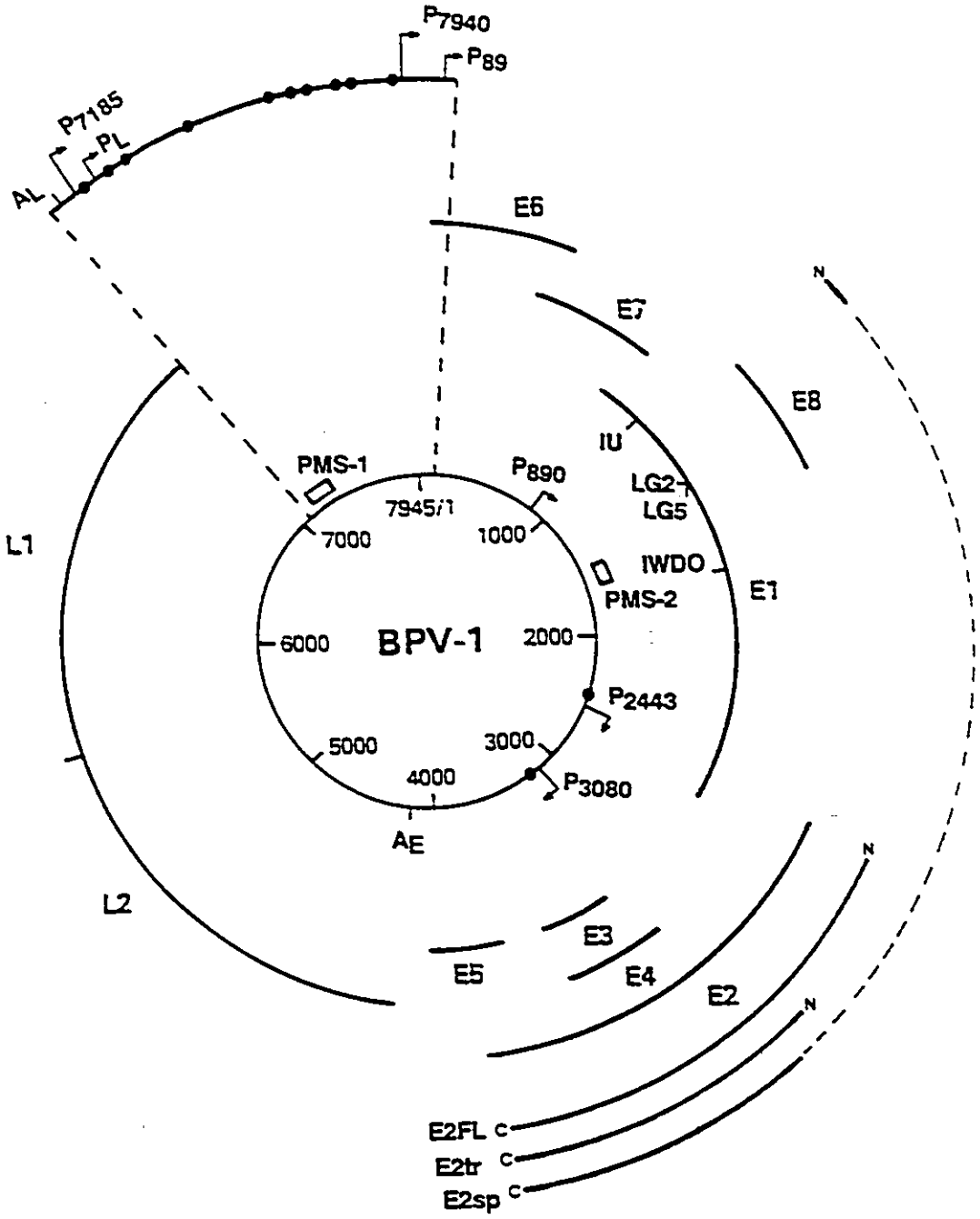
Since there is no in vitro system to propagate PVs for serologic identification, molecular hybridization has been used to differentiate PV isolates. Liquid phase hybridization under standard conditions of stringency (Tm-25^{o}C) has been used to determine the degree of sequence homology between genomes of various isolates. Viral genomes from the same species that share less than 50% homology, shown by less than 50% cross-hybridization, are considered different types. When greater than 50% but less than 100% of the sequences cross-hybridize, they are defined as subtypes. If two sequences show 100% homology by hybridization but vary in restriction endonuclease patterns, they are defined as variants of the same type (Coggin and zur Hausen, 1979). To date, there are more than 60 different types of HPV and at least 7 different types of BPV recognized based on the above criteria (de Villiers, 1989). The HPVs identified thus far can be divided into three groups based on their preferred site of infection. One group is associated with cutaneous infections of immunocompetent individuals, a second group appears to be associated with cutaneous infections of immunocompromised patients, while the third group preferentially infects mucosal or mucocutaneous surfaces. Furthermore, within a group there generally is a preferential but not exclusive site of infection. For example HPV 1 is preferentially found in deep plantar warts but only rarely in common digital warts; conversely, HPV 2 is more commonly found in digital warts than in plantar warts (Jenson et al., 1982).

C. Genomic Organization

The PV genome is approximately 8,000 basepairs (bp) arranged in a circular, double-stranded DNA structure. Within the virion, the genome is complexed with cell-derived histones (Favre et al., 1977). All papillomaviruses whose DNA sequences are known share a common genomic organization. All potential open reading frames (ORF), with a coding capacity of around 100 amino acids, are located on one strand. The genome is divided into the noncoding region (NCR), early region (E) and late region (L). The E and L regions were originally designated based on the biological activity in tissue culture of subgenomic fragments of the regions. This designation still holds from studies of the temporal expression during the infectious cycle. The E4 ORF is the only exception and is expressed late in infection. The genomic organization of BPV 1 is shown in Figure 1A.

In general, genes expressed before the initiation of replication are identified as early genes but since there is no in vitro system to study virus production, little is known about the replication of the virus. Therefore, the early region has been defined by exclusion of the region of the genome that is not needed for in vitro transformation which will be discussed in later sections. In BPV 1 the E region contains eight overlapping ORFs approximately 4,500 bp in length and is located downstream of the NCR. Although the BPV 1 E region contains eight ORFs (E1 to E8), the E1, E2, E4, E5, E6, E7 ORFs are present in all PVs and their position in relation to each other is constant.

FIGURE 1A. Circular representation of bovine papillomavirus type 1 (BPV-1) genomic organization. Numbers within the circle refer to nucleotide positions. Open reading frames for the early region (E1 through E8) and the late region (L1 and L2) are shown as arcs outside of the genomic circle. Promoters are indicaed as P_{89}, P_{890}, P_{2443}, P_{3080}, P_{7185}, P_L and P_{7940}. The early and late polyadenylation signals are indicated as A_E and A_L, respectively. The two boxed areas represent the plasmid maintenance sequences PMS-1 and PMS-2. The 11 E2 binding motifs are indicated as O on the genomic circle. The three E2 open reading frame protein products (E2FL, E2tr and E2sp) are indicated from the N to C terminus. The relative locations of the IU, LG2, LG5 and IWDO in the E1 open reading frame are indicated.


P7940
P89
P7185
PL
AL
E6
E7
E8
IU
LG2
LG5
IWDO
E1
PMS-1
P890
7945/1
7000
1000
PMS-2
L1
6000
BPV-1
2000
P2443
5000
3000
4000
P3080
AE
L2
E3
E5
E4
E2
E2FL
E2tr
E2sp
N

Putative ORFs E3 and E8 have been identified only in BPV.

The late region encompasses about 3,000 nucleotides (nt) and is located immediately downstream of the E region. This region encodes the structural proteins L1 and L2 which appear to be transcribed as functional mRNAs only in productive infection.

The noncoding region (NCR), also called the long control region (LCR) or upstream regulatory region (URR), is located upstream of the first initiation codon of the E6 early gene and downstream of the last stop codon of L1. In most PVs, the NCR is approximately 1,000 bp in length. It does not harbor any ORF but carries a significant number of regulatory elements such as transcriptional promoters (P), enhancers (E) and suppressor elements (S) of transcription, as well as an origin of replication. In addition, other elements, such as the plasmid maintenance sequences (PMS-1) involved in maintenance of the genome as a plasmid in infected cells, are also present.

Most of the work done to define and understand the functions of the three regions and of the different ORFs has used wild-type BPV 1 as well as a variety of genetically engineered BPV 1 mutants.

1. Early region

a. E1 ORF. The E1 ORF, from nt 850 to nt 2800 in BPV 1, is the largest (Figure 1A) and the most conserved ORF among the PVs (Sousa et al., 1990). This ORF codes for proteins that are expressed at very low concentrations and have short half-lives (Santucci et al., 1990), making the peptides difficult to

characterize. By immunochemical studies, two polypeptides originating from the E1 ORF have been identified. One appears to be coded for by the entire reading frame with a molecular weight of 68 Kd (E1 68 Kd) (Santucci et al., 1990; Sun et al.; 1990) and the other, a 23 Kd protein, corresponds to the 5' end of the ORF (E1 23 Kd) (Thorner et al., 1988; Sun et al., 1990). Both proteins are phosphorylated and have been shown to be located in nuclei of BPV1 transformed cells. This location is in agreement with the function of E1 proteins in replication.

The truncated E1 product (E1 23 Kd), corresponding to the amino terminus of the E1 68 Kd polypeptide, is speculated to play a role in viral DNA replication. However, mutations within this coding region have failed to demonstrate changes in DNA replication (Lambert et al., 1990; Ustav and Stenlund, 1991).

The E1 68 Kd protein shares some homology at the amino acid level with the simian virus 40 (SV40) large T antigen (T Ag) (Clertant and Seif, 1984). Because of this similarity, it has been speculated that the E1 68 Kd protein could be functionally related to T Ag. It is known that both proteins are located in the nucleus (Santucci et al., 1990; Sun et al., 1990; Blitz and Laimins, 1991), phosphorylated (Thorner et al., 1988; Santucci et al., 1990), bind and hydrolyze adenosine triphosphate (ATP) (Thorner et al., 1988; Santucci et al., 1990; Sun et al., 1990) and finally, play a role in viral DNA replication (Ustav and Stenlund, 1991; Lusky and Botcham, 1986a).

In previous genetic studies (Lusky et al., 1985;

Berg et al., 1986a; Lusky and Botchan, 1986b) the E1 ORF was described as carrying two functions. The 3' end of the ORF, referred to as R, was involved in positive regulation of replication and the 5' end of the ORF, referred to as the M or modulation function, was involved in negative regulation of replication (Lusky and Botchan, 1986). No R protein or mRNA has been identified as yet. Also in these studies, the E1 ORF was reported to be sufficient in itself to carry out the replication functions. This was in agreement with the findings of other investigators (Sarver et al., 1984; Groff and Lancaster, 1986; Rabson et al., 1986b; DiMaio and Settleman, 1988) who demonstrated that mutations in the E1 ORF R region resulted in virus DNA integration.

Recent studies (Mohr et al., 1990; Ustav and Stenlund, 1991) disagree with previous reports that suggest E1 encodes two functions. Complementation studies have failed to distinguish two functions coded by the E1 ORF (Ustav and Stenlund 1991). In addition there is evidence for the requirement for full-length E2 protein interaction with products from an intact E1 ORF for viral DNA replication (Mohr et al., 1990; Ustav and Stenlund, 1991).

The mechanism of action of the E1 products in replication is unknown. Recent studies have shown that the E1 68 Kd protein, expressed in a baculovirus vector, does not bind specifically to virus DNA (Santucci et al., 1990; Blitz and Laimins, 1991). However, when expressed in bacterial vectors it binds specifically to the BPV 1 origin of replication (Lambert,

1991). Recently, it has been shown by immunoprecipitation that a complex can form between E1 and E2 proteins that increases specific DNA binding to BPV 1 DNA (Mohr et al., 1990). The protein interaction occurs through the amino terminus of the E2 full-length product (48 Kd) with the carboxyl terminus of the E1 68 Kd protein. DNA binding appears to be in the vicinity of the origin of replication located around nt 1 (Yang and Botchan, 1990) downstream of PMS-1 at nt 6945 to nt 7476 (Figure 1A). This binding probably occurs at an E2 binding site which is also located in the vicinity of the origin of replication (Yang and Botchan, 1990). The initiation of replication is not understood as yet but it is speculated that the E1-E2 complex binds specifically to DNA through the carboxyl terminus of E2 at the E2 binding site and that the complex acts as a helicase driven by the E1 ATPase activity (Mohr et al., 1990).

The E1 ORF also carries a plasmid maintenance sequence (PMS-2). PMS-2 with PMS-1 can support the replication of extrachromosomal BPV DNA (Lusky and Botchan, 1984).

b. E2 ORF. Located from nt 2700 to nt 3800 on the BPV 1 genome, the E2 ORF is translated into three polypeptides: a 48 Kd full length E2 protein (E2FL) transcribed from promoter P_{2443} (Spalholz et al., 1985; Stenlund et al., 1985; Yang et al., 1985b), a 31 Kd truncated form (E2tr) transcribed from the internal promoter P_{3080} (Stenlund et al., 1985; Ahola et al., 1987; Baker and Howley, 1987; Cripe et al., 1987; Lambert et al., 1987; Lambert et al., 1989) and a 28 Kd spliced form (E2sp) which

appears to be transcribed from promoter P_{89} (Choe et al., 1989). E2sp has the amino terminus of the E8 ORF spliced to the 3' end of the E2 ORF (Stenlund et al., 1985; Yang et al., 1985b; Ahola et al., 1987; Lambert et al., 1987; Choe et al., 1989; Lambert et al., 1989). These three forms have been identified in BPV 1 transformed cells (Hubbert et al., 1988).

The E2 full length polypeptide is divided into three domains to which functions have been assigned (Figure 1A). The amino terminus transactivates transcription from promoters P_{89} and P_{7940} as well as P_{2443} and probably P_{3080}. The carboxyl terminus allows for dimerization (Landschultz et al., 1988; O'Shea et al., 1989) of the protein and for its binding (Giri and Yaniv, 1988; McBride et al., 1988; Moskaluk and Bastia, 1988) to the consensus E2 binding site ACCNNNNNNGGT (Androphy et al., 1987b; Haugen et al., 1987; Moskaluk and Bastia, 1987; Spalholz et al., 1987; McBride et al., 1988). The hinge, the most divergent domain based on amino acid sequences, separates the amino terminus from the carboxyl terminus.

E2tr as well as E2sp contain only the carboxyl terminus (McBride et al., 1988). These two truncated forms, lacking the transactivating domain, can dimerize through the carboxyl terminus and bind to the same consensus sequence as full length E2 thus competing with E2FL for the same binding sites. The high concentration of truncated and spliced forms over the full length form favors the binding of repressor thus down regulating transcription. Repression of transcription can also occur due to the formation of heterodimers creating complexes

inactive for transactivation (Lambert et al., 1987; Haugen et al., 1988; Lambert et al., 1988; McBride et al, 1989).

The level of E2 polypeptides in BPV infected cells have a ratio of E2FL:E2tr:E2sp of 1:10:3 (McBride et al., 1988). This would suggest a constitutive down regulation of the expression of early transcripts originating from promoters P_{89} and P_{7940}, especially for ORFs E6 and E7, as well as autoregulation of E2FL at P_{2443}.

Transactivation is significantly increased when two homodimers of E2FL are bound to two adjacent E2 binding sites which represents the E2 responsive element (E2RE). Two close binding sites are the minimum requirement for expression of the transactivation activity (Harrison et al., 1987; Gius et al., 1988; Hawley-Nelson et al., 1988; Spalholz et al., 1988; Sowden et al., 1989). Two adjacent E2 binding sites have been shown to work equally well independent of orientation or position (Hawley-Nelson et al., 1988; Sowden et al., 1989) making the E2RE an E2FL-dependent enhancer. The mechanism by which transcriptional transactivation occurs from interaction of E2FL with E2RE is not understood.

c. E3 ORF. The E3 ORF, which is present only in the BPV genome and overlaps the E2 ORF but in a different reading frame, is located from nt 3260 to nt 3550. This ORF has a potential coding capacity of about 100 amino acids but it is not believed to be transcribed. No E3 ORF product has been identified and no function has been assigned based on mutational

studies of the ORF (Hermonat and Howley, 1987).

d. E4 ORF. The E4 ORF, located at position nt 3300 to nt 3600, overlaps with the hinge domain of E2 ORF and is the most divergent region among all PVs. E4 mRNA transcripts have been found in naturally occurring fibropapilloma but absent in BPV 1 transformed cells. By primer extension analysis, E4 transcripts appear to originate at nt 7246 indicating the use of the late promoter (P_L). P_L, which is active in terminally differentiated epithelial cells, is also used to transcribe the structural proteins encoded by the late region. This indicates that the E4 ORF could be a late gene although its location is within the early region (Baker and Howley, 1987).

HPV 1-induced warts have been found to harbor a 16-17 Kd protein which represents as high as 30% of the total protein of the lesion (Doorbar et al., 1986). By extensive biochemical analysis and peptide sequencing, the protein has been identified as the E4 gene product. By immunological assay, the product is not a structural protein of the intact virus. Also because of its high concentration, the E4 polypeptide is not believed to be a classical tumor viral early gene product. Due to its presence in productive infection, it could possibly have a role in virus maturation.

e. E5 ORF. The E5 ORF, present in all PVs (Bubb et al., 1988; DiMaio and Neary, 1990), is located at the 3′ end of the early region either overlapping the 3′ end of the E2 ORF in a different reading frame or downstream of the former. In BPV 1

transformed cells, the E5 ORF has been shown to be present in several RNA transcripts. It is not clear, as of today, which one or if all mRNAs translate the E5 product. It is tempting to speculate that P_{2443} transcribes the E5 mRNA since deletion of P_{2443} inhibits expression of E5 (Yang et al., 1985a; Baker and Howley, 1987; Prakash et al., 1988).

Studies to elucidate the function of the E5 protein have been done using BPV 1 wild type and mutants with mouse cell lines C127 and NIH 3T3. E5 synthesis directed from heterologous promoters can induce focus formation in these cells (Yang et al., 1985; Schiller et al., 1986; Bergman et al., 1988). When E5 is expressed from a recombinant retrovirus under the control of a strong promoter, the cells become fully transformed based on morphology, colony formation in soft agar and tumorigenicity in nude mice (Bergman et al., 1988). When expressed from its own promoter in the absence of other BPV ORFs, E5-induced foci take longer to develop and the cells are less tumorigenic than cells transformed with the entire BPV genome (Howley and Schlegel, 1987). The BPV 1 E5 ORF, approximately 200 nucleotides in length, codes for a 44 amino acid product and is the smallest oncoprotein identified to date. The amino terminus two thirds of the protein contains hydrophobic amino acids which are responsible for its location at the Golgi apparatus as well as at the plasma transmembrane (Burkhardt et al., 1989). The extracellular carboxyl terminus contains a cysteine-X-cysteine motif which allows for dimerization (Schlegel et al., 1986; Burkhardt et al., 1987; Green and Loewenstein, 1987). The

transforming activity has been shown to be carried by the carboxyl terminus as determined by mutation of specific amino acids (Horwitz et al. 1988). Amino acid substitutions result in a decrease in the efficiency of focus formation (Yang et al., 1985; DiMaio et al., 1986; Schiller et al., 1986, Burkhardt et al., 1987; Horwitz et al., 1988).

BPV 1 infection as well as microinjection of the BPV 1 genome into the nucleus induces cellular DNA synthesis in quiescent cells (DiMaio et al., 1987; Jaskulski et al., 1987). Microinjection of the E5 product into nuclei leads to the same observation (Green and Lowenstein, 1987). Although the E5 product is associated with the plasma membrane, its action appears to be at the level of the nucleus (Schlegel et al., 1986; Burkhardt et al., 1989). One function of E5 may be associated with viral DNA replication since mutational inactivation of the gene interferes with replication and maintenance of the BPV genome as a plasmid in cell lines derived from these foci (Groff and Lancaster, 1986; Rabson et al., 1986b).

The mechanism of transformation by E5 oncoprotein can be explained in two ways (Martin et al., 1989; Petti et al., 1991). Experiments have shown that cotransfection of human epidermal growth factor receptor (EGF-rec) and the cloned BPV 1 E5 ORF expressed under the control of a retroviral long terminal repeat (LTR) into C127 mouse cells results in an increase in the half-life of human EGF-rec. Once activated by EGF, receptors remain on the surface for an extended period of time (Martin et al., 1989). The second relevant experiment proposes that

expression of the BPV 1 E5 ORF in C127 cells increases the phosphorylation pattern of the platelet dependent growth factor receptor (PDGF-rec) which leads to its activation. However, there is no increase in the PDGF-rec half-life (Petti et al., 1991). In normal cells, when activated by their respective ligands, both the EGF and PDGF receptors become phosphorylated followed by internalization resulting in fusion with lysozymes and subsequent degradation. Once phosphorylated, these receptors stimulate DNA synthesis and cell proliferation, both characteristics of transformed cells. The experiments of Martin et al. (1989) suggest that in the presence of E5, activated EGF-rec remains at the cell surface longer resulting in an increase in cellular activity leading to cell transformation. PDGF-rec, on the other hand, is phosphorylated in the absence of PDGF in E5 transformed cells (Petti et al., 1991). In both studies the involvement of the E5 product and growth factor receptors are necessary for continued cell proliferation but the exact mechanism how the E5 protein acts upon the receptors is not understood. The recent demonstration that the E5 protein interacts with a 16 Kd cellular polypeptide suggests another level of complexity in cell transformation by E5 (Goldstein and Schlegel, 1990). The cellular protein 16Kd was later identified as the pore-forming constituent of vacuolar H+-ATPases. The interaction of E5 oncoprotein to 16 Kd could indirectly activate PDGF-rec as well as EGF-rec (Golstein et al. 1992).

Until recently, the BPV 1 E5 product was thought

to be specific for fibroblast proliferation. However, the BPV 1 E5 ORF under the control of an LTR can induce morphological transformation in a line of murine epidermal keratinocytes. These transformed cells induce in nude mice what appear to be squamous cell carcinomas (Leptak et al., 1991). More investigations are needed to understand the role of the BPV 1 E5 polypeptide in keratinocytes.

The E5 ORF is present in all PVs but its expression has only been detected in BPV 1 transformed cells (Halbert and Galloway, 1988). Although HPV 16 E5 mRNA has been found in HPV 16 immortalized murine epidermal keratinocytes, the protein has not been detected (Leptak et al., 1991). These HPV 16 infected cells expressing E5 transcripts failed to exhibit morphological changes but were tumorigenic (Leptak et al., 1991). These results suggest that the HPV 16 E5 product has transforming potential in keratinocytes.

f. E6 and E7 ORFs. The E6 and E7 ORFs are located at the 5′ end of the early region immediately downstream of promoter P_{89} in BPV 1. The E6 reading frame overlaps the E7 ORF and both total approximately 800 bp. Several proteins could be obtained from these two ORFs based on mRNAs isolated from BPV infected cells (E6, E6 short, E6 spliced to E7, E6 spliced to E4, E7) (DiMaio and Neary, 1990). By primer extension analysis, P_{89} of BPV 1 seems to be the preferred promoter to transcribe the E6 and E7 ORFs (Ahola et al., 1983; Stenlund et al., 1985; Yang et al., 1985a); however, mRNAs originating from promoters P_{7940} and P_{7185}

have also been found.

BPV 1 E6 mRNA carries two initiation codons to give a 136 amino acid or 104 amino acid product depending on which AUG is used (DiMaio and Neary, 1990). Antisera raised against E6 expressed in bacteria precipitate a 15.5 Kd E6 protein from BPV 1 transformed C127 cells (Androphy et al., 1985). Also, cell fractionation has localized low concentrations of E6 protein within the nucleus and non-nuclear membrane fraction of BPV 1 transformed cells (Androphy et al., 1985). All PVs sequenced thus far, predict the E6 product to contain four CYS-X-X-CYS motifs (Cole and Danos, 1987) suggesting a zinc finger type of DNA binding protein. The CYS-X-X-CYS motif has been identified in DNA binding proteins such as transcription factors (TFIII) in Xenopus laevis (Giri et al., 1985). This motif could also be involved in other interactions such as protein-protein complexes (Arthur et al., 1988). The nuclear localization of E6 is in agreement with its potential role as a DNA binding protein.

The E7 ORF is located immediately 3' and overlaps the E6 ORF. BPV 1 E7 transcripts carry a single initiation codon and would encode a protein of 127 amino acids based on the coding capacity of the gene. Cell fractionation analysis has shown that E7 is a phosphoprotein located in the cytoplasm (Smotkin and Wettstein, 1987). Later work using a gentle sequential fractionation procedure in addition to biochemical and immunocytochemical methods associated the HPV 16 E7 protein with the nuclear matrix (Greenfield et al., 1991). The E7 ORF of all PVs sequenced so far encode similar E7 products harboring two

CYS-X-X-CYS motifs. This suggests E7 may also interact with other proteins (Arthur et al., 1988) or is a DNA binding protein (Barbosa et al., 1989; Barbosa et al., 1990). BPV 1 E7 protein plays a role in replication to control the copy number of episomal BPV genomes (Lusky and Botchan, 1985; Berg et al., 1986b).

At the amino acid level, the HPV E7 products, but not the BPV 1 E7 product share some homology with the adenovirus E1A protein (Walker et al., 1983; Figge et al., 1988). This suggests that E7 may be similar in oncogenic function and location to E1A (Phelps et al., 1988). E1A acts as a transcriptional transactivator in the absence of DNA binding through interactions with cellular proteins (Ferguson et al., 1985). E1A, in cooperation with an activated ras oncogene, can transform primary rat cells (Graham, 1984). The same functions of transcriptional transactivation as well as transformation of primary rat cells in cooperation with ras have been attributed to HPV E7 oncoprotein (Phelps et al., 1988; Phelps et al., 1991).

Biological activity of the E6 and E7 products has been studied using wild type and mutants genomic BPV 1 as well as subgenomic constructs expressed in C127 and NIH 3T3 cells. The full length BPV 1 genome induces foci in both C127 and NIH 3T3. However, when subgenomic E6 and E7 ORFs are expressed under the control of the murine sarcoma virus LTR, foci develop only on C127 cells (Schiller et al., 1984). This difference is due to the E5 ORF which carries transforming activity for both cell lines. Cell lines established from E6/E7-induced foci can form

colonies in soft agar and induce tumors in nude mice. Mutations in subgenomic constructs, such as frame shift or premature termination codons within E6 gene, abolish transformation (Schiller et al., 1984; Neary and DiMaio, 1989).

Frame shift mutations or premature termination codons within the E7 gene do not affect focus formation in the presence of an intact E6 but seem to show a decrease in their ability to grow in soft agar (Rabson et al., 1986a; Neary and DiMaio, 1989). This implies that E6 is sufficient to induce foci but both E6 and E7 are needed for complete cell transformation. Furthermore, in the context of the entire BPV genome, mutations within the E7 ORF have been linked to a decrease in copy number of the viral genome from 100/cell in wild type transformed C127 cells to 1-5 copies/cell in cells transformed by E7 mutants (Berg et al., 1986b; Lusky and Botchan, 1986b).

Extrapolation of the E6 and E7 products as participants in oncogenesis from BPV to HPVs has been based on homology of the predicted amino acid sequence of the E6 and E7 products as well as on the presence of specific mRNAs in cervical cancer derived cell lines. This has led to the belief that some HPVs may play a role in human cervical cancer. Cell lines established from cervical carcinomas that harbor HPV DNA invariably show integration of the viral genome with a break point downstream of the E6 and E7 ORFs (Schwartz et al., 1985; Yee et al., 1985; Schneider-Gadicke and Schwartz, 1986; von Knebel Doeberitz et al., 1988). mRNAs containing the E6 and E7 coding regions are found at high levels in these cell lines

(Smotkin and Wettstein, 1986). By immunoprecipitation, the E7 protein has been detected in Caski and Siha, cell lines which harbor HPV 16 sequences (Smotkin and Wettstein, 1986; Seedorf et al., 1987) as well as HeLa, C4-1 and SW756, cell lines that contain HPV 18 sequences (Seedorf et al., 1987). Cervical dysplasias and squamous cell carcinomas also contain the protein (Walker et al., 1983; Smotkin and Wettstein, 1986).

The E6 and E7 products are believed to play a role in the maintenance of the transformed phenotype in certain human cancers. In vitro, the E7 products of all anogenital HPVs have been shown to bind, albeit with different affinities, to the retinoblastoma gene product (Rb) (Munger et al., 1989b). Rb is known to play a role in cell growth regulation as a tumor suppressor gene product which normally complexes with E2F cellular transcription factor. This interaction of E7-pRB proteins eliminates E2F-pRB complexes, changing the tight regulation control of the cell, leading to an additional step towards carcinogenesis (Pagano et al. 1992).

E6 protein of all HPVs binds p53 cellular protein, in vitro (Werness et al., 1990; Crook et al., 1991). p53 is believed to play an important role in regulation of cell growth (Finlay et al., 1989). In addition, the interaction of E6 from oncogenic HPVs (types 16 and 18) with p53 promotes degradation of p53 while E6 from benign HPVs does not (Scheffner et al., 1990). Thus, the reduction of p53 by the viral E6 gene product may play a role in carcinogenesis.

g. E8 ORF. So far, the E8 reading frame has been shown only to be present in the BPV 1 genome. This potential ORF is about 400 nucleotides long and is enccmpassed within E1 ORF in a different reading frame. No function has been assigned to this ORF.

2. Late region

a. L1 ORF. The L1 ORF is located downstream of the early region from nt 5597 to 7093 in BPV 1. This structural protein is transcribed from the late promoter P_L which is active only when the infected cells are committed to terminal differentiation. Analysis of the L1 ORF of sequenced PVs shows numerous regions of amino acid homology (Baker, 1987). However, Ab raised against intact PV do not cross-react. Monoclonal Ab raised against disrupted BPV virions, on the other hand, show either a type specific reactivity or reactivity to a wide range of PV (Gorra et al., 1985). These results indicate that the L1 55 Kd protein (Engel et al., 1983) carries internalized viral group specific epitopes.

b. L2 ORF. The L2 ORF is located downstream of the early region and upstream of the L1 ORF at nt 4172 to 5593 in BPV 1. This structural protein is also translated from the late promoter and encodes the minor capsid polypeptide. This protein shows little amino acid homology among the PVs and may represent a type-specific protein (Konly et al., 1986; Doorbar and Gallimore, 1987). However, cells expressing the 3′ end of the HPV

1a L2 ORF reacted with antisera directed against disrupted BPV virions (Campione-Piccardo et al., 1985). This suggests that some PVs may have conserved and internalized epitopes in the L2 protein.

D. Transcription and Translation

1. BPV

In BPV 1, the NCR contains three promoters that are used for transcription of the early region (P_{89}, P_{7940}, P_{7185}) and one (P_{7250} or P_L) that is preferentially used for transcription of the late region. P_{89} has the features of a conventional promoter containing a TATA box and a CAAT box and is active in in vitro transcription assays. However, in vivo this promoter is dependent on the presence of a transactivator derived from the early region E2 ORF (Stenlund et al., 1987; Linz and Baker, 1988). By primer extension analysis, P_{89} is thought to transcribe the E6 and E7 ORFs as well as the spliced E8-E2 product of the early region (Stenlund et al., 1985; Yang et al., 1985; Ahola et al., 1987). P_{7940}, a promoter without a consensus TATA box (Baker and Howley, 1987), is used efficiently in vivo and appears to transcribe the E6 and E7 ORFs during infection (Stenlund et al., 1985; Yang et al., 1985b; Ahola et al., 1987) but has no transcriptional activity in vitro (Stenlund et al., 1987; Linz and Baker, 1988). P_{7185} has a TATA box-like element and has been shown to carry a very active transcriptional function in in vitro assays; however, in vivo it seems to be a

very weak promoter (Baker and Howley, 1987). P_{7185} is located within the plasmid maintenance sequence (PMS-1) involved in replication of the virus genome. The E1 ORF, which carries a regulatory function for replication, appears to be transcribed by this promoter (Stenlund et al., 1987).

Transcription of the late region originates from a promoter located within the NCR, at nt positions 7214-56. P_{7250}, also named the late promoter or P_L, becomes activated relative to the state of differentiation of infected cells. This promoter transcribes the late genes L1 and L2 as well as the E4 ORF (Stoler and Broker, 1986; Baker and Howley, 1987).

Additional promoters outside the NCR are used to transcribe ORFs from the early region. In BPV 1, P_{2443} is located near position nt 2443, upstream of the E2 ORF. This promoter, which contains a TATA box, is very active in vivo and generates two different mRNAs. The first one corresponds to the entire E2 ORF, the second and most abundant is spliced from the E2 ORF (nt 2505) to the E5 ORF (nt 3225) and could express the E5 protein (Ahola et al., 1986).

P_{3080} is located within the E2 ORF. Even though it does not contain any conventional promoter elements, it appears to generate in vivo an abundant level of mRNA (Baker and Howley, 1987) for translation of a truncated form of the E2 product (Lambert et al., 1987).

Very little is known about P_{890}; however, it appears to be used for transcription of an E8-E2 ORF spliced product (Choe et al., 1989). P_{2443}, P_{3080} and P_{890} have all been found

to be very active in in vitro transcription assays (Linz and Baker, 1988; Choe et al., 1989).

In BPV transformed cells, 0.01% of poly A^+ RNA represents BPV early region transcripts (Yang et al., 1985). The 5′ end of the mRNA is capped and the poly A tail is coded for by the unique early poly A signal located at nt 4180 at the 3′ end of the early region.

Two mRNAs isolated from productive BPV infections encompassing the L1 and L2 ORFs have been shown to originate from P_{7250}, or P_L. These mRNAs are capped and terminate at a unique late poly A signal located at 3′ end of the late region at nt 7156 (Pettersson et al., 1987).

Both in BPV transformed cells and BPV fibropapillomas, mRNA transcripts vary in size from 1 to 4 kb. They appear to be generated using splice donor sites at nt 304, 864, 1235, 2505, 3764, 7385 with acceptor sites at nt 528, 3225, 3605 and 5609 (Baker, 1987).

2. HPV

Since no tissue culture system is available for HPV replication and tumor-derived material is in limited amounts, viral transcripts have mainly been studied from HPV-containing cell lines derived from cervical carcinomas. In the HPV 16-containing cell line, Caski, the viral promoter P_{97} (homologous to P_{89} in BPV) transcribes the E6, E7, and E1 ORFs (Smotkin and Wettstein, 1986). In the HPV 18-containing cell lines HeLa, C4-1 and SW-56 the corresponding promoter is located at nt 105

(Schneider-Gadicke and Schwarz, 1986) and transcribes the same ORFs. In Caski, a transcript corresponding to the E2 ORF is also present and appears to originate from a promoter analogous to BPV 1 P_{2443} or P_{3080} (Baker et al., 1987).

RNA studies have demonstrated transcription pattern differences between cervical intraepithelial neoplasia (CIN) and invasive carcinoma. In the former, mRNAs hybridize to all parts of the early region indicating transcription of all early genes. In the latter, where HPV is present in an integrated form, only E6 and E7 transcripts are observed (Shirasawa et al., 1988). Even though no transcripts from the late region have been detected in CIN and cervical carcinoma, it is likely that a late promoter must also be present.

3. Regulation of transcription

Regulation of expression of the viral promoters, as currently understood, is under the control of several cellular factors as well as at least one viral protein (E2). By DNAse foot-printing analysis, cellular proteins have been identified that bind specifically to sequences within the NCR. It is important to mention that the binding of these ubiquitous cellular factors does not explain the tissue specificity of HPV. It has been speculated that a difference in concentration or quality of these cellular factors may be responsible for this specificity (Chong et al., 1991).

a. Cellular factors. The cellular factor AP1 binding

site, with its consensus sequence TGACT(A/C)A, is present in at least one copy in the NCR of all HPVs analyzed thus far but absent in all animal PV (Garcia-Carranca et al., 1988; Gloss et al., 1989a). Two AP1 binding sites have been shown by footprinting analysis to be at conserved locations within the NCR of the HPV 16, 18 and 33 genomes (Chan et al., 1988; Garcia-Carranca et al., 1988; Jones et al., 1988). It is believed that these two binding sites play an important role in the transcription of early genes, specifically E6 and E7 (Chan et al., 1988; Garcia-Carranca et al., 1988; Jones et al., 1988).

The cytokeratin octamer, AANCCAAA, is present in cytokeratin genes expressed in the epidermis. This consensus sequence has also been identified in all PVs in the NCR immediately upstream of the E6 ORF and residing within a constitutive enhancer (Blessing et al., 1987). Using the constitutive enhancer harboring the octamer in transient reporter gene assays, some experiments have observed a cell type specificity (Cripe et al., 1987; Garcia-Carranca et al., 1988; Chin et al., 1989). However, the constitutive enhancer of HPV 11 without the octamer has also shown tissue specificity (Chin et al., 1989). Although the octamer is present in all PVs, its function is not clear.

Nuclear factor 1 (NF1) stimulates transcription by RNA polymerase II from promoters containing a CAAT motif. Although the consensus sequence, TGGCTNNNAGCCA (Jones et al., 1988), does not appear to be present in all PVs, protein DNA binding assays have shown that half of the consensus motif,

PyGGCA, is sufficient to bind NF1. PyGGCA is found in the NCR of all PVs (Leegwater et al., 1986; Jones et al., 1988). At least for HPV 16 and 18 (Gloss et al., 1989a; Gloss et al., 1989b), TGGCA has been demonstrated by foot-printing analysis to be within the constitutive enhancer, and it is suggested to play an important role in the enhancer's activity (Gloss et al., 1989a; Gloss et al., 1989b).

A glucocorticoid responsive element (GRE), GGT(A/T)CA(A/C)NNTGT(C/T)CT, is present in the NCR of all PVs infecting the mucosa (Pater et al., 1988). Transcription can be induced by dexamethasone (Gloss et al., 1987) and the elements respond to glucocorticoids (Pater et al., 1988). The role of the GRE in the regulation of transcription of the viral genome is not understood.

b. Viral factor. The BPV 1 E2 protein, the only viral protein known, so far, to play a role in regulation of transcription, has been shown to bind to the sequence ACCNNNNNNGGT located in the NCR of BPV (Androphy et al., 1987b). Several copies of the same motif have since been demonstrated within the NCR of all PVs. The E2 transactivation and transrepression functions have been discussed in a previous section.

i. BPV. As discussed earlier, the BPV 1 E2 ORF yields three different forms of E2 products: the E2FL functions as a transactivator of transcription and the two truncated forms as repressors; all function by binding to DNA through the

consensus sequence. There are eleven E2 binding sites within the NCR of BPV 1 (Li et al., 1989). However, only two E2 responsive element (E2RE) have been identified based on their transcriptional enhancer activity on heterologous promoters in the presence of E2 (Spalholz et al., 1987). E2RE1 constitutes two pairs of binding sites, one pair is at each end of the fragment located between nt 7600 to 7800. The two pairs of elements cooperate to transactivate P_{89} and P_{7940} (Haugen et al, 1987; Spalholz et al., 1987). The second E2RE is located at the 5' end of the NCR at nt 7200 to 7386 (Spalholz et al., 1987). It is not required for transactivation of P_{89} and P_{7940} but must have a function within the context of the entire NCR.

Single binding sites for E2 are also present upstream of both P_{2443} and P_{3080}. Although P_{2443} has been shown to be E2 dependent (Hermonat et al. 1988, Prakash et al. 1988), these single sites are not sufficient to transactivate their respective promoters. E2 binding at these sites through an interaction with E2 proteins bound to the E2RE1 could transactivate, although this has not been proven (Hermonat et al., 1988; Prakash et al., 1988).

E2RE1 of BPV 1 has been shown to be activated by E2 proteins from any PV (Cripe et al., 1987; Hirochika et al., 1987; Phelps and Howley, 1987; Giri and Yaniv, 1988). Also, the BPV 1 E2FL protein can transactivate heterologous promoters without the specific consensus binding sequence (Haugen et al., 1987; Turek and Haugen, 1987).

ii. HPV. All PVs sequenced to date carry E2 binding sites (Dartmann et al., 1986). Even though 11 motifs have been found in BPV 1, none of the HPVs harbor more than four sites within the NCR (Figure 1A). They appear to be arranged in a similar manner in all genital HPVs. There are two sites located close to the first initiation codon of the E6 ORF between the TATA and CAAT boxes of the proximal promoter (Sousa et al., 1990). The third binding site is a perfect palindrome only in HPVs associated with benign lesions; in oncogenic HPVs it is not. Its location is approximately 100 nt upstream of the promoter (Sousa et al., 1990). By foot-printing analysis, the last E2 protected site (Garcia-Carranca et al., 1988) is located about 400 nt further upstream. Although this single site binds E2 protein it does not exhibit any increase in transcriptional activity for HPV 16 (Phelps and Howley, 1987), HPV 18 (Gius et al., 1988) or HPV 11 (Hirochika et al., 1988).

The two proximal binding sites have been shown to fill the function of enhancer when they are cloned upstream of heterologous promoters as well as being transactivated by heterologous sources of E2 proteins (Cripe et al., 1987; Hirochika et al., 1987; Phelps and Howley, 1987; Thierry and Yaniv, 1987; Gius et al., 1988; Hirochika et al., 1988; Bernard et al., 1989). BPV 1 E2tr or E2sp can bind to these sites and repress transcriptional activity (Cripe et al., 1987; Chin et al., 1988). However, when these two proximal binding sites are used with their autologous promoter, repression of the promoter is observed regardless of the source of E2 protein (Thierry and

Yaniv, 1987; Bernard et al., 1989; Chin et al., 1989).

It is noteworthy that although three different E2 products have been demonstrated in BPV 1, only the full length form has been identified thus far in HPVs. The transcriptional suppressor activity by E2FL observed in HPVs, in the context of their own promoter, can be explained by steric hindrance of cellular transcription factors (Thierry and Yaniv, 1987; Bernard et al., 1989; Chin et al., 1989).

Several cellular factors as well as the E2 viral protein in combination with the state of differentiation of the cell play a important role in the regulation of PVs through the presence of responsive sequences located within the viral NCR.

E. Papillomavirus Oncogenicity

HPV infections are usually benign proliferations of the epithelium. However, with time and with the presence of cofactor(s), some HPV-induced lesions progress to carcinoma. For example, epidermodysplasia verruciformis (EV) patients who have flat or pityriasis-like HPV-induced lesions exposed to sunlight will develop skin carcinoma (Jablonska et al., 1972). Therapeutic X-irradiation of tumors in patients with laryngeal papillomatosis frequently progressed into carcinoma (Galloway et al., 1960). HPV lesions of the anogenital tract can also develop into carcinomas. Although several factors have been associated with the development of anogenital cancers to some degree, such as smoking, age at first intercourse, number of sexual partners,

use of oral contraceptives, etc. (Slattery et al., 1989; Hildesheim et al., 1990; Ley et al., 1991), other co-factors have yet to be defined. Recently, it has been reported that the host genetic background may play an important role in progression (Wank and Thomssen, 1991).

No in vitro system for PV cultivation exists. However, early work revealed that BPV could transform rodent fibroblasts. Therefore early studies to delineate the structure and function of the PV genome concentrated on BPV.

1. BPV cell transformation

a. Focus formation. As early as 1963, the potential for BPV to transform cells in tissue culture was observed for bovine conjunctival cells and murine fibroblasts (Black et al., 1963; Boiron et al., 1964; Thomas et al., 1964). Focus formation was also observed when BPV 1 DNA cloned into bacterial vectors was transfected, by calcium phosphate co-precipitation, into established mouse fibroblastic cell lines such as C127 and NIH 3T3 (Lowy et al., 1980; Lancaster, 1981; Moar et al., 1981), in established rat cells (Binetruy et al., 1984) and in hamster cells (Amtmann and Sauer, 1982). The mouse fibroblast cell lines C127 and NIH 3T3 have been the most extensively used to study BPV 1-induced morphological transformation. In these transformed cells the BPV 1 genome remains as an episome within the nucleus at 50 to 100 plasmids per cell (Lancaster, 1981; Law et al., 1981; Moar et al., 1981).

b. Transforming region. Transfection of restriction enzyme digested BPV 1 DNA into mouse cells revealed that focus formation could be attributed to about 5437 nt (69% of the viral genome) encompassing the NCR and early region (Lowy et al., 1980). This subgenomic fragment is referred to as BPV_{69T}.

c. Characteristics of transformed cells. Transformed cells harbor specific biological and biochemical characteristics. Different authors have used the term transformed cells to describe cells that have one or a subset of the following biological or biochemical characteristics: (i)spindle-shaped and refractile appearance; (ii) increased acidity of media; (iii) loss of density dependent growth inhibition; (iv) increased saturation density; (v) increased growth rate; (vi) colony formation in soft agar; (vii) growth at low serum concentrations; (viii) tumorigenicity in immunocompromised hosts; (ix) immortalization of primary cells; (x) disorganization of cytoskeletal components; (xi) increased secretion of plasminogen activator; (xii) increased pp60 tyrosylkinase activity.

It is important to mention there have been reports of transformed cells that do not seem to harbor any of the expected biological characteristics of BPV 1 cells transformed in vitro. A cell line established from an equine sarcoid, a naturally occurring BPV 1-induced fibrosarcoma-like lesion of the horse, is contact inhibited, unable to form colonies in soft agar and does not form tumors in nude mice (Lancaster, 1981).

d. Transcripts. BPV 1 transcripts in transformed cells represent approximately 0.01% of total mRNA or around 15 to 30 copies per cell. The transcripts are capped, polyadenylated and are transcribed from the early region (Yang et al., 1985b).

e. Early genes responsible for transformation. Genetic manipulation has been used to delineate the genes responsible for BPV 1 transforming activity. A series of deletion mutants (Nakabayashi et al., 1983) was created from BPV_{69T} and showed that the transforming activity was between nt 2113 to 4451 at the 3′ end of the early region. This region needed to be linked to the NCR (or upstream regulatory region, URR) or a long terminal repeat (LTR) from retroviral vectors to successfully transform cells. Linker insertion mutations (Schiller et al., 1984) identified a second region of the genome located at the 5′ end of the early region. Each region alone can transform murine fibroblasts. In cotransfection assays, their effect is additive (Turek and Haugen, 1987).

i. <u>E6 gene</u>. The 5′ transforming region corresponds to the E6 and E7 ORFs and could allow for transcription of E6 alone as well as spliced E7 products (Schiller et al., 1984; Neary and DiMaio, 1989). Another series of mutants confirmed that E6 (Yang et al., 1985a) was sufficient to induce focus formation in the established murine cell line C127 but not in NIH 3T3 cells (Schiller et al., 1984). However, to successfully immortalize primary rat embryo fibroblasts, E6 required the E2 or E5 gene to be expressed (Cerni et al., 1989).

ii. E5 gene. The region located at the 3' end of the early region capable of inducing morphological transformation has been identified by different engineered mutants as the E5ORF. The BPV 1 E5 ORF has been shown to induce foci on established murine fibroblast cell lines. Recently, the oncogenic potential of this ORF has been described for established murine keratinocyte lines suggesting that the E5 product alone may not be sufficient to induce transformation but requires previous cellular mutations (Leptak et al., 1991).

2. HPV transformation

Major differences exist between BPV and the oncogenic HPVs with respect to cell transformation. Mouse cells transformed by BPV retain the viral genome as an episome. HPV sequences in carcinoma-derived cell lines, such as Hela and SiHa, are integrated into the host genome.

a. Oncogenic HPVs. Initial studies have focused on the transforming capacity of potential oncogenic HPVs based on their association with cervical cancer. These studies utilized those systems established for BPV cell transformation. Cell transformation by HPVs is defined as any cellular behavior different from the control cell.

Transfection of HPV 16 DNA into NIH 3T3 cells results in morphological transformation with concomitant integration of the viral genome. These cells are tumorigenic in nude mice (Yasumoto et al., 1986). Transfection of the HPV 18

genome into NIH 3T3 and rat-1 cells induces focus formation after a delay of 5 to 6 weeks. These cells grow in soft agar and will induce tumors in nude mice. The viral sequences in these cells are integrated into the host genome (Bedell et al., 1987). Subgenomic fragments of the HPV-18 genome carrying the URR and E6 and E7 are sufficient for cell transformation.

Matlashewski et al. (1987) co-transfected primary baby rat kidney (BRK) cells with HPV 16 E6 and E7 under the control of the Moloney murine leukemia virus long terminal repeat (MoMuLv-LTR) with the neomycin resistance gene as a selectable marker. Very few colonies developed 3 to 4 weeks post transfection with G418 selection. In other experiments, in addition to MoMuLv-LTR HPV 16, an activated ras oncogene was co-transfected into primary BRK cells resulting in an increase in the efficiency of cell immortalization. Mutational studies showed that the HPV 16 E7 ORF, under the control of a heterologous promoter was necessary and sufficient to: (i) immortalize BRK cells when co-transfected with activated ras (Storey et al., 1988); (ii) induce morphological transformation (focus formation) in NIH 3T3 cells (Phelps et al., 1988).

The target cell for natural HPV infection is the epidermal keratinocyte. In vitro systems attempting to mimic natural infection have also been investigated. Normal human fibroblasts transfected with HPV 16 were found to have an increased life-span but did not become immortalized (Pirisi et al., 1987). Normal human keratinocytes on the other hand, were immortalized after transfection with HPV 16 DNA. These cells are

nontumorigenic in nude mice suggesting that human cells are more resistant to transformation to malignancy as compared to mouse cells (Pirisi et al., 1987). Human primary foreskin keratinocytes transfected with HPV 16 or 18 DNA are immortalized and are resistant to induction of differentiation after treatment with calcium and serum as compared to HPV 6 or 11 transfected keratinocytes (Schlegel et al., 1988).

To understand the mechanism of immortalization, several investigators have used different constructs of the HPV 16 genome and subgenomic fragments, under the control of either autologous or heterologous promoters such as SV40, beta-actin or MoMuLTR, to transfect primary human keratinocytes or fibroblasts. These studies all demonstrated that both ORF E6 and E7 were necessary and sufficient to immortalize primary human keratinocytes (Hawley-Nelson et al., 1989; Munger et al., 1989a; Watanabe et al., 1989). These HPV 16 or HPV 18 immortalized keratinocytes lines fail to show any morphological changes as monolayers. However, when cultivated in organotypic or raft culture, which allows for cellular stratification and differentiation, the cells show a pattern of cellular disturbance similar to that observed in the histopathology for cervical dysplasia. Untransfected primary keratinocytes showed ordered maturation and differentiation similar to epithelial cells seen in tissue sections of normal skin (McCance et al., 1988; Pecoraro et al., 1989; Hudson et al., 1990).

Although HPV DNA is integrated and transcriptionally active in immortalized human keratinocyte cell

lines, it has been shown repeatedly that these cells cannot induce tumors in nude mice (Pirisi et al., 1987; Kaur and McDougall, 1988; Schlegel et al., 1988). Subsequent changes must occur for the cell to become tumorigenic. DiPaolo et al. (1990) have reported that cervical keratinocytes immortalized by HPV 16 become tumorigenic in nude mice when transfected with the herpesvirus type 2 BglII N transforming DNA fragment. Interestingly, the herpesvirus DNA sequences were not detected by Southern blot analysis in these cells. This experiment is a further indication that the HPV genome is not sufficient for malignant transformation but requires subsequent injuries to the cell. To support this observation, McDougall and co-workers (Hurlin et al., 1991) have reported that HPV 18 immortalized human keratinocytes are nontumorigenic at low passage (passage 12) and fail to form colonies in agar. At passage 32, some cells could form colonies in agarose and produce tumors that regressed in nude mice. At passage 59, however, the cells could efficiently grow in agarose and produce squamous cell carcinomas in nude mice. Chromosome analysis at the different passages showed an increase in aneuploidy suggesting that the progression of this cell line to tumorigenicity was due to chromosomal alterations and not to changes in expression of E6 and E7.

Recent studies have shown that the HPV 16 E5 ORF alone, under the control of a strong promoter, transfected into established murine keratinocytes failed to induce morphological transformation but these transfected cells could induce tumors in nude mice (Leptak et al., 1991).

b. Nononcogenic HPVs. Recent studies have used HPV 6 or 11 to investigate their potential for transformation, although these virus types are rarely associated with high grade lesion or cervical cancer.

C127 cells transfected with the HPV 6b genome do not exhibit any altered morphology but can form microcolonies in soft agarose and even induce tumors in nude mice (Morgan et al., 1990). When HPV 6 E7 was cotransfected with an activated ras oncogene into primary BRK cells, immortalization occurred at a much lower efficiency than cells transfected with HPV 16 E7. These immortalized cells exhibited integration of the viral DNA and were as efficient as HPV 16 immortalized cells in inducing tumors in nude mice (Storey et al., 1990).

The HPV 6 E5a ORF has been investigated for its transforming potential in both C127 and NIH 3T3 cells. C127 cells do not show any morphological transformation as compared to NIH 3T3 cells which exhibited characteristics of transformed cells such as focus formation and anchorage independence (Chen and Mounts, 1990). The significance of HPV 6 transforming activity in an in vitro system is not clear since very rarely if at all is HPV 6 present in cervical cancers.

In cell lines established from cervical carcinomas containing HPV 16 or 18 sequences, the E6 and E7 ORF products are believed to be necessary to maintain the transformed phenotype. Transfection of the HPV 16 or 18 E7 gene alone is sufficient to immortalize primary rodent cell lines whereas the E6 gene is necessary for tumor induction in nude mice. Cells

transfected with the HPV 6 or 16 E5 ORF have been shown to induce tumors in nude mice as well. The mechanism of cellular transformation probably involves a cascade of events such as cellular mutations concomitant with viral expression of certain ORFs, leading to formation of protein-protein interactions such as the p53-E6 complex and the pRB-E7 complex which plays an important role in disruption of the cell cycle (to be discussed in a later section).

c. Infection of the skin with HPVs.

i. Immunocompetent individuals. Cutaneous warts have been recognized for millenia. Their transmissibility was shown as early as 1907 by Ciuffo's experiments, through inoculation of wart emulsion into volunteers. The only reservoir of transmission remains a contact with an individual carrying a wart which manifests itself as an epidermal proliferation of different clinical appearances varying from verruca vulgaris (increased elevation with rough, irregular and hyperkeratotic surface), to myrmecia (deeply seeded in the skin of the palm or the sole) to verrucae planae (small, slightly elevated, skin colored papules present on the hands and face). Lesions usually appear after a period of incubation from 1 to 20 months.

Although there are more than 60 different HPVs, only a few types (1-4, 7, 10, 26 to 28) are specifically associated with skin lesions in the general population. Based on cytopathic effects (Croissant et al., 1985, Jablonska et al., 1985), certains types have been specifically linked to some

lesions (Table 1). In the general population, these benign infections usually regress spontaneously presumably due to either humoral or cell mediated immunity or both.

ii. Immunocompromised individuals. Cutaneous warts are a major problem for a subgroup of patients with the rare genetic defect, epidermodysplasia verruciformis (EV) (Table 2). These patients can be infected with several types of HPV at once with the most common types being 5, 8, 17, 20 (Jablonska and Orth, 1985). These virus types are not usually found, by conventional means, in the general population. When these lesions occur on sun-exposed areas of the body, UV irradiation apparently acts as a cofactor to induce progression to squamous cell carcinoma, usually within the second decade of life. In these carcinomas, Southern blot hybridization has demonstrated the presence of episomal form of HPV most commonly typed as 5, 8, or 14 (Jablonska and Orth, 1985).

The mechanism of malignant transformation is not understood but some experimental work suggests that these EV patients lack a "suppressor gene activity" (Pfister et al., 1990). Presumably, this gene does not allow oncogenic expression of these HPVs in the normal population.

The HPVs associated with EV patients have also been detected in immunocompromised patients such as renal allograft recipients (Lutzner et al., 1980); in some instances these infections can lead to carcinoma.

d. Infection of the mucosa with HPVs. HPV is highly

TABLE 1. Association of cutaneous HPV infections in the immunocompetent host with specific virus types.

HPV type	Lesion morphology	Location
1, 4	myrmecia (benign)	plantar and palmar surfaces
2, 26, 28, 29	verruca vulgaris (benign)	hands and extremities
3, 10, 27	verruca plana (benign)	face
7	verruca vulgaris (benign)	hands of meat handlers

TABLE 2. Association of cutaneous HPV infections in epidermodysplasia verruciformis and the immunocompromised host with specific virus types.

HPV type	Lesion morphology	Location
5, 8	macular (high malignant potential)	generalized
9, 12, 14, 15, 17, 19-25, 36, 46-50	macular or verruca plana (benign or low malignant potential)	generalized

tissue specific and some 40 types have been associated with skin lesions, the majority of which have been identified in EV patients. About 25 types are found in mucosal sites, either anogenital tract, conjunctiva, respiratory tract or oral cavity (Table 3). Only two types, HPV 13 and 32, appear to be restricted to the oral cavity. All other HPVs found in the anogenital tract have been demonstrated at other mucosal sites. The routes of transmission can only be speculated since there is no in vitro system or animal model for testing (Shah, 1990).

i. Respiratory tract. The respiratory tract is susceptible to infection by HPV. Even though papillomatosis has been observed in all parts of the respiratory tract, the most commonly affected site is the vocal cords. Some laryngeal papillomatoses can be life threatening due to airway obstruction and require surgical intervention for their removal at frequent intervals due to tumor recurrence (Shah, 1990). The virus is believed to be acquired during passage of the fetus through a birth canal contaminated with condyloma. Transmission in utero does not seem to play a role (Shah, 1990). The incubation period may vary from a few months to several years with approximately 50% of cases developing during the first five years of life. In about one third of the cases, the onset of illness will occur after their twentieth birthday (Shah, 1990).

HPV type 6 or 11 DNA is present as an episome within these lesions. There are reports of eventual progression of these benign lesions to carcinoma over a period of time as

TABLE 3. Association of mucosal HPV infections with specific virus types.

HPV type	Lesion morphology	Location
6, 11, 34, 39 41-44, 51, 53-55	condylomata (benign or low malignant potential)	anogenital and respiratory tract
13, 32	focal epithelial neoplasia (benign)	oral cavity
16, 18	condylomata, intra-epithelial neoplasia (high malignant potential)	anogenital tract
30, 31 33, 35, 45, 52 56	condylomata, intra-epithelial neoplasia (intermediate malignant potential)	anogenital tract

long as 30 years (Crissman et al., 1988). The mechanism of benign to malignant conversion is not known; however, the viral genome remains as an episome and is transcriptionally active in the few cases that have been studied.

ii. Anogenital tract. Genital warts were recognized almost a century ago as being sexually transmitted (Jenson and Lancaster, 1991). Due to their similarity to skin lesions, they were thought to be benign lesions and thus did not receive attention (Campion, 1987). It was not until the 1970's that cytopathologists noticed specific cellular changes attributable to HPV infection in cervical dysplasia (Meisels and Fortin, 1976; Purola and Savia, 1977; Laverty et al., 1978). With the advent of molecular biology, the presence of HPV DNA has been demonstrated in a high percentage of benign anogenital warts as well as lesions showing dysplastic changes.

Of the more than 65 types of HPV that have been identified, about 30% have been associated with anogenital lesions (de Villiers, 1989). Based on their association with malignancy, these HPVs have been further classified into low, intermediate and high risk groups (Lorincz et al., 1987c).

HPV types 6 and 11, representative of the low risk group, are the most common isolates found in genital warts or condyloma acuminata, although HPV types 42, 43, 44 have also been found (Lorincz et al., 1991). Condylomas very rarely progress to malignancy. In the rare cases where progression has been observed, several point mutations and duplications in the

NCR of the viral genome had occurred (Rando et al. 1986). Similar if not identical mutations have been detected in a condyloma at an extragenital site (Kulke et al., 1989).

The intermediate risk group includes HPV 31, 33, 35, 51 and 52 which are found in moderate and severe cervical dysplasias and in some cervical cancers. It is important to mention that most of these types have also been demonstrated in cervical tissue exhibiting no cytological or histopathological evidence of HPV infection (Schneider et al., 1985; Toon et al., 1986; Lorincz et al., 1986b). These so-called "normal" tissues may harbor viral genomes for undefined periods of time before initiation of a lesion. The presence of HPV alone is not sufficient to induce cellular changes. Some extra-cellular factors, still unidentified, seem to be needed to allow a latent HPV infection to progress to a lesion with the specific histologic features of active HPV infection (zurHausen, 1989).

Approximately 85% of cervical carcinomas harbor HPV DNA (Riou et al., 1990) and almost 70% contain HPV 16 or 18 DNA sequences (zur Hausen and Schneider, 1987). The majority of remaining cases contain HPV type 45 or 56 (Lorincz et al., 1992). The biology of a specific type may also play a role in progression. For example, HPV 18 containing lesions appear to progress faster than lesions associated with HPV 16 (Barnes et al., 1988).

In benign and all grades of dysplastic lesions, the viral genome is present as an episome. In invasive cancers, HPV DNA is found integrated in the majority of lesions

(Cullen et al., 1991). However, HPV 16 has been reported present as a dimeric episomal form in vulvar carcinoma (Kennedy et al., 1987). When integration occurs, the viral genome appears to be randomly located in chromosomes, although in some cell lines, integration occurs in the vicinity of cellular oncogenes (Durst et al., 1987). The viral genome is consistently broken at the E1-E2 ORF (Schwarz et al., 1985, Baker et al., 1987). It is felt that the loss of the E2 product, coding for the transactivator and transrepressor of viral transcription, leads to loss of down regulation of the early proteins, E6 and E7 (Schneider-Gadicke and Schwarz, 1986; Smotkin and Wettstein, 1986; Baker et al., 1987). It is speculated that an increase in expression of the viral oncogenes E6 and E7 contribute to cellular transformation (Schneider-Gadicke and Schwarz, 1986; Smotkin and Wettstein, 1986; Baker et al., 1987).

A possible mechanism of cell transformation by E6 and E7 has been recently described. In vitro the E7 gene product of all anogenital HPVs can bind the retinoblastoma gene product (pRb), known to be a tumor suppressor gene. However, the E7 protein from the high risk HPV group has a 10-fold greater affinity for pRb product compared to the E7 expressed from the low risk group (Munger et al., 1989b). It has been speculated that, in vivo, in the presence of a high risk virus, and E7-pRb interaction, there may be a decrease in the concentration of free pRb product leading to a loss of cellular growth control.

A second interaction of a viral protein with a cellular suppressor gene product has been observed in vitro

(Werness et al. 1990). The E6 product of HPV 16 and 18 binds to the p53 protein, another product of a tumor suppressor gene. This binding has also been observed with other viral oncoproteins such as SV40 T Ag and E1b of adenovirus 5, although the mechanisms involved appear to be different. For SV40 T Ag or E1b of adenovirus transformed cells, there is an increase in the half-life of p53 (Oren et al., 1981; Reich et al., 1983). Whereas in the presence of the E6 gene product from oncogenic HPVs, a decrease in concentration of p53 is observed. In vitro, E6 binding to p53 promotes the degradation of p53 through ubiquitin mediated pathway (Scheffner et al., 1990). This is consistent with the absence of p53 reported in Hela cells Matlashewski et al., 1986).

Carcinogenesis is a multistep process. In cervical carcinoma, the interaction of viral products with cell growth regulatory proteins is a plausible mechanism to explain the oncogenic potential of some HPVs but is not sufficient to explain the phenomenon in its totality. Several questions have not yet been answered. Is integration required for carcinogenesis to occur or is it the result of carcinogenesis? What triggers integration? Is the integration targeted to specific sequences on the human chromosome? How does integration change viral gene regulation? Are any cellular proteins, related to the state of differentiation of the cell, involved in viral gene regulation? What are the co-factors involved in benign to malignant progression? Until these questions can be answered, it is of importance to be able to identify patients at risk to

develop cancer by identifying the presence of HPVs from the high risk group. As of today, no in vitro system can support the growth of HPV for use in the development of diagnostic aids. Other than the cytological or histopathological changes specific for HPV, molecular biology techniques are the only reliable means to detect HPV infection in clinical samples.

F. Detection of HPV Sequences

Considerable work has been done investigating ways of detecting HPV infections, especially of the anogenital tract, since it appears to be the site where HPV infections are the most likely to progress to cancer.

The conventional method of screening cervical lesions has been and still remains Papanicolaou staining (Pap smear) for the detection of HPV specific cellular changes such as the presence of koilocytes, cells exhibiting nuclear atypia with cytoplasmic vacuoles. The presence of koilocytes correlate with the presence of HPV infection but its absence does not exclude HPV (Jenson and Lancaster, 1990). It is estimated that as many as 20% of the Pap smears are inadequately reported (Coppelson and Brown, 1974; Jenson and Lancaster, 1990).

Electron microscopy (EM) has been used by a few investigators. Among the histopathological lesions studied, approximately 50% harbored virions within nuclei (Koss, 1987). This high number of false negatives and the cumbersome technique makes EM undesirable for HPV screening.

Antibodies raised against disrupted BPV virions cross-

react with structural antigens of all PVs (Jenson et al., 1980). This antiserum is used in immunocytochemistry to identify lesions containing PV virions. Approximately 50% of condyloma acuminata and low grade cervical dysplasias studied show a positive reaction for HPV structural antigen (Kurman et al., 1984). The drawback to this antigen detection technique is a decrease in the positivity rate as the severity of the lesion increases. All cervical carcinoma tested gave a negative result for the detection of capsid antigen (Pfister, 1990). The reason for lack of positive staining in high grade lesions and cervical cancers is due to the absence of differentiated cells which are required for viral maturation.

Several investigators have directed efforts into developing an assay where the main target would be detection of the E6 and E7 early proteins (Smotkin and Wettstein, 1986; Androphy et al., 1987a; Banks et al., 1987; Oltersdorf et al., 1987; Seedorf et al., 1987). These two polypeptides are apparently expressed throughout viral HPV infections. In view of the diversity of HPV types and the lack of cross-reactivity among the different E6 and E7 proteins, the complexity of the task increases because of the multitude of type specific reagents. Furthermore, E6 and E7 proteins have not been consistently demonstrated in tumor material probably due to the very low concentration or stability of these oncoproteins (Pfister, 1990).

Evaluation of the immune status of patients infected with HPVs is still at an early stage. The plurality in HPV types combined with the number of early proteins being expressed in an

infection together with the inability to grow the virus contribute to the difficulty in selecting the appropriate antigens for a diagnostic test. The level of antibody against the HPV 16 E7 protein has been investigated and may be of some relevance for HPV 16 screening (Dillner et al., 1990; Jochmus-Kudielka et al., 1990). The lack of concordance among the various serology studies for detection of HPV-specific antibodies suggests that more information has to be known about the host immune response and the viral antigens eliciting the response. It is likely that an elaborate immunological test with an extensive panel of reagents will be needed to address this question.

The Pap smear is the standard procedure for detecting cellular changes associated with HPV infections. However, it lacks the capacity to identify latent HPV infections. As of today, the only definitive method to diagnose an HPV infection, either latent or active, is the detection of virus-specific nucleic acids by molecular hybridization.

1. Southern blot hybridization

This technique was first described by Southern (1975). For biopsies, the samples have to be digested with proteinase K in the presence of detergent to ensure efficient lysis of cells. Contaminating RNA is removed by RNAse treatment. Five to ten μg of purified DNA are digested with restriction endonucleases followed by electrophoresis through agarose gels. After adequate separation is obtained, the agarose gel is soaked

in alkali to denature the DNA followed by neutralization in high salt. The denatured DNA is transferred onto a support such as nitrocellulose or nylon membranes by capillary action. HPV sequences are localized by hybridization to labeled HPV probes. Radioactive labelling is used most commonly but nonradioactive detection methods are gaining popularity. Standard conditions of hybridization are at 25°C below the melting temperature of the probe (Tm-25°C). Because PVs contain regions of conserved homology it is possible to detect sequences from one virus type using another virus type as probe. However, the conditions of hybridization are such that heteroduplexes between different types remain stable. This requires reducing the stringency of hybridization to Tm-40°C in most cases. Because of the cross-hybridization between closely related virus types, it is frequently necessary to increase the stringency of the hybridization reaction to Tm-10°C for purposes of identifying a given HPV type. After 12 to 24 hours hybridization, the filters are washed at a temperature equivalent to the hybridization stringency to remove the unbound radioactive probe and exposed to X-ray film for autoradiography from 12 hours to several days. The probe hybridizes to targets, when present on the filter. PVs have characteristic restriction enzyme cleavage patterns which aid in their identification on autoradiograms. The sensitivity of Southern blotting is about 10^4 copies of a given DNA (Lancaster and Norrild, 1989). Therefore, this test requires relatively large amounts of total cellular DNA (10 μg) to have reasonable success in detecting viral sequences in a clinical

sample. Using HPV types most often found in anogenital lesions, studies have demonstrated HPV present in about 10% of normal Pap smears (Schneider et al., 1985; Lorincz et al., 1986; Macnab et al., 1986; Toon et al., 1986; de Villiers et al., 1987; Fuchs et al., 1988) and up to 85% of cervical carcinomas (Riou et al., 1990; Lorincz et al., 1992).

Southern blot hybridization is useful for screening for the presence of HPV, identifying new types, and for typing with the help of specific restriction enzymes patterns obtained after electrophoresis. This technique also provides information on the state of the viral genome. Aberrant restriction patterns raise the possibility of integration of the viral genome, inadequate cleavage, a mutation within a restriction enzyme site or cross-hybridization with a new HPV type.

The disadvantages of this technique are time considerations and the involvement of numerous manipulations, as well as the need for a relatively high concentration of cellular material required to obtain successful results.

2. Reverse Southern blot hybridization

This technique is similar to the Southern blot except cellular DNA is labelled and used as a probe against a panel of cloned HPV DNAs which are electrophoresed and transferred onto a filter. This technique makes possible the detection of more than one type in a given sample, by comparison of the intensity of signal between different HPV types on the filter. The sensitivity of this assay is around 10 genomes per cell if all of

the cells are infected (Schneider, 1987). This technique is mainly used in an attempt to confirm a new virus type.

3. Dot blot hybridization

Purified cellular DNA from about 2 x 10^5 cells is spotted or dotted directly onto a filter membrane followed by denaturation by alkali (Wickenden et al., 1985; Schneider, 1987). Several samples can be applied at once in a relatively short period of time without extensive manipulations. Radioactive cloned HPV probes are used under conditions of stringent hybridization. This assay facilitates the typing of several samples at one time. The level of sensitivity is comparable to the Southern blot. A major drawback is that closely related HPV types cannot be differentiated. Nonstringent hybridization cannot be done due to non-specific background signal, new types cannot be detected and no information on the state of the viral DNA can be determined.

4. Filter <u>in situ</u> hybridization

In this HPV detection system, cells in suspension are filtered onto a membrane, nitrocellulose or nylon, where they are lysed <u>in situ</u>. The DNA is denatured by alkali treatment of the filter (Wagner et al., 1984; Schneider et al., 1985). Labelled HPV probes are used only under stringent conditions of hybridization. This technique allows for the detection of 10^4 to 10^5 HPV copies within a sample provided the genomes are clustered in one area of the filter. If viral genomes are present at low

copy number in several cells, contrary to the dot blot hybridization, this technique will not provide an unequivocal signal.

In theory, the filter in situ hybridization should be comparable to dot blot hybridization and have the same disadvantages. However, false positive results have been reported more often, probably due to abundant cellular debris present on the membrane (Lorincz, 1987a).

5. In situ hybridization

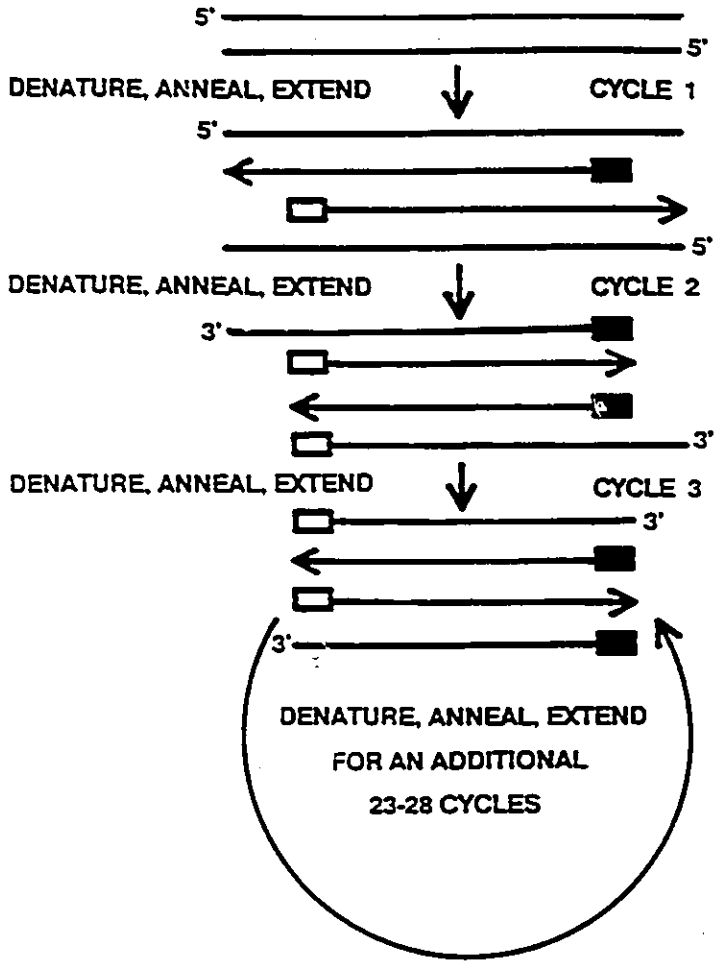
This is the only technique that can correlate histopathology with the presence of HPV sequences. The technique consists in having a tissue section attached onto a slide. The cells are made permeable by proteinase K digestion and the DNA is heat denatured. The probe can be radioactive or labelled by nonisotopic methods, and can consist of either DNA or RNA; the probe most often used is labelled with tritium. (Beckman et al., 1985; Gupta et al., 1985; Stoler and Broker, 1986). For radioactive probes, a photographic emulsion overlaying the specimen after hybridization is used for detection of signal. Hybridization occurs directly on the slide, under stringent conditions (Ostrow et al., 1987). Nonstringent conditions have also been used; however, background noise is increased making the interpretation of the results more difficult but specific reactions can be detected due to the cellular location of the signal. The level of detection of HPV DNA sequences is about 10-20 copies per cell. Antisense RNA probes can also be used to

detect virus-specific transcripts. This technique is inherently more sensitive since there are more viral specific RNA transcripts than DNA copies/cell (Stoler and Broker, 1986). However, the tissue has to be processed differently than for normal histopathology and the region of the viral genome used as probe must be represented in the transcripts in the sample.

6. Polymerase chain reaction

A more recent technique has been described (Saiki et al., 1985) which is superior to any other technique in terms of sensitivity. The polymerase chain reaction (PCR) consists of amplifying a defined region of DNA using oligonucleotides primers complementary to the DNA target on opposite strands. The primers must have their 3′ ends facing each other to allow extension to occur. A thermostable DNA polymerase is used to synthesize new DNA from the 3′ end of the primers. Several cycles of DNA denaturation followed by primer annealing and extension yield an exponential amplification of the target sequence up to 10^6 copies from the one original template (Figure 1B). The amplified products can be visualized on ethidium bromide-stained agarose gels. To increase the sensitivity, Southern blot hybridization can be performed on the amplified product. This technique was first used to detect HPV in 1988 by Shibata et al. using type specific primers. Several reports have taken advantage of this new technique (Gregoire et al., 1989; Manos et al., 1989; Snijders et al., 1990) to detect the presence of HPV DNA in a variety of clinical specimens.

FIGURE 1B. Polymerase chain reaction. The first cycle requires DNA denaturation allowing for annealing of two primers (open and closed boxes) followed by extension from the 3′ ends of the primers (indicated by arrows). Subsequent cycles of denaturation, annealing and extension will yield an exponential increase of the the products.



II. OBJECTIVES

A major problem with detection of HPV sequences in clinical samples is the plurality of the virus group. Of the approximately 60 identified HPV types about 1/3 are associated with anogenital tract infections. Use of a large number of probes to detect HPV sequences would be too cumbersome for large numbers of specimens. Further complicating the detection of HPV sequences is the geographic variation for given HPV types in anogenital lesions as well as evidence suggesting differences in prevalence of certain HPVs in ethnic groups. In addition, numerous reports have indicated the presence of unknown HPV types in cervical samples using nonstringent Southern blot hybridization on total cellular DNA. Illustrative of this problem is a recent study by Lorincz et al. (1992) in which 15 of the common anogenital tract HPVs were used as probes. Testing cervical specimens ranging from normal to invasive cervical cancer, they found that a total of 14% of 792 HPV positive samples contained HPV sequences that could not be classified. Unknown types were detected in about 30% of samples classified by histopathology as normal or unspecified atypia. The frequency of unknown types dropped to 13.4% in low grade squamous intraepithelial neoplasia (SIL) to 6.6% in high grade SIL to 5.8% in invasive cancers. Since this study used Southern blot analysis, the percentage of samples containing unknown types may be underestimated. More sensitive techniques for detection of HPV sequences, such as PCR with the appropriate primers, may

reveal higher proportions of samples containing unknown HPV types. To gain insights into the natural history of HPV infection of the anogenital tract it is important to develop a test that can detect these viruses and provide a more accurate measurement of the frequency of samples containing any HPV DNA sequence.

The overall objective of this work was to develop a technique that would allow for the detection of all human papillomaviruses in a variety of clinical samples. Emphasis was placed on anogenital tract specimens since this is one region harboring HPV-induced lesions with a high potential to progress to cancer. Cutaneous lesions as well as lesions of the respiratory tract were also considered. The limited amount of clinical material available for viral detection was a criterion taken in consideration to develop an assay.

In light of the advantages and the limitations of the different techniques described above for detection of HPV sequences in clinical material, PCR in conjunction with Southern blot was the method of choice to develop for detection of low copy numbers of viral DNA. This technique should be applicable to the detection of any HPV, latent HPV infections and samples too small to be analyzed by other methods.

Since HPV DNA sequence amplification by PCR would be limited by the potentially vast number of known and unknown types infecting the anogenital tract, the main objective of this work was to develop PCR primers that would be specific for any HPV sequence. The approach was to: (i) identify regions of homology

in the genomes of HPVs whose sequences were known; (ii) design HPV genus-specific PCR primers based on these homologies and to optimize the conditions for their use in PCR; and (iii) show their utility for amplification of HPV DNA in clinical samples from both histologically normal tissues and diseased tissues.

III. MATERIALS AND METHODS

A. Primer Design

Early hybridization studies using different PV probes showed cross-reaction between a number of PVs in two discontiguous segments of the PV genome (Law et al., 1979). This was later confirmed after the sequences for a number of PVs became available. Computer analysis showed that ORFs E1, E6, E7 and L1 contained homologous sequences (Baker, 1987).

For initial primer design, the genome sequences of the anogenital tract HPVs available in the literature to 1987 were used. These viruses were HPV 6, 11, 16, 18, and 33 (Cole and Streeck, 1986; Cole and Danos, 1987; Dartmann et al., 1986; Schwarz et al., 1983; Seedorf et al., 1985). A computer search using pair-wise comparisons revealed that the longest conserved nucleotide string was in the E1 ORF. The sequence dodecanucleotide string of TAAAACGAAAGT, termed UNI, was located in the 5' half of the E1 ORF and showed perfect homology among the five anogenital HPVs tested. This was a fortunate finding since integration of HPV sequences in carcinomas frequently occurs downstream of this ORF thus rendering this sequence applicable for use in PCR amplification of HPV sequences in cancers. More recently, perfect homology to the sequence was shown to be present about 200 nt downstream of the first AUG codon in the E1 ORF of all sequenced PVs of human origin; animal PVs showed only 67 to 75% homology to the sequence (Campione-Piccardo et al., 1991).

Since a dodecanucleotide is too short to ensure specific binding to its target, it was necessary to extend the length of the sequence for use in PCR. A prerequisite for fidelity of chain elongation in PCR requires a perfect match at the 3′ end of the primer (Innis et al. 1990). Therefore, nucleotides were added to the 5′ end of the dodecanucleotide to create a 21 nt sequence. Considerable degeneracy was observed 5′ to the dodecanucleotide in the sequenced PV genomes. To keep the total number of sequences to a minimum and to ensure maximum binding to target, all two base degeneracies were maintained and inosine was substituted at four base degeneracies. Inosine facilitates stabilization of primers during annealing (Ohtsuka et al., 1985). The final primer design was termed IU with the sequence: 5′-TII(A/G)I(A/G)II(C/T)TAAAACGAAAGT-3′.

The second primer was selected using pair-wise comparisons of translated E1 ORFs of sequenced PV genomes. A region having amino acid homology containing methionine and tryptophan residues (i.e single codons) was selected for further analysis. The greatest degree of nucleotide homology with a stretch of perfectly paired nucleotides at the 3′ end occurred about 850 nt downstream of IU in mucosotropic HPVs and 800-750 nt downstream of IU in cutaneotropic HPVs. Two and four base degeneracies were handled in the same fashion as IU. The final design for the 21 nt primer was termed IWDO with the sequence: 5′-(A/G)TC(A/G)(A/T)AIGCCCA(C/T)TGIACCAT-3′.

The location of these primers on the genomes (Figure 1A) of sequenced HPVs are given in Table 4.

TABLE 4. Nucleotide positions for the primary annealing sites of the E1 ORF consensus primers IU and IWDO.*

Virus	Nucleotide position IU	IWDO	Size (bp)	Reference
1a	1019	1798	779	Danos et al., 1982
5	1180	1929	749	Zachow et al., 1987
6b	1066	1932	866	Schwarz et al., 1983
11	1164	1910	746	Dartmann et al., 1986
8	1066	1932	866	Fuchs et al., 1986
16	1111	1962	851	Seedorf et al., 1985
18	1167	2032	865	Cole and Danos, 1987
33	1122	1955	833	Cole and Streeck, 1986

*IU anneals to the coding strand and IWDO anneals to the noncoding strand.

B. Polymerase Chain Reaction

1. PCR reaction mixture

Amplification reactions were carried out in a volume of 100 µl, except where otherwise specified, in 0.5 ml microcentrifuge tubes. The reaction mixture consisted of deoxynucleotides at a final concentration of 200 µM each and primers at 1 µM each in 67 mM Tris-HCl (pH 8.8), 31 mM KCl, 2 mM $MgCl_2$ and 2 µg/ml BSA. For the template, the concentration of cloned HPV DNAs varied from 0.1 to 1 ng. For clinical samples 50 to 500 ng of cellular DNA was used. Two units (U) of thermostable Taq DNA polymerase (Cetus, Perkin-Elmer) per reaction were used for synthesis of DNA from the 3′ ends of the primers.

To optimize PCR using the HPV consensus primers, concentrations of magnesium ion, primers and enzyme were varied; $MgCl_2$ from 0.5 to 10 mM, primers from 0.1 to 5 µM, and Taq DNA polymerase from 0.5 to 3.5 U.

Different compositions of buffers were tested to determine the most efficient for amplification. The buffers were: (i) 10 mM Tris-HCl (pH 8.3), 50 mM KCl and 1.5 mM $MgCl_2$ (Saiki, 1990); (ii) 67 mM Tris-HCl (pH 8.8), 31 mM KCl, 2.0 mM $MgCl_2$ and 2 µg/ml bovine serum albumin (Paabo, 1990).

2. PCR cycle

PCR consists of several cycles of denaturing the template(s), annealing of the primers followed by their

extension. All experiments of PCR were run on a DNA thermal cycler from a single manufacturer (Cetus, Perkin-Elmer). Specifications of the manufacturer recommended maintaining all temperatures for a minimum of 1 minute. The temperature of denaturation used was 94°C for a 2 minute period for all studies. For PCR using HPV consensus primers, the optimum temperature of annealing was determined by varying the temperature from 37°C to 55°C for 2 minutes. A period of extension of 3 minutes at 72°C was used in initial studies with IU and IWDO primers. Subsequently, the extension temperature was lowered and the length of time shortened to increase the efficiency of amplification.

For PCR using different sets of primers, the temperature of denaturation at 94°C for 2 minutes remained unchanged. The temperature of dissociation (Td) was calculated according to the G+C ratio (Mason and Williams, 1985) and the initial temperature of annealing was set at Td-5°C for 2 minutes. The temperature of extension will be indicated for each specific set of primers as well as the length of time needed for polymerization according to the size of the fragment predicted by the target sites.

3. Samples

a. Fresh biopsies. Fresh biopsies of the anogenital tract were obtained from patients in the Washington, DC, USA, as well as patients from Lima, Peru. Samples consisted of either penile, vulvar or cervical biopsies of abnormal

appearing tissue and of normal tissue. Histopathologic diagnosis was obtained either by frozen section on the biopsy from which DNA was extracted or from half of the lesion processed for standard histopathology. These samples had been previously analyzed for the presence of HPV sequences by Southern blot hybridization (Lorincz et al., 1987b; Lorincz et al., 1992).

b. Cervical scrapings. Two different populations were selected for collection of the clinical samples: series F was from two family practices located in Ann Arbor, MI, USA; series R was collected from patients visiting a referral clinic for HPV infection in Southfield, MI, USA.

Series F was exclusively caucasian women who visited for a routine gynecological examination or because of complaints of vaginal discharge, discomfort or itching. Series R was collected from caucasian women identified as previously having recurrent HPV infection of the anogenital tract. At the time of sample collection they were free of any visible lesion.

c. Formalin-fixed, paraffin-embedded tissues. Twenty-six paraffin-embedded tissues identified as cervical intraepithelial neoplasia or carcinoma <u>in situ</u> by histopathology were obtained from the archival collection of the Department of Pathology, Wayne State University School of Medicine, Detroit, Michigan, USA. The specimens were processed for HPV PCR to evaluate the efficiency of the E1 consensus primers with samples processed for routine pathologic examination.

C. DNA preparation for PCR

1. Cloned HPV sequences

HPV types whose sequences were known were released from flanking vector sequences by cleavage at the unique restriction enzyme site of insertion. All of the cleavage sites were outside of the E1 ORF. HPV 6 (de Villiers et al., 1981), HPV 11 (Gissmann et al., 1982) and HPV 16 (Durst et al., 1983) were released by Bam HI cleavage. HPV 18 (Boshart et al., 1984) was released by digestion with Eco RI and HPV 33 (Beaudenon et al., 1986) by digestion with Bgl II. Virus DNA from animal and human PVs whose sequences have not been published were released from flanking vector sequences by cleavage at the unique cloning site. HPV 2b (Heilman et al., 1980), HPV 31 (Lorincz et al., 1986a), HPV 52 (Shimoda et al., 1988) and canine oral papillomavirus (COPV) (W. D. Lancaster unpublished data) were released from vector sequences by cleavage with Eco RI. Bam HI was used to release HPV 4 (Heilman et al., 1980), the two subgenomic HPV 35 fragments (Lorincz et al., 1987b) and bovine papillomavirus type 7 (BPV 7) (R. Olson and W. D. Lancaster, unpublished data) from their vectors.

2. Fresh biopsies

Total cellular DNA from clinical samples was isolated as described previously (Lancaster et al., 1986) by lysing cells in 0.6% sodium dodecyl sulfate, 0.01 M EDTA with 100 μg/ml of proteinase K and incubated overnight at 37°C. Proteins

were removed by two extractions with phenol, followed by two chloroform-isoamyl alcohol (24:1, vol/vol) extractions. Nucleic acids were precipitated with ethanol. RNA was removed by treatment with RNAse, followed by proteinase K digestion and phenol and chloroform extractions as above. DNA was precipitated with ethanol, suspended in H_2O, and digested with Bam HI and Hind III.

3. Cervical scrapings

For series F, at the time of the visit, a sample for the Pap smear was taken and the cotton swab used for harvesting the cells was emulsified in ViraPap DNA hybridization collection medium (Digene, Silver Spring, MD, USA). This medium lyses the cells and releases the DNA into solution. Two hundred μl of the DNA solution was precipitated using 80 μl 7.5 M ammonium acetate and 400 μl of isopropanol. The mixture was left at room temperature for a minimum of 10 minutes followed by centrifugation at 4°C for 30 minutes. The supernatant was discarded and the pellet was dried overnight at room temperature before being resuspended in 60 to 100 μl of deionized, distilled H_2O (Gibco Laboratories, Grand Island, NY, USA). DNA samples were stored at -20°C until PCR analysis.

For series R, the samples were harvested using a cotton swab which was placed in a solution of phosphate buffered saline with 10 μg/ml of fungizone. The samples were stored up to 30 days at 4°C until DNA extraction. For processing, the samples were centrifuged at low speed for 5 minutes at room temperature,

the supernatant discarded and the cell pellet washed three times in 1.5 ml of TE [10 mM Tris-HCl (pH 7.5), 1 mM EDTA] before lysing the cells in 1x PCR buffer [67 mM Tris-HCl (pH 8.8), 35 mM KCl, 2.0 mM $MgCl_2$] containing 100 µg/ml proteinase K at 55°C for 1 hour. The temperature was increased to 95°C for 10 minutes to inactivate the proteinase K. Aliquots were stored at -20°C until PCR analysis (Kawasaki, 1990).

4. Paraffin sections

From each block, five 7 µ sections were collected and placed in a 1.5 ml microcentrifuge tube. A fresh disposable microtome blade was used for each specimen to eliminate block to block contamination. The sections were deparaffinized by extraction with xylene, followed by an ethanol wash to remove any trace of xylene (Wright and Manos, 1990). The tissues were air dried and then rehydrated in 1x PCR buffer containing 100 µg/ml of proteinase K. The samples were incubated at 37°C overnight or 55°C for one hour to release the cellular DNA before being heated to 95°C for 10 minutes to inactivate the proteinase K. Samples were stored at -20°C until tested by PCR.

5. Controls for PCR

a. Positive controls. Clones of HPV types 2, 4, 6, 11, 16, 18, 31, 33, 35, 37, 38, 45, 51 and 52 were submitted to PCR using HPV consensus primers. These reactions were performed to confirm the property of the primers as being genus specific. These experiments were performed prior to amplifying

any clinical samples.

HPV type 4 was selected as a positive control to assess reagent quality when testing clinical samples. This cutaneous virus type has never been demonstrated in any anogenital sample. In addition, if contamination of samples were to occur from the positive control, the source could be identified more readily.

b. Negative controls. A variety of negative controls were performed each time a series of clinical samples were tested. To verify that the reagents did not carry any contamination, a reaction mixture consisting of all reagents but without any DNA was submitted to PCR as well as hybridization. If the reagent controls were positive by hybridization, the reagents were contaminated and the results would be discarded and the series retested with fresh reagents. Reagent controls remain negative through out the study.

To ascertain whether the reagents used in the preparation of DNA for clinical samples were free of HPV DNAs as well as to verify if sample to sample contamination had occurred, several blank controls were included in the series. These blank controls consisted of empty tubes treated in parallel with samples using the same reagents and manipulations for DNA extraction. If blank controls were positive and reagent controls were negative, contamination occurred during handling of samples. The results would be disregarded and the DNA samples not reassayed since the contamination was within the clinical

samples. Blank controls remain negative through out the study.

The last control to be included was human cell line 293 (American Type Culture Collection, Rockville, MD, USA). This is an adenovirus transformed kidney cell line known to be free of HPV DNA. This control would determine if any contamination could have occurred during reaction set up.

D. Southern Blot Hybridization

1. Transfer

To detect HPV amplified products and to type when necessary, a 20 µl sample of the amplification mixture was electrophoresed at 4 volts/cm for 3 hours through a 2% agarose gel, in 1x TAE buffer with 0.5 µg/ml of ethidium bromide. The gel was photographed before the 30 minute DNA denaturation step (0.5 M NaOH, 1.5 M NaCl). The gel was neutralized for 30 minutes in 0.5 M Tris-HCl (pH 7.4), 1.5 M NaCl. The denatured DNAs were then transferred by capillarity using 20X SSC (0.3 M Na citrate and 3.0 M NaCl, pH 7.0) onto a pre-soaked nylon membrane (Amersham). After overnight transfer, the membranes were rinsed briefly in water, air dried and the DNAs cross-linked onto the membrane by exposure to ultra-violet irradiation on a transilluminator for 3 minutes.

2. Labelling of probe

DNAs used as probe were either full-length viral genomes or amplimers obtained after HPV PCR amplification. The

genomes were released from vector sequences by cleavage with the appropriate restriction endonucleases as described above. To isolate amplimers, PCR products were electrophoresed through 1% low melting point agarose gels (LMA). The expected size fragment was excised and labelled directly within the LMA. The labelling of all probes was done using alpha-^{32}P-dATP by the random primer technique using the Klenow fragment of DNA polymerase I (Feinberg and Vogelstein, 1983). Unincorporated deoxynucleotides were separated from the probe by chromatography through Sephadex G-50. Specific activities of about 10^9 counts/min/μg DNA were routinely achieved for DNAs labelled in the absence of LMA. Amplimers labelled in LMA were of lower specific activity (10^7 to 10^8 counts/min/μg DNA). All hybridization reactions used 5 x 10^5 counts/min of probe per ml of hybridization solution and 1 ml of hybridization solution/12 cm^2 of membrane.

3. Hybridization reaction

Filters were pre-hybridized for a minimum of 2 hours at 60^{o}C in 6X SSC containing 5% Denhardt's solution, 0.5% SDS with 0.5 mg/ml of RNA (Torula yeast, Sigma). All hybridization reactions were incubated at 60^{o}C overnight. Hybridizations were performed either at Tm-40^{o}C for a non-stringent reaction or Tm-10^{o}C for stringent condition. The concentration of salt needed to establish the stringency was calculated according to the following equation (Anderson and Young, 1985):

$$Tm = 81.5 + 16.6 \log(M) + 0.41 (G + C \%)$$

where Tm is the temperature at which 50% of the DNA denatures, M is the molar concentration of Na^+ and G+C is the guanine and cytosine content of the DNA probe.

The hybridization mixture contained SSC at a concentration to achieve the desired Na^+ concentration, 5% Denhardt's solution, 0.5% SDS, 0.5 mg/ml of RNA to prevent non specific binding of the probe, and an adequate volume of radioactively labelled probe to obtain 5×10^5 counts/min/ml.

After hybridization, free probe was removed by washing filters in 2X SSC at room temperature. Two additional washes of 30 minutes each were done at the appropriate temperature and concentration of Na^+ necessary to achieve either Tm-40°C, Tm-25°C or Tm-10°C. The hybridized membranes were wrapped in plastic to prevent drying and exposed to X-ray film. When necessary, the filters were stripped of probe by soaking in 0.4 M NaOH for 30 minutes at 45°C followed by washing in 0.1% SDS for 30 minutes at 45°C. To verify the membranes were completely free of labelled probe, they were exposed to X-ray film overnight at -70°C with intensifying screens before being used in the next hybridization.

4. Probes

Routinely, all hybridization reactions were first carried out under non-stringent conditions (Tm-40°C) against three different probe mixes: probe mix A consisted of HPV types

6 and 11, probe mix B contained HPV types 16 and 18 and probe mix C HPV types 31, 33, 35. This provided for detection of all HPVs since the conditions of hybridization allowed for cross-hybridization. The membranes were then washed at Tm-25°C and then Tm-10°C to eliminate cross-hybridization among different probes. The membranes were exposed to X-ray film after each wash. Samples showing a positive signal with probe mix A were not differentiated any further. Samples positive with probe mix B and/or C were then dot blotted and hybridized under stringent conditions against single probes HPV types 16, 18, 31, 33, and 35. When necessary, additional probes such as types 45 and 52 were also used.

5. Hybridization controls

HPV types 6, 11, 16, 18, 31, 33, 35 were used in all hybridization reactions against all probes to ascertain the specificity of the probe as well as to verify its cross-reactivity. The positive controls were either cloned HPV sequences or amplimers generated using consensus primers. The positive controls were electrophoresed through agarose gels using a gel apparatus different from the one used to test samples to eliminate possible well leakage of known HPV amplimers to test samples.

E. Contamination

A major problem with PCR is the potential for contamination. Due to its extreme sensitivity, in theory, the

introduction of a single target in any given sample can yield up to 10^6 molecules after 30 cycles thus giving a false positive result. Extreme precautions must be used to prevent this problem from occurring. The most common source of contamination is from previously amplified DNAs.

In an effort to prevent contamination, all reagents were prepared in an adjacent laboratory where no HPV sequences have been amplified and no manipulation of HPV-containing samples or plasmids has been performed. All materials used in PCR were disposable when possible. All disposable materials (0.5 ml Eppendorf tubes, pipet tips, Pasteur pipets) were autoclaved before PCR.

A set of pipets was dedicated for PCR use. A 200 µl pipettor was dedicated solely for manipulation of pre-amplification reagents, no DNAs (either cloned HPV or clinical samples) were pipetted with this pipet. A 20 µl pipettor was used for clinical samples and cloned HPV DNAs. A 0.5 to 10 µl Eppendorf pipet was used only to measure Taq DNA polymerase and enzymatic dilution.

1. Sodium hypochlorite

Fragments of DNA may persist after autoclaving and in some cases can provide target for two primers which will allow for amplification. The use of ethanol is absolutely not advised since it preserves DNA instead of destroying it. Therefore a solution of 1.5% sodium hypochlorite was used to oxidize and thus destroy any DNA that could contaminate nondisposable material.

Before preparing any solution for PCR or handling any clinical samples that would later be submitted to PCR or before setting up any PCR reactions, the bench top was wiped with a solution of 1.5% sodium hypochlorite. The day PCR reactions were set up, pipettors to be used with reagents and samples were first soaked in a solution of 1.5% sodium hypochlorite for a minimum of 15 minutes followed by a rinse in distilled water.

2. Reagents

a. Buffer. Tris buffers were made from reagents purchased for PCR use only. The reagents were stored in an adjacent laboratory. The day the buffer was prepared, aliquots of 800 μl were stored at -20°C. A sample of the buffer was assayed for efficiency of amplification and possible contamination.

b. Deoxynucleotide triphosphates. Deoxynucleotide triphosphates (dNTPs, Sigma) were stored sealed at -20°C. Working solutions at a concentration of 1.25 mM of each dNTP were prepared in deionized distilled H_2O and 500 μl aliquots stored at -20°C. An aliquot of the freshly prepared dNTP solution was assayed the day of the preparation to ensure its quality with respect to efficiency of amplification and possible contamination.

c. Primers. Primers were synthesized in the Molecular Biology Core facility of Wayne State University and received either dry or in solution in H_2O; they were stored at -

20°C. When necessary, the primers were solubilized in water. The concentration of the solution was estimated by absorbance at 260 nm (1 A_{260}=20 µg/ml of primer). The stock solution of primers were then aliquoted in small volumes to prevent contamination as well as to minimize freeze-thaw cycles. Primer solutions were never thawed more than five times. Primers were assayed on the day of preparation in the PCR reaction to evaluate their efficiency of amplification.

d. Water. To further eliminate any contamination from the water source in the same lab PCR was performed, deionized, distilled water was purchased from Gibco in 100 ml quantities. Approximatively 7 ml were distributed in disposable tubes and stored at 4°C. One tube was used only the day needed and the remainder discarded.

e. Paraffin oil. Paraffin oil (Sigma) was used to limit condensation in microcentrifuge tubes during thermal cycling. Volumes of 2 to 3 ml were distributed in disposable tubes and stored at 4°C. Four drops of oil (approximately 100 µl) were distributed on top of each reaction mixture and the remainder of the tube discarded.

3. Ultra-violet irradiation

Ultra-violet irradiation creates pyrimidine dimers in DNA generating a stop signal for DNA polymerase. To reduce any contaminating target sequences in PCR amplifications, microcentrifuge tubes containing PCR reagents (water, buffer,

dNTPs, primers) were irradiated on a transilluminator for 20 minutes prior to addition of samples DNA and Taq DNA polymerase (Sarkar and Sommer, 1990; Cimino et al., 1990). No change was observed in the efficiency of amplification with irradiated reagents compared to unirradiated reagents.

4. Negative controls

a. Cell line 293. Known negative controls were included each time a series of samples were submitted to PCR. DNA from cell line 293, human kidney cells immortalized with adenovirus (Graham, 1984), have been extracted using the method described for fresh biopsies. At least two reactions containing DNA from cell line 293 were included with a PCR series. These reactions were processed along with the clinical samples for electrophoresis and Southern blot hybridization.

b. Reagent control. In an attempt to ascertain the absence of any contamination in the reagents, at least two reaction mixtures where no DNA was added were submitted to PCR. These reactions were also submitted to gel electrophoresis as well as Southern blot hybridization.

c. Blank control. To determine whether the reagents used in the DNA extraction of the clinical samples may be contaminated, tubes without any sample were interspersed among test samples and were submitted to the same manipulations and the same reagents as the clinical samples. These samples were processed after PCR for gel electrophoresis and Southern blot

hybridization.

5. Taq DNA polymerase

DNA polymerase, isolated from Thermus aquaticus, is a thermostable enzyme with an optimal temperature of activity at 72°C which incorporates approximatively 2,000 nt per minute. However, at lower temperatures, polymerization occurs at a reduced rate (Gelfand, 1989).

F. Cloning of Potential New HPV Type

Amplification reactions using IU and IWDO on clinical samples gave rise to amplified products which could not be assigned to a given virus type. These sequences represent putative new HPV types. To further characterize these sequences, amplified product from one sample, termed R15, was cloned and sequenced.

Several researchers have reported difficulties in cloning PCR products (Crowe et al., 1991; Denney and Weissman, 1990; Jung et al., 1990; Sardelli, 1991). The cause of the problems has been speculated to be that: (i) Taq DNA polymerase does not complete strand synthesis leaving a 5′ overhang (Sardelli, 1991); (ii) Taq DNA polymerase amplified fragments may have a non-template 3′ overhang, usually a deoxyadenosine triphosphate (Denney and Weissman, 1990); (iii) Taq DNA polymerase remains bound to the 3′ end of the extended DNA strand, preventing any further enzymatic modifications (Crowe et al., 1991).

Because of these potential problems in cloning amplified fragments, several approaches were used to ensure success. The first approach was to repair potential overhangs with T4 DNA polymerase, ligation of Bam HI linkers to the repaired ends, followed by Bam HI digestion for cloning into the unique Bam HI site of the dephosphorylated vector pUC 19.

The second approach was to add the sequence GGTGGATCC, which contains the Bam HI restriction enzyme site, at the 5' end of the primers during oligonucleotide synthesis. The 5' overhang was repaired with the Klenow fragment of DNA polymerase followed by digestion with Bam HI and cloning into the unique Bam HI site of dephosphorylated pUC 19.

The last approach was to use the Klenow fragment of DNA polymerase to repair the fragment ends after amplification with primers containing a Bam HI restriction site. The blunt end products were cloned at the Sma I site in dephosphorylated pUC 19.

After transformation of E. coli, colonies with potential clones would be screened by hybridization, under stringent conditions, to probe R15. The colonies with a positive signal would be expanded and the resident plasmid characterized by Bam HI digestion and release of the 850 bp fragment.

1. Cloning fragments with added linkers.

a. End repair with T4 DNA polymerase. Twenty μl of the amplification mixture was precipitated using 1/10 volume of 3.0 M sodium acetate (pH 5.3) and two volumes of ethanol. The

DNA pellet was resuspended in 20 μl of water. Repair was done using 2.5 U of T4 DNA polymerase in the presence of 100 μM each of the dNTP and approximately 2 μg of amplified fragment. The reaction was carried out in 33 mM Tris-acetate (pH 7.9), 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol and 100 μg/ml BSA at 37°C for 30 minutes. DNA polymerase was inactivated by heating to 65°C for 10 minutes (Maniatis et al., 1982). The reaction was extracted with equal volumes of phenol and chloroform and precipitated using 1/2 volume of 7.5 M ammonium acetate (pH 7.5) and two volumes of isopropanol. The pellet was air dried and the DNA dissolved in 15 μl of water.

b. Ligation of the Bam HI linkers. One μg of phosphorylated linkers, d(pCGGATCCG) (New England Biolabs), were ligated to the repaired amplified fragments in 66 mM Tris-HCl (pH 7.6) 5 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM ATP and incubated overnight at 22°C.

c. Digestion of the Bam HI-tailed fragments. The Bam HI linker ligated amplified fragments were digested directly in the ligation mixture by adding concentrated Bam HI buffer to a final concentration of 1X and 15 U of Bam HI. The reaction was incubated at 37°C overnight before extraction with equal volumes of phenol and chloroform and precipitated using ammonium acetate isopropanol as described above. The precipitated air dried fragments were resuspended in 10 μl of water and 1 μl was electrophoresed on a 2% agarose gel in parallel with DNA of known concentration to estimate the concentration of the fragment.

d. Vector pUC 19. pUC 19 (2 μg) was digested to completion with Bam HI and dephosphorylated using 0.1 U of calf intestinal phosphatase (CIP, Amersham) in 50 mM Tris-HCl (pH 9.0), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1.0 mM spermidine and the reaction incubated at 37°C for 15 minutes. An additional 0.1 U of CIP was added and incubated an additional 15 minutes at 37°C. The enzyme was heat inactivated at 68°C for 15 minutes before extraction with phenol and chloroform. The vector was precipitated using ammonium acetate and isopropanol as above.

e. Ligation of fragments to vector. An insert:vector ratio of 5:1 at a final concentration of 0.28 pmole (150 ng) of insert to 0.056 pmole (89.6 ng) of vector were ligated in 20 μl of buffer consisting of 66 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM ATP and 1 U of T4 DNA ligase. The reaction was incubated at 16°C overnight.

2. Cloning fragments with Bam HI-tailed primers.

The ends of the amplification products using consensus primers modified at their 5′ ends with a Bam HI restriction site were repaired using the Klenow fragment of DNA polymerase. The reaction was carried out in a volume of 25 μl in 50 mM Tris-HCl(pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol and 50 μg/ml of BSA in the presence of 2 μM of each dNTP, 6 U of DNA polymerase and 2 μg of DNA (Maniatis et al., 1982). The reaction was incubated at 30°C for 30 minutes followed by phenol and chloroform extraction. The repaired, amplified fragments were

precipitated using sodium acetate and ethanol as described above, resuspended in water and digested with 15 U of Bam HI in the appropriate buffer in a volume of 25 μl overnight at 37^{o}C, followed by phenol and chloroform extraction and precipitation using ammonium acetate and isopropanol. The digested fragments were resuspended in 10 μl of water and 1 μl was electrophoresed in a 2% agarose gel along with DNA of known concentration to evaluate fragment concentration. Fragments were cloned into Bam HI-digested, dephosphorylated pUC 19 as described above.

3. Cloning fragments with blunt ends.

a. End repair with Klenow DNA polymerase. In an attempt to clone the fragment with blunt ends, the ends were repaired using the 3′ to 5′ exonuclease activity of the Klenow fragment of DNA polymerase. Repair was done in the amplification mixture after adjusting the Mg^{2+} concentration from 2 mM to 5 mM. Prior to addition of the Klenow fragment, the amplification mixture was heated at 99^{o}C for 10 minutes to destroy Taq DNA polymerase. The reaction mixture was brought to room temperature over a period of 1 minute. Two units of the Klenow fragment were added as well as 0.8 μl of gamma-^{32}P-ATP and 10 U of T4 DNA ligase to phosphorylate the amplified fragments. The reaction was incubated at 25^{o}C for 30 minutes and the temperature increased to 37^{o}C for 10 minutes. The reaction was terminated by heating to 65^{o}C for 10 minutes.

b. Purification of repaired fragments. To purify the fragment to be cloned, oil overlaying the amplification mixture was removed by extraction with chloroform:isoamyl alcohol (24:1 vol/vol), followed by one phenol:chloroform (1:1) extraction. The amplification mixture was run in a 1% LMA gel in 1X TAE buffer, at 4 volts/cm to separate the nucleotides and primers from the 850 bp fragment. The 850 bp band was cut from the agarose and purified using syneresis. Briefly, a 80 mm^3 piece of gel carrying the amplified fragment was placed in 0.5 ml microcentrifuge tube. A hole was punctured at the bottom of the tube which in turn was inserted into a 1.5 ml microcentrifuge tube and the content of the gel recovered by centrifuging at high speed for 5 minutes. One hundred μl of 0.3 M sodium acetate was added to the DNA/agarose mixture and the contents transferred to a 0.5 ml microcentrifuge tube containing glass wool. The tube was frozen at -70°C for one hour. A hole was punctured at the bottom of the tube and the contents collected into a 1.5 ml microcentrifuge tube by centrifuging in a microcentrifuge. The recovered solution was extracted three times with isobutanol saturated with water. Two volumes of ethanol was added to the recovered solution to precipitate the DNA. The resulting pellet was air dried and resuspended in water. The concentration of DNA was evaluated by gel electrophoresis as described above.

c. Vector pUC 19. pUC 19 was digested to completion with Sma I. An aliquot was saved, to be used as a positive control for ligation. The remainder of the reaction was

dephosphorylated using CIP as described above. The phosphorylated and dephosphorylated Sma I-digested pUC 19 samples, were electrophoresed in a 0.8% agarose gel and purified using a commercially available kit (Prep-A-Gene, BioRad) per the vendor's instructions. After purification, small aliquots were electrophoresed on agarose gels to evaluate the concentration.

d. Ligation of fragment to vector. A ratio of 8:1 (fragment:vector) at a concentration of 0.55 pmole (280 ng) of insert to 0.06 pmole (98 ng) of vector were ligated in 20 μl of buffer consisting of 40 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT and 1 mM ATP. After the addition of 1.5 U of T4 DNA ligase, the reaction was incubated at 22°C overnight. For the positive ligation control, 1 μg of Sma I-digested pUC 19 was self ligated. For the negative ligation control, 1 μg of deposhorylated Sma I-digested pUC 19 was self ligated. Also, as an additional control to determine if the ligation reaction contained inhibitors, 1 μg of Sma I-digested pUC 19 was ligated in the presence of 1 μg of Sma I-digested and dephosphorylated pUC 19.

4. Transformation

Competent E. coli cells were prepared using the $CaCl_2$ procedure as described by Maniatis et al. (1982). Briefly, E. coli DH 5 alpha were grown in 100 ml of L broth to a density of approximatively 5 x 10^7 cells/ml. The culture was chilled on ice for 10 minutes and the cells pelleted at 4000 x g for 5 minutes at 4°C. The bacteria were gently resuspended in 50 ml of

ice-cold, freshly prepared, filter sterilized 50 mM $CaCl_2$. The cells were incubated in an ice-bath for 15 minutes before being pelleted at 4000 x g for 5 minutes at 4°C. The supernatant was removed and the cells resuspended by gentle pipetting in 6.6 ml of 50 mM $CaCl_2$ and 10 mM Tris-HCl (pH 8.0). The resuspended cells were stored for 24 hours at 4°C prior to use.

Transformation of competent E. coli DH 5 alpha was done as described elsewhere (Maniatis et al., 1982). Approximately 15 ng of ligated DNA was added to 200 µl of competent cells. The cells were heat shocked at 42°C for 2 minutes, 1 ml of L broth added and incubated at 37°C for 1 hour. One hundred µl and 30 µl were then spread on L agarose plates containing 50 µg/ml of ampicillin. After overnight incubation at 37°C, the colonies were screened for the presence of the inserts by colony hybridization under stringent conditions using R15 amplifed fragment as a probe. The colonies were lifted from the plate using nylon membranes (Amersham). The filters, colony side up, were placed on a sheet of Whatman 3 MM filter paper saturated with 0.5 M NaOH, 1.5 M NaCl for 5 minutes to lyse the cells and denature the DNA. The filters, colony side up, were then transferred to a second sheet of filter paper saturated with 1.5 M NaCl, 0.5 M Tris-HCl (pH 8.0). The filters were in contact with the neutralizing solution for 5 minutes before being air dried. The DNA was fixed by ultra-violet irradiation for 3 minutes. The membranes were hybridized under stringent conditions (Tm-10°C) to labelled R15 amplimer. Colonies showing a positive signal on the autoradiograph were grown in a small

amount of L broth and the resident plasmid characterized by digestion with Bam HI to release the amplified fragment from the vector.

5. DNA Sequencing

Inserts were sequenced using a commercially available T7 DNA polymerase sequencing kit (Pharmacia) according to the manufacturer's recommendations.

a. Template preparation. Plasmids containing the R15 insert were purified by cesium chloride, ethidium bromide density gradient centrifugation. The supercoiled fraction was harvested, extracted with water-saturated butanol to remove ethidium bromide and dialyzed overnight against 1X TE buffer. Two μg of plasmid DNA, in a volume of 8 μl, was denatured using 2 μl of 2 M NaOH. After 10 minutes incubation at room temperature, 3 μl of 3 M sodium acetate (pH 4.8) and 7 μl of water was added along with 60 μl of 100% ethanol. The reaction was stored at -70 $^{35V}{}^{o}$C for 30 minutes before pelleting the DNA by centrifugation. The DNA pellet was washed twice with 70% ethanol, air dried and resuspended in 10 μl of water.

b. Annealing reaction. To 10 μl of denatured DNA, 2 μl of annealing buffer and 2 μl of sequencing buffer were added. The solution was brought to 95^{o}C for 5 minutes followed by an overnight decrease of temperature to room temperature to allow the primer to anneal to template.

c. Sequencing reaction. To the annealed template and primer, 6 μl of a mixture of alpha-^{35}S-dATP (10 μCi) and T7 DNA polymerase (3 U) was added. The labelling reaction was incubated at room temperature for 5 minutes and 4.5 μl were distributed to 2.5 μl of pre-warmed sequencing mixes containing either ddATP, ddGTP, ddCTG or ddTTP. These reactions were incubated at 37°C for 5 minutes. Five μl of a stop solution containing formamide, EDTA, xylene cyanol and bromophenol blue was added.

d. Electrophoresis and autoradiography. After heating the terminated sequencing mixtures to 75-80°C, 2.5 μl of each mixture was electrophoresed through a sequencing gel (8% polyacrylamide, 0.28% methylene bis-acrylamide, 28% urea in 90 mM Tris-HCl, 90 mM boric acid, 2 mM EDTA) at 30 mA, 40 W for 2.5 hours. The gel was first soaked for 15 minutes in an aqueous solution of 10% acetic acid and 10% methanol, transferred on Whatman 3MM filter paper to dry before autoradiography.

IV. RESULTS

A. HPV Amplification

1. Sequenced HPVs

To test whether the consensus primers IU and IWDO were capable of amplification of a broad range of HPVs using PCR, known genital HPV type DNAs were submitted to 30 cycles of amplification. A cycle consisted of a denaturation step at 94°C for 2 minutes, annealing at 37°C followed by a first extension at 55°C for 1 minute and a second extension period of 3 minutes at 72°C. Under these conditions of amplification, the consensus primers generated the expected 850 bp fragment for HPV types 6, 11, 16, 18 and 33 although HPV 18 consistently gave an additional band at 550 bp (Figure 2).

To determine the origin of the additional band seen when HPV 18 was amplified with IU and IWDO, PCR was run with only one of the two primers for the HPV types mentioned above. When IWDO was used as the primer, HPV types 6 and 18 DNAs showed fragments of about 850 and 550 bp, respectively. No amplification was observed with HPV types 11, 16 or 33. When IU was used as the single primer, a very faint band at about 850 bp was detected only for HPV type 33 (Figure 3). The extra fragments for HPV types 6 and 33 observed after single-primer amplification were not detected in dual-primer reactions because of comigration with the expected 850 bp fragment.

A computer search of the viral sequences revealed

potential alternative annealing sites with a minimum of 69% homology with IWDO only for HPV types 6 and 18. This degree of mismatch would be tolerated under the conditions of primer annealing at 37°C.

The expected target site for IWDO on HPV 18 DNA is at position 2012 on the coding (positive) strand. A potential alternative binding site for IWDO in the correct orientation for amplification was detected on the negative strand 545 nucleotides upstream of the target site. Other annealing sites were also identified, but only one additional set was properly oriented to permit amplification. One site was at position 3783 on the positive strand, and the other site was 575 bp nucleotides downstream on the negative strand. Annealing of IWDO to the alternative binding sites would generate the size fragment seen in Figure 3.

For HPV 6, only one set of additional alternative annealing sites was identified. One site was located at position 3006 on the positive strand, and the other was located 859 bp downstream on the negative strand. These alternative binding sites are likely to be responsible for the 850 bp fragment seen in Figure 3.

For HPV 33, the target site for IU is at position 1122 on the coding strand; however, no alternative binding site in the correct orientation that would yield a fragment of about 850 bp was detected on the noncoding strand. A set of alternative binding sites was located, however, at position 976 on the positive strand and 845 bp downstream in the correct

FIGURE 2. Amplification of HPV DNAs with E1 consensus primers. Denatured HPV DNAs were annealed to IU and IWDO at 37°C, followed by 25 cycles of PCR. Bands of the expected size (850 bp) were readily detected. An additional band was observed with HPV-18 (arrow). HindIII-digested lambda DNA was used for size markers. Lane C contained amplified lambda DNA (500 bp).

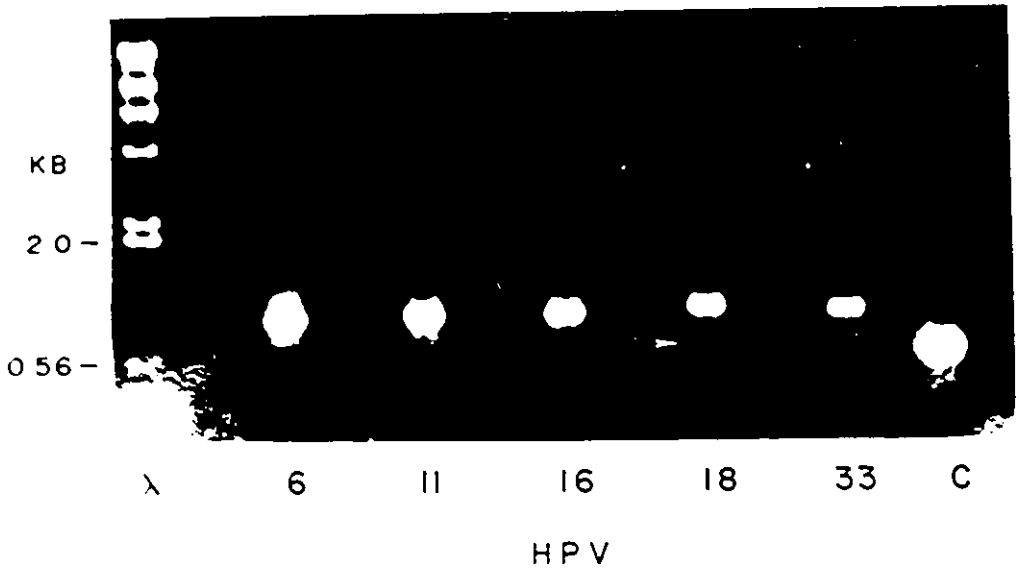
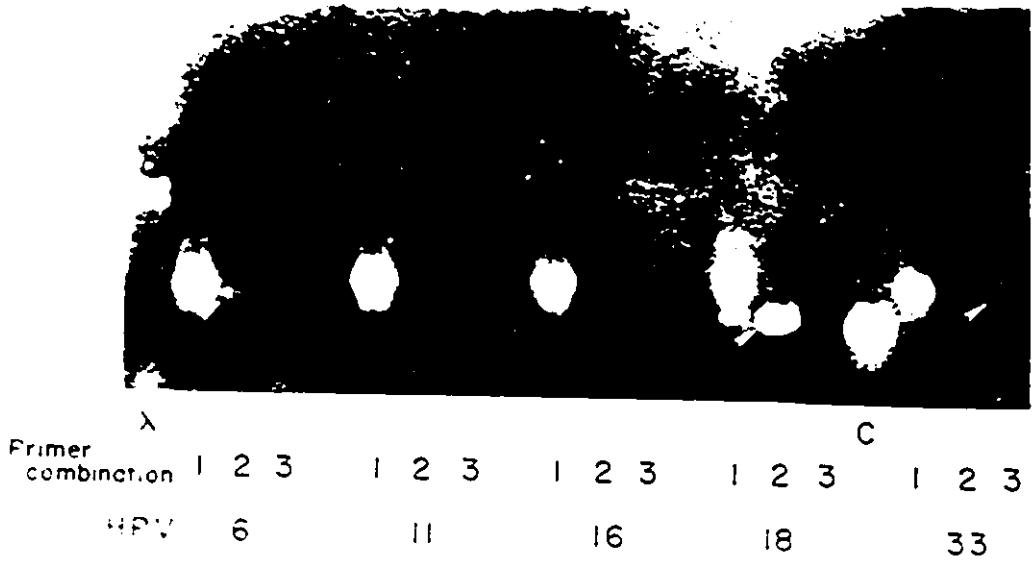


FIGURE 3. Amplification of HPV DNAs using different consensus primer combinations. Denatured HPV DNAs were annealed to both IU and IWDO (primer combination 1), IWDO alone (primer combination 2) and IU alone (primer combination 3) at 37°C, followed by 25 cycles of PCR. Single primer combinations for HPV 6, 18 and 33 showed amplification (arrows). HindIII-digested lambda DNA was used for size markers. Lane C contained amplified lambda DNA (500 bp).



orientation on the negative strand. This again could account for the fragment seen in Figure 3.

To eliminate the alternative primer-binding sites, the temperature of annealing was increased. PCR was repeated for HPV types 6, 18 and 33 with only one primer, and the annealing temperature was increased from 37°C to 46°C. HPV type 6 and 33 did not show amplification at this temperature, but a 550 bp band was still present for HPV type 18.

When the temperature of annealing was increased from 46°C to 52°C, HPV type 18 failed to amplify with a single primer. At an annealing temperature of 52°C, no extension time was included but rather a slow increase in temperature from 52°C to 72°C over a period of 90 seconds completed the cycle. By increasing the temperature to 52°C the annealing of either IU or IWDO to secondary sites was prevented as demonstrated by the disappearance of these additional bands in HPV types 6, 18 and 33 (Figure 4), or the annealing was drastically reduced. Amplification with the primers was successful even though annealing was carried out at a temperature 4°C higher than the lowest Td for IU calculated with the use of the formula:

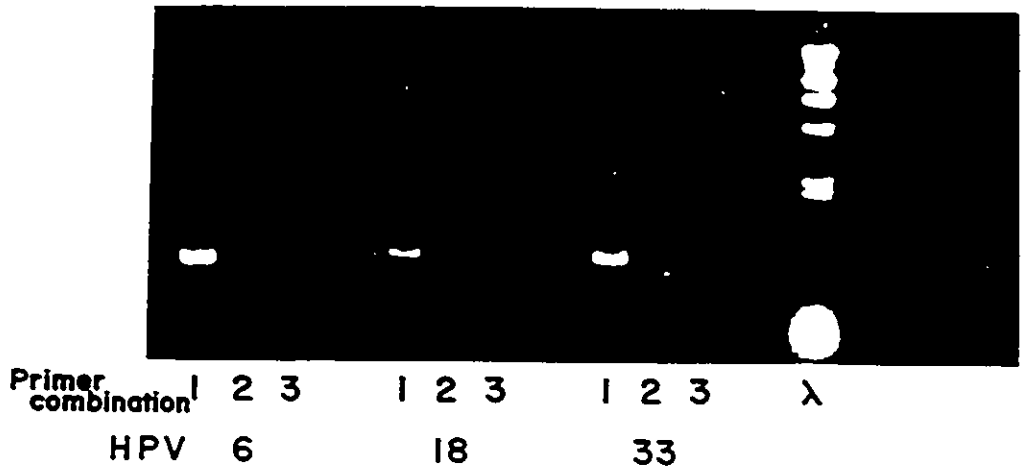
$$Td = 4\ (G+C) + 2\ (A+T)$$

(Mason and Williams, 1985) where an inosine residue was calculated as an A or T.

2. Unsequenced HPVs and animal papillomaviruses

To evaluate the utility of the primers as HPV universal primers, amplifications were attempted on a variety of

FIGURE 4. Effect of temperature of annealing on amplification of HPV DNA with E1 consensus primer combinations. Denatured HPV DNAs were annealed to both IU and IWDO (primer combination 1), IWDO alone (primer combination 2), or IU (primer combination 3) at 52°C, followed by 25 cycles of PCR. HindIII-digested lambda DNA was used for size markers.



human and animal papillomavirus DNAs whose sequences were not available at the time of analysis. At a temperature of annealing of 46°C, amplification of genital HPVs (types 31, 35, 45, 51, 52) as well as cutaneous HPVs (types 2, 4, 37, 38) produced fragments about 850 bp in length (Figure 5). Also, at an annealing temperature of 46°C, canine oral PV (COPV) gave an amplification product at 850 bp where the amplified fragment from bovine PV (BPV) type 1 was larger in size and absent for BPV type 7 (Figure 6).

3. Origin of the amplified products

To confirm that the amplified product originates from the E1 ORF, cloned HPV type 6, 11, 16, 18 and 33 DNAs were digested to completion with Pst I followed by gel electrophoresis and transfer onto nylon membranes. The 850 bp amplified fragments of each HPV was labeled and used as a probe against its respective viral template. Hybridization reactions were carried out under standard conditions of stringency (Tm-25°C). Only the restriction fragment of the viral genome containing the target sites for the consensus primers hybridized to the PCR product (Figure 7).

The amplified fragments were digested with restriction endonucleases Bam HI, Acc I and Hae III to confirm the E1 ORF origin of the products (Figure 8A). In addition, HPV types 6, 16, and 33 were digested with Alu I and HPV 33 with Hpa I. All reactions gave the expected bands with the exception of HPV type 33 which failed to cleave with Hae II. However, Acc I,

FIGURE 5. Amplification of unsequenced HPV DNAs using consensus primers IU-IWDO. Cloned HPV DNAs were denaturated and annealing at a temperature of 46°C to the genus-specific IU-IWDO primers, followed by 25 cycles of PCR. All HPV DNAs gave the expected size fragments of approximately 850 bp. Lanes 1 and 2 represent HPV types 6 and 11, lanes 4 and 5 are HPV types 16 and 18, lanes 7, 8 and 9 are HPV types 31, 33, and 35, lanes 11 and 12 are HPV types 37 and 38, lanes 14, 15, and 16 show HPV types 45, 51, and 52. Lane 18 contains phiX 174 RF Hae III-digested DNA as size markers.

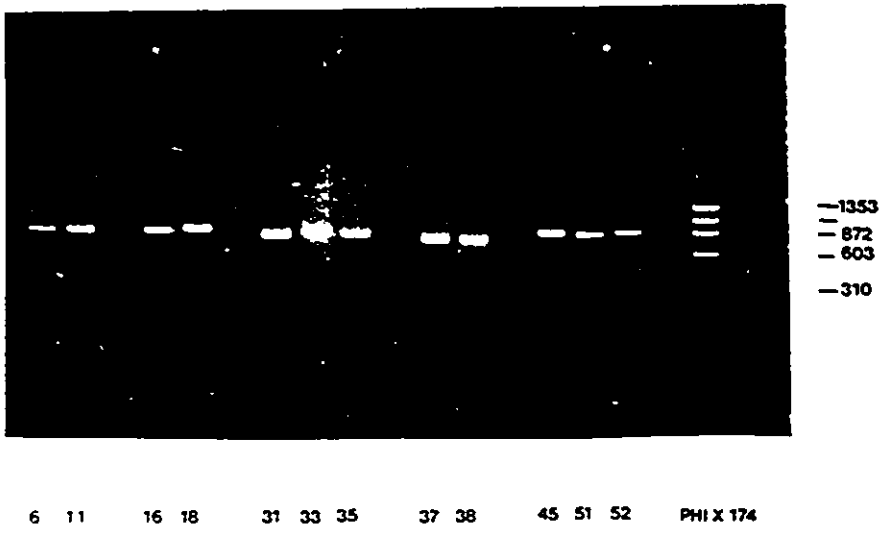


FIGURE 6. Amplification of different papillomavirus DNAs using consensus primers IU-IWDO. DNAs from HPV types 2, 4, 31, 35 and 52, BPV type 7 and COPV were denatured and annealed to IU and IWDO at a temperature of 46°C, followed by 25 cycles of PCR. All DNAs were successfully amplified with the exception of BPV type 7. HPV type 2 produced a weak band (arrow). Two different preparations of HPV types 31 and 52 were used (A and B). HPV type 35 S and L (L contains E1 ORF) represent the 3.75 and 4.1 kb fragment respectively (Lorincz et al., 1989). HindIII-digested lambda DNA was used for size markers.

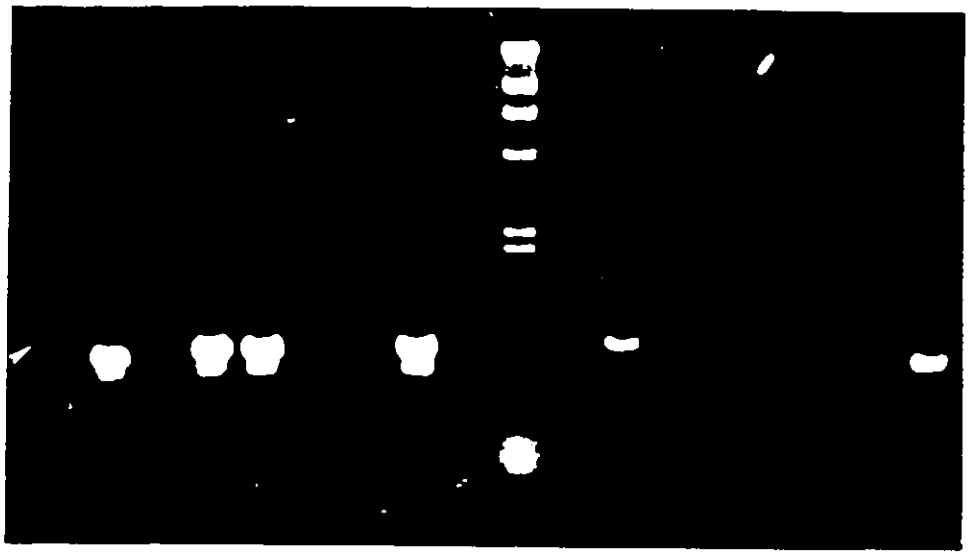
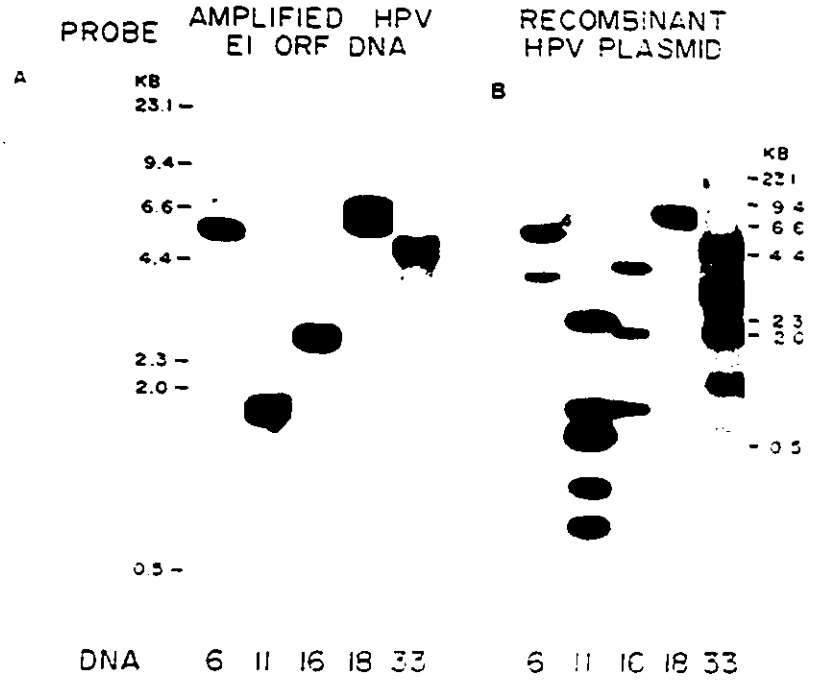


FIGURE 7. Specificity of amplified E1 ORF HPV DNA with universal primers IU-IWDO. Denatured HPV DNAs were annealed to IU and IWDO at 52°C, followed by 25 cycles of PCR. Amplified fragments were excised from the gel, labeled and hybridized to recombinant HPV DNAs digested with PstI. (B) Digested recombinant HPV plasmid DNA probed with full-length HPV DNA to indicate the location of the restriction fragments and represents the positive control. (A) Digested recombinant HPV DNA probed with amplified HPV DNA fragments. Each amplified DNA hybridized to the restriction fragment carrying the E1 ORF of the respective virus type. HindIII-digested lambda was used for markers. The marker in panel A is for the gel in which HPV types 6, 11, 16 and 18 were run, whereas the marker in panel B is for HPV type 33, which was run in a different gel.



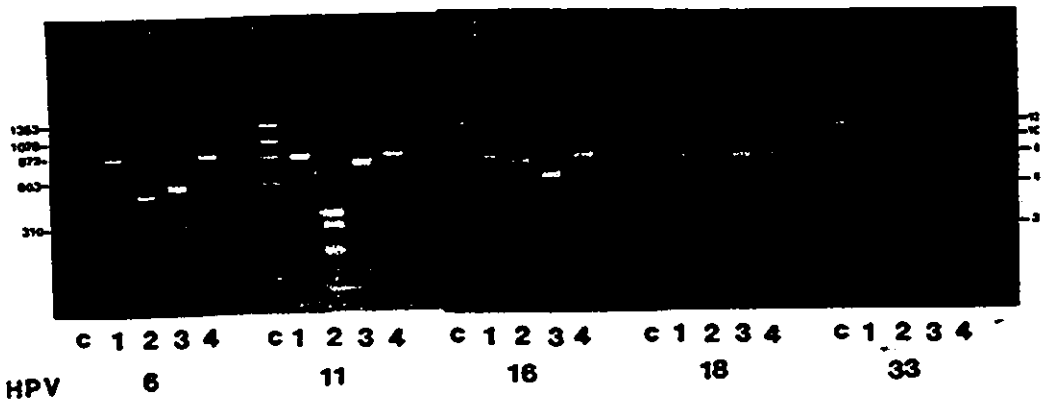
Alu I and Hpa I yielded the expected bands suggesting that misincorporation of a nucleotide inactivated the Hae II site (Figure 8B).

4. Sensitivity of amplification

To determine the lower limit for target concentrations, amplification was done at an annealing temperature of 52°C which removed aberrant bands from all HPV DNAs submitted to PCR. This was compared to an annealing temperature of 46°C in which the only viral DNA showing an extra band was HPV 18. Amplification reactions containing serial dilutions of HPV type 6 and 16 templates indicated a difference in the amount of amplified product between 46°C and 52°C annealing temperatures of about 2 orders of magnitude at limiting amounts of template (Figure 9). On ethidium bromide-stained gels amplified product resulting from an annealing temperature of 46°C was readily detected when 0.01 pg (2×10^3 molecules) of target was used. However, 1.0 pg (2×10^5 molecules) of target was required to generate a detectable fragment at an annealing temperature of 52°C. Southern blot analysis of these gels increased the sensitivity of detection by approximately 2 orders of magnitude over that of ethidium bromide staining. A positive hybridization signal was detected for the products of amplification performed at an annealing temperature of 46°C from an initial concentration of as little as 0.001 fg (1 to 10 molecules) of HPV 6 target DNA as compared to 0.01 pg (2×10^3 molecules) at an annealing temperature of 52°C.

FIGURE 8. Restriction endonuclease cleavages of the IU-IWDO amplified products. (A) Amplified products were digested with Bam HI, Acc I and Hae III for HPV types 6, 11, 16, 18, and 33. Lane 1 is uncut amplified products, lanes 2, 3 and 4 represent AccI, HaeII and BamHI digested fragments respectively. No BamHI sites are present on any of the amplified fragments. AccI-digested DNA gave the expected size bands for all the amplified products. HaeII gave the expected bands for HPV 6, 11, 16, no HaeII site are present on HPV 18. HaeII site appears to be inactive in HPV 33 since the amplified fragment did not yield the expected bands. Lane C is phiX 174 RF Hae III-digested DNA as size marker. (B) HPV types 6, 16, and 33 amplified fragments were digested with AluI. Lane 1 is uncut amplified products, lane 2 represents AluI-digested fragments with bands at the expected size. Lane 3 shows HpaI-digested HPV 33 with expected size bands. Lane C is phiX 174 RF Hae III-digested DNA as size marker.

A



B

FIGURE 9. Effect of temperature on the efficiency of amplification of HPV DNA with universal primers IU-IWDO. HPV 6 DNA was denatured and allowed to anneal to the universal primers at 46°C or 52°C, followed by 40 cycles of PCR.

B. Reaction optimization

1. Buffer composition

The buffer for the polymerase chain reaction as described by Saiki et al. (1985) was designed for use with the Klenow fragment of E. coli DNA polymerase I. Although the thermostable DNA polymerase (Taq) obtained from the thermophilic bacteria Thermus aquaticus is now used in PCR, the buffer designed for Klenow polymerase [10 mM Tris-HCl (pH 8.3)] is still widely used. However, a second buffer that uses 67 mM Tris-HCl (pH 8.8) has been designed that is closer to the pH optimum of Taq polymerase. The concentration of KCl is decreased in this buffer from 50 mM to 31 mM. The optimal Mg^{2+} concentration must be determined for each primer set. This buffer formulation also contains bovine serum albumin (BSA). This protein stabilizes Taq polymerase and is known to nonspecifically bind some drugs and proteins. This property of BSA has been used in an attempt to remove inhibitors of Taq DNA polymerase present in some clinical samples.

2. Magnesium ion concentration

The concentration of magnesium ion has been reported to be very critical for efficient amplification and appears to vary as a function of the primer set (Saiki et al., 1989). To determine the optimal Mg^{2+} concentration for the HPV consensus primers in the 10 mM Tris-HCl (pH 8.3 buffer), the Mg^{2+} concentration was varied from 0.5 to 10.0 mM keeping the

concentration of the HPV DNA template constant at 1 ng and primers at 1 μM each. For the concentration range from 1.5 to 10 mM of Mg^{2+}, no differences in the amount of amplified product were observed on ethidium bromide-stained gels (Figure 10A). However, faint additional bands were present at Mg^{2+} concentrations of 4.0 mM and higher.

Concentrations of Mg^{2+}, from 0.5 to 3.0 mM, were also performed in 67 mM Tris-HCl (pH 8.8) buffer. The yield of amplified products appeared to be comparable to the lower Tris molarity and pH buffer as seen on ethidium bromide-stained gels (Figure 10B). Thus, no major differences were noted when the two buffers [10 mM Tris-HCl (pH 8.3) versus 67 mM Tris-HCl (pH 8.8)] were compared in terms of yield of amplified products.

Since DNA can bind Mg^{2+}, different concentrations of the ion were assayed in PCR in the presence of 1 μg of human cellular DNA originating from cell line 293. The concentration of Mg^{2+} varied from 0.5 to 3.0 mM in 67 mM Tris-HCl (pH 8.8) (Figure 10C). The lowest concentration of Mg^{2+} needed to obtain amplification was 1.0 mM. At a concentration of magnesium ion greater than 1.5 mM, the yield of amplified product appeared similar to that obtained at 1 mM.

Even though the two formulations of Tris buffer appeared to yield similar amounts of amplified product, buffer with 67 mM Tris-HCl (pH 8.8), 31 mM KCl, 2.0 mM $MgCl_2$ and 2 μg/ml of BSA was used in further experiments because of the potential benefits of BSA and the pH optimum of Taq polymerase.

FIGURE 10A. Optimization of magnesium ion concentration in 10 mM Tris-HCl buffer. The concentration of Mg^{2+} was varied from 0 to 10 mM in 10 mM Tris-HCl buffer (pH 8.3) in the presence of 1 ng of HPV 16 DNA. Twenty five cycles of amplification were carried out using 1 μM of each of the consensus primers IU-IWDO. Lane 9 contains 4 mM Mg^{2+} decreasing in concentration by 0.5 mM increments to lane 1 which contains no Mg^{2+}. Lanes 10, 11, and 12 represent Mg^{2+} concentrations at 6, 8 and 10 mM, respectively. Lane C is phiX 174 RF Hae III-digested DNA as size marker.

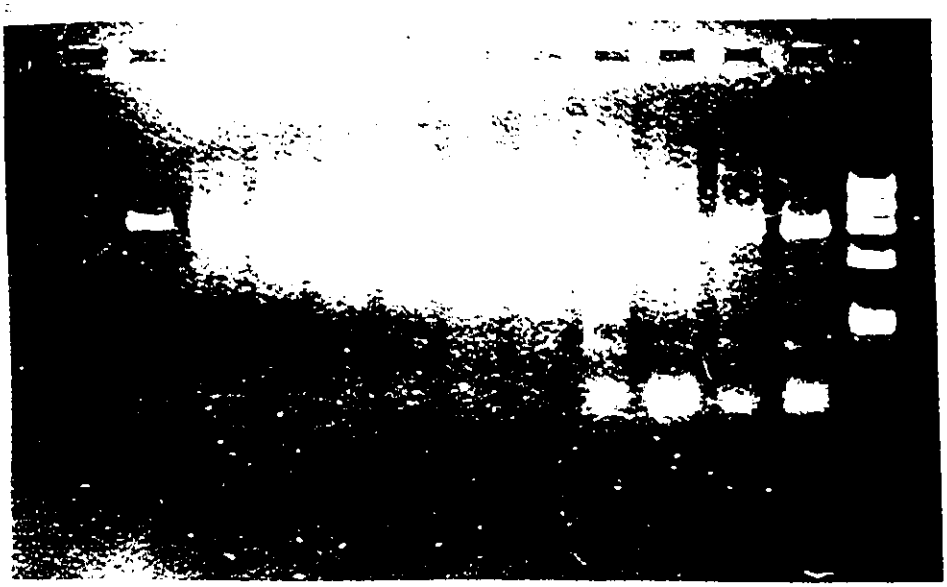


FIGURE 10B. Optimization of magnesiun ion concentration in 67 mM Tris-HCl buffer. The concentration of Mg^{2+} was varied from 0 to 3.0 mM in 67 mM Tris-HCl buffer (pH 8.8) in the presence of 1 ng of HPV 16 DNA. Twenty five cycles of amplification were carried out using 1 μM of each of the consensus primers IU-IWDO. Lane 2 represents Mg^{2+} at a concentration of 0.5 mM. Each following lane was increased in 0.5 mM increments to reach a concentration of 3.0 mM Mg^{2+} in lane 7. Lane C is phiX 174 RF Hae III-digested DNA as size marker.

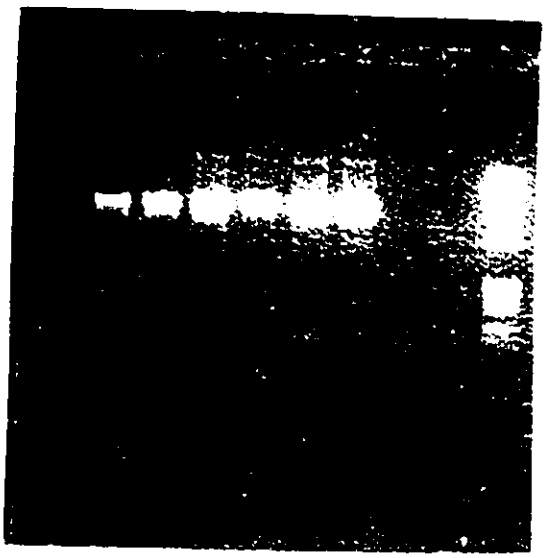
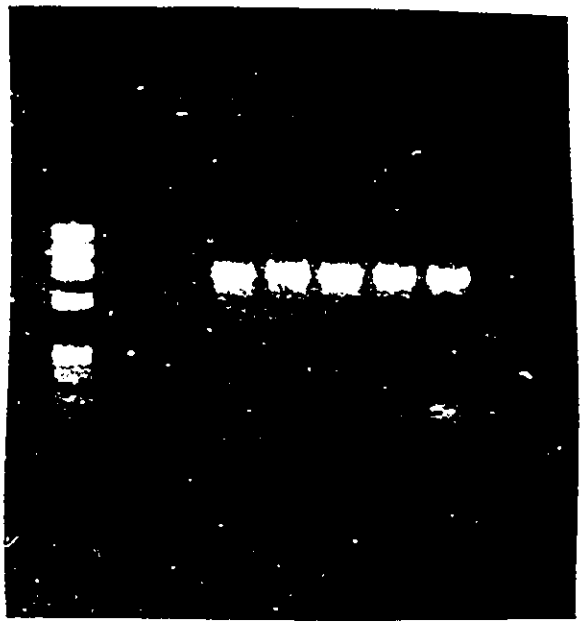


FIGURE 10C. Optimization of magnesium ion concentration in the presence of 1 μg cellular DNA. The concentration of Mg^{2+} was varied from 0 to 3.0 mM in 67 mM Tris-HCl buffer (pH 8.8) in the presence of 1 ng of HPV 16 DNA and 1 μg of cellular DNA extracted from cell line 293. Twenty five cycles of amplification were carried out using 1 μM of each of the consensus primers IU-IWDO. Lane 2 represents Mg^{2+} at a concentration of 0.5 mM. Each following lane was increased in 0.5 mM increments to reach a concentration of 3.0 mM Mg^{2+} in lane 7. Lane 8 contains 1 μg of cell line 293 DNA without any HPV DNA. Lane C is phiX 174 RF Hae III-digested DNA as size marker.



3. Concentration of cellular DNA

To evaluate whether the concentration of cellular DNA had any effect on amplification of HPV DNA, different concentrations of cellular DNA originating from cell line 293 were used in amplification reactions of HPV DNA using consensus primers. The reactions contained DNA from cell line 293 at concentrations ranging from 1 μg to 20 μg with 1 ng of HPV 16 DNA (Figure 11). A decrease in the efficiency of amplification on ethidium bromide-stained gels was observed when the concentration of cellular DNA was 10 μg. At concentrations greater than 10 μg, the amplification product could not be visualized.

4. Concentration of primer

To evaluate if primer concentration would alter the yield of amplified product, PCR was performed varying the concentration of primers from 0.2 to 1 μM; Figure 12 shows no difference in the efficiency of the reaction within the concentration range tested. Since a primer concentration of 1 μM was as efficient as lower concentrations, the 1 μM concentration was used throughout.

5. Concentration of enzyme

It has previously been reported that large amounts of enzyme (>4 U per 100 μl of reaction) yield aberrant products, probably from nonspecific binding of the polymerase to DNA (Saiki et al., 1989). To determine the optimal enzyme concentration for

FIGURE 11. Effect of the concentration of cellular DNA on the efficiency of amplification of HPV DNA with universal primers IU-IWDO. In the presence of 1 ng of HPV 16 DNA, cellular DNA extracted from cell line 293 was assayed at concentration of 1 μg (lane 3), 10 μg (lane 5), 20 μg (lane 7) and 40 μg (lane 9) by PCR using consensus primers IU-IWDO. Lanes 4, 6, 8, 10 represent the same concentration of cellular DNA, respectively, amplified in the absence of HPV 16. Lane 4 shows a band slightly larger than 850 bp, the origin of this band cannot be explained and was negative in Southern blot hybridization. Lane C is phiX 174 RF Hae III-digested DNA as size markers. Lanes 1 and 2 represent HPV types 16 and 18, respectively.

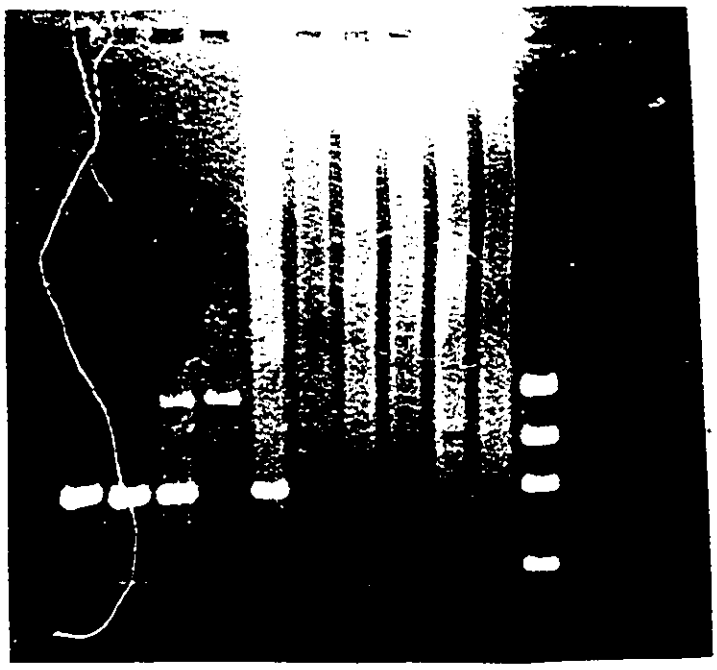
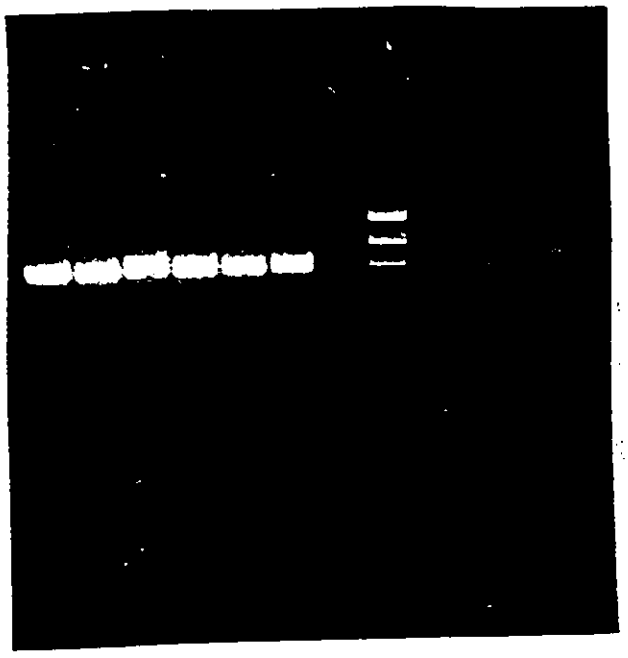


FIGURE 12. Effect of the concentration of primers IU and IWDO in the efficiency of amplification. The concentration of primers was varied from 0.1 μM to 1 μM in 67 mM Tris-HCl buffer (pH 8.8), 2.0 mM $MgCl_2$, 2.5 U of Taq DNA polymerase, 1 ng of HPV type 16 DNA. Lanes 1 to 6 contained each primer at 1 μM, 0.8 μM, 0.6 μM, 0.4 μM, 0.2 μM and 0.1 μM, respectively. All primer concentrations assayed yielded similar amounts of amplified product detected by ethidium bromide staining. Lane C is phiX 174 RF Hae III-digested DNA as size marker.

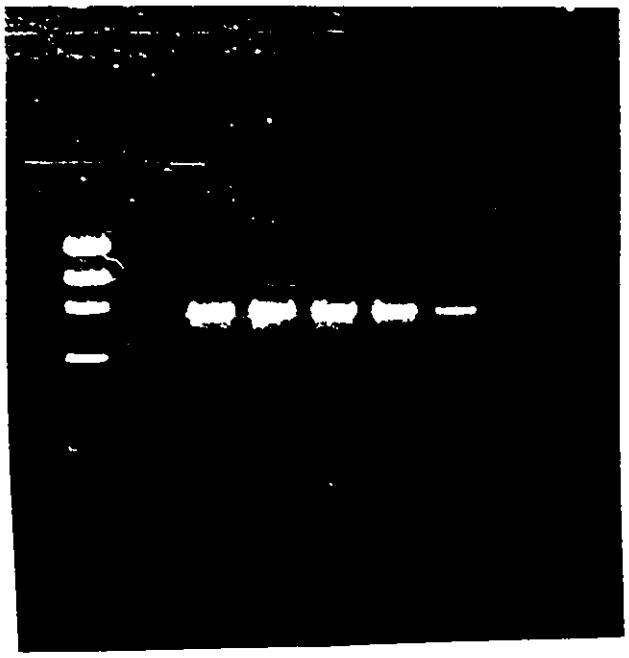


1 2 3 4 5 6 C

use with the consensus primers, the amount of Taq DNA polymerase was varied from 0.5 to 2.5 U in the reaction. Figure 13 shows that at low concentrations of enzyme, the yield of amplified product was lower than reactions using 2 to 2.5 U of enzyme. Therefore, the concentration of enzyme used in the remainder of the studies was 2 U per reaction for 30 cycles.

To summarize, optimal PCR using the HPV consensus primers consists of 30 cycles: where one cycle has a denaturation step at 94°C for 2 minutes, one step of annealing for a 2 minute period at 46°C followed by a slow increase of temperature to 55°C over a period of 1 minute with a polymerization period of one minute at 55°C. The reactions contain, in a volume of 100 μl, 67 mM Tris-HCl (pH 8.8), 2.0 mM $MgCl_2$, 31 mM KCl, 2.0 μg/ml of BSA, deoxynucleotides at 200 μM each, primers at 1 μM each, and 2.0 U of Taq DNA polymerase. DNA concentration from clinical specimens was not evaluated, but to ensure that the concentration of DNA was sufficient, clinical samples were submitted to PCR using primers located in moderately repetitive sequences of the human genome. To increase the level of sensitivity of the assay, the detection of amplified products is done by Southern blot hybridization under nonstringent conditions using mixed probes representing the HPV types most commonly found in cervical samples. Samples giving a positive signal on autoradiography are submitted to a second hybridization under stringent conditions (Tm-10°C) using the appropriate single probe.

FIGURE 13. Effect of the concentration of Taq DNA polymerase on the amplication of HPV 16 with the consensus primers IU-IWDO. Concentration of polymerase varied from 0 to 2.5 U. Lane 1 represents 2.5 U of DNA polymerase. Each following lane represents a 0.5 U incremental decrease with lane 6 containing no enzyme. Lane C is phiX 174 RF Hae III-digested DNA as size marker.



C. Clinical samples

1. Comparison of PCR to Southern blot hybridization

The next objective of this work was to show the application of HPV E1 ORF consensus primers in the amplification of HPV sequences in clinical samples.

One hundred and twenty six anogenital biopsies, classified by histopathology as either normal, benign, dysplastic or malignant, and HPV type previously determined by conventional Southern blot hybridization (Lorincz et al., 1987a; Lorincz et al., 1992) were analyzed by PCR followed by Southern blot hybridization (PCR/SB) of amplified product using a variety of HPV probes.

Of the 126 samples tested, the two techniques were concordant for 109 (86.5%) samples with respect to the presence or absence of HPV DNA sequences. Six samples were discordant for Southern blot positivity and 11 samples discordant for PCR/SB positivity (Table 5).

Comparison of HPV typing was done on the 83 samples that were HPV DNA positive for both Southern blot and PCR/SB. Three different groups of samples were studied. Group I represented dysplastic or malignant cervical biopsies from women in the Washington DC, area, Group II consisted of biopsies of benign condylomas from women in the Washington, DC, area and group III contained samples of invasive cervical cancer from women from Lima, Peru. Of the 83 samples found positive by conventional Southern blot, the HPV type could be determined in

TABLE 5. Comparison of Southern blot to PCR/SB for detection of HPV sequences in anogenital tissues (n=126).*

		PCR/SB +	PCR/SB -	Totals
Southern blot	+	83	6	89
	-	11	26	37
	Totals	94	32	126

*Biopsies consisted of normal anogenital tissue, condylomas, micropapillary condylomas, intraepithelial neoplasias, and primary and metastatic vulvar and cervical cancers.

only 73 samples. However, an HPV type designation could be assigned to 82 of the 83 samples using PCR/SB. Of the 73 samples that were typable by both Southern blot and PCR/SB, only 63 (86%) of the samples were in agreement with respect to the HPV type (Table 6).

The results of Southern blot and PCR/SB with respect to HPV positivity were compared to the histopathological reports of the 126 samples. Of the eight normal anogenital tissues, all negative by Southern blot, one sample was positive by PCR/SB (Table 7). For the 88 benign and dysplastic lesions, 91% (80) of the samples agreed by both techniques; 66 samples were positive and 14 negative, but eight samples were positive by PCR/SB only (Table 8). Twenty-two (73%) of the 30 carcinomas were concordant with 17 samples being positive for HPV and five negative. Six of these were positive by Southern blot only and two samples were positive by PCR/SB only (Table 9).

Of the six carcinoma samples positive by Southern blot only, four samples were submitted to PCR using different sets of primers (Table 10). One set of primers was specific for HPV types 16 and 18 and amplified sequences from the E6 ORF. Primers specific for HPV 16 are designated H1 (5'-ATTAGTGAGTATAGACATTA) and H2 (5'-GGCTTTTGACAGTTAATACA) and for HPV 18 H1 and H3 (5'-GGTTTCTGGCACCGCAGGCA); the primers generate fragments about 120 bp in length (Shibata et al., 1988). The conditions of amplification were: denaturation at 94°C for 1 minute, annealing at 37°C for 2 minutes and an extension time of 2 minutes at 55°C for 30 cycles. These primers were used because

TABLE 6. Comparison of HPV typing using Southern blot versus PCR/SB.

	No. Typable by Southern blot	No. Typable by PCR/SB	% Concordance
GROUP I (n=30)	29	30	97 (28/29)
GROUP II (n=30)	29	30	83 (24/29)
GROUP III (n=23)	15	22	73 (11/15)

Total samples typable by Southern blot and PCR/SB = 73

Total samples with same type by Southern blot and PCR/SB = 63 (86%)

TABLE 7. Comparison of Southern blot to PCR/SB for detection of HPV sequences in normal anogenital tissues (n=8).

		PCR/SB		
		+	-	Totals
Southern blot	+	0	0	0
	-	1	7	8
	Totals	1	7	8

TABLE 8. Comparison of Southern blot to PCR/SB for detection of HPV sequences in condylomas, micropapillary condylomas and intraepithelial neoplasias (n=88).

		PCR/SB +	PCR/SB -	Totals
Southern blot	+	66	0	66
	-	8	14	22
	Totals	74	14	88

TABLE 9. Comparison of Southern blot to PCR/SB for detection of HPV sequences in primary and metastatic vulvar and cervical cancers (n=30).

		PCR/SB +	PCR/SB -	Totals
Southern blot	+	17	6	23
	-	2	5	7
	Totals	19	11	30

the E6 ORF appears to always be present in cervical carcinomas and HPV DNA positive cervical cancer cell lines (Wettstein, 1990). A second set of primers was derived from a 45 nt highly conserved region detected in the E1 ORF identified by pair-wise computer searches of anogenital HPV sequences. The sequence lies midway between the IU and IWDO annealing sites. This region of homology allowed for the design of two oligonucleotides (Figure 1A) termed LG2 [5'-AAAGTGATAAA(A/T)C(A/C)A(C/G)ITGT) and LG5 [5'-(T/G)AAATGGTCT(A/T)ACTAA(A/T)TCI(A/G)IAAA] that in combination with IWDO (LG2) and IU (LG5) will both yield an amplified fragment of about 425 bp (Figure 14). The conditions of amplification were the same as those used for IU and IWDO alone. These two sets of primers were used in an attempt to ascertain whether the target sites for IU or IWDO were lost in certain carcinomas.

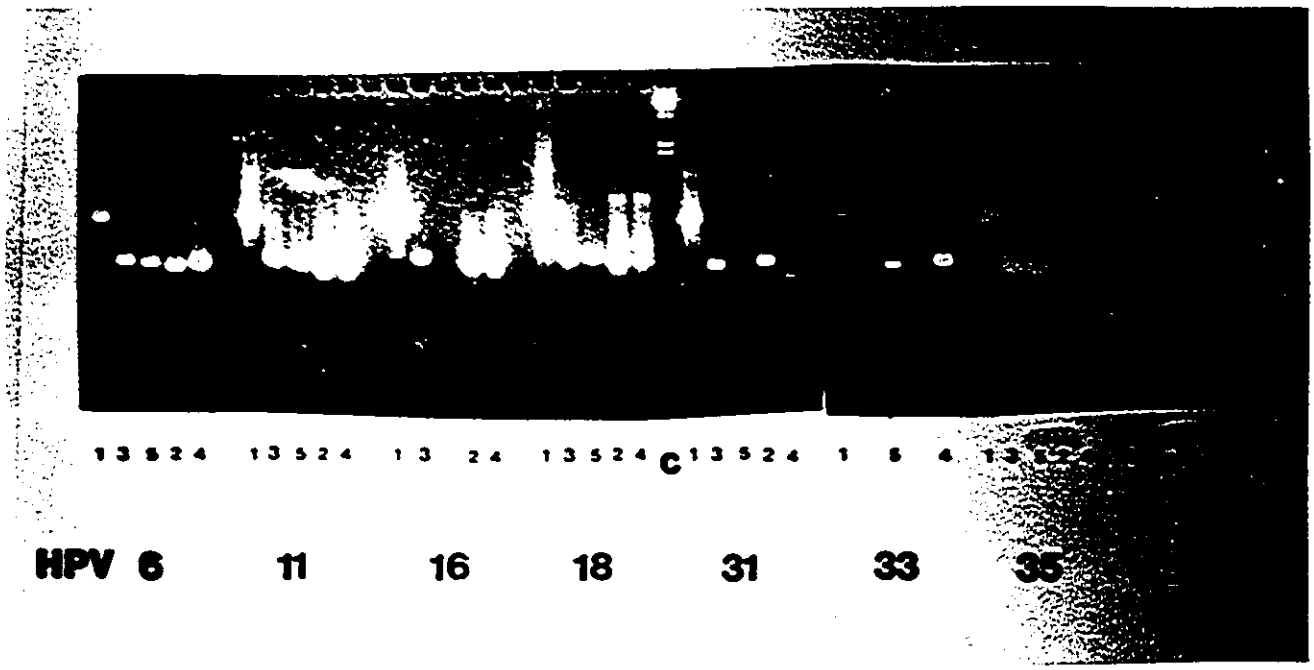
Amplification performed with primers H1-H2 and H1-H3 on four of the PCR/SB carcinomas using IU and IWDO demonstrated the presence of HPV 16 in two samples and HPV 18 in one thus confirming the Southern blot results (Table 10). One sample that was positive for HPV 16 using H1-H2 did not amplify with either IWDO- LG2 or IU-LG5 suggesting the absence of target sites for the IU and IWDO consensus primers. Two samples that were positive with either H1-H2 or H1-H3 were also found positive with the combination IWDO-LG2 but negative with IU-LG5 suggesting loss of the IU annealing site. This is somewhat surprising since the IU target site is located upstream to IWDO in the E1 ORF near the E6 ORF. A second explanation could be that the IU site has been

TABLE 10. Comparison of HPV typing of Southern blot-positive, IU-IWDO consensus primer-negative anogenital carcinomas using other primer sets.

Sample no.	Southern blot type	PRIMERS			
		IU-IWDO	H1,H2,H3	LG2-IWDO	LG5-IU
28	35	neg	nd*	nd	nd
31	35	neg	nd	nd	nd
35	16	neg	16	neg	neg
44	16	neg	16	16	neg
49	18-related	neg	18	18	neg
77	16	neg	neg	16	16

*nd, not done

FIGURE 14. Amplification of HPV types using alternative E1 consensus primer sets IU-LG5 and IWDO-LG2. Denatured HPV DNAs were annealed to different combinations of consensus primers: IU and LG5, IWDO and LG2, IWDO and oligonucleotide complementary to LG5 (termed LG4), IU and oligonucleotide complementary to LG2 (termed LG3). The amplified products gave bands at the expected size of 425 bp for all reactions. Lane 1 represents amplification using primers IU-WDO and yield a fragment at 850 bp. Lane 2 represents PCR using IWDO-LG2, lane 3 is PCR using IU-LG3, lane 4 is PCR using IWDO-LG4, lane 5 is PCR using IU-LG5. Lane C is lambda HindIII-digested DNA as size marker.



mutated in these viral genomes where it could no longer allow the annealing of the primer.

One sample that was negative by PCR using primers located in the E6 ORF, yielded amplified products with both IWDO-LG2 and IU-LG5 and the virus type was the same as determined by Southern blot. For this sample, the negative PCR result with IWDO-IU cannot readily be explained since the two targets appear to be present as shown by positive amplification using IWDO-LG2 and IU-LG5.

The HPV genome is present in these four samples since it was detected by PCR/SB with the use of different sets of primers. The negative results obtained after amplification using IU-IWDO can be explained in three of these samples in that one of the target sites for annealing of a consensus primer appears to be either absent or mutated to a point that does not allow annealing to occur. Sequencing of the viral genomes in these tumors would be necessary to confirm any mutation.

In summary, PCR/SB was in agreement with Southern blot in 86.5% of all samples ranging in pathology from normal to invasive cervical cancer. However, the correlation increases to 90.6% when cervical cancers are excluded (Table 11). Only 73% of the cervical carcinomas agreed by both techniques and PCR/SB failed to detect viral genomes in six carcinomas when IU and IWDO were used as primers. In carcinomas the viral genome is frequently integrated disrupting the E1 and/or E2 ORFs followed very often by rearrangements and deletion of portions of the viral sequences. Viral sequences, therefore, may lose one or

TABLE 11. Summary of HPV typing of anogenital tissues comparing Southern blot and PCR/SB.

		% Concordant	No. PCR/SB and Southern blot positive
Normal tissues	(n=8)	88	7
Benign and dysplastic lesions	(n=88)	91	80
Carcinomas	(n=30)	73	22
Totals	n=126	86.5	109

both consensus primer annealing sites explaining the negative signal by PCR/SB. However, four of the six carcinomas tested by PCR/SB using different sets of primers were found positive.

2. Formalin-fixed, paraffin-embedded tissues

Formalin-fixed, paraffin embedded tissues reported by histopathology as cervical carcinoma (26 cases) were used to determine whether the E1 consensus primers could function with archival material. Based on our previous results using fresh tissues, 73% of the carcinomas were positive for HPV DNA sequences after PCR/SB using IU and IWDO as primers. In this collection of specimens, one would expect that a similar percentage, or 19 of 26 samples, should be positive for HPV DNA sequences with 12 positive for HPV 16.

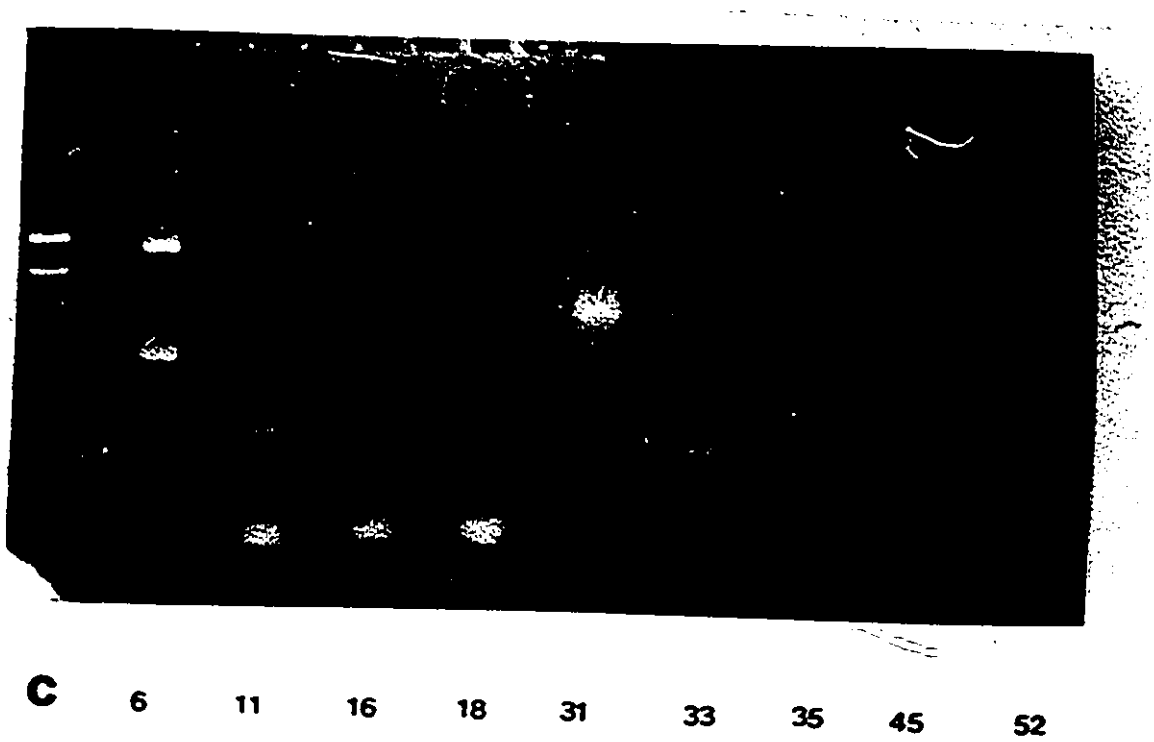
Analysis of DNA extracted from sections of the carcinomas revealed that none showed inhibition of PCR since they exhibited the expected 350 bp band on ethidium bromide-stained gels after amplification using primers in moderately repetitive human genomic sequences. These primers were derived from a human genomic clone and are designated HG1 (5'-GTGTTCTCAATATATTTTGGATG) and HG2 (5'-AAAAACATCTGACTTGGTCTGGG) (W. D. Lancaster, unpublished results). These primers were used rather than the beta-globin primers designed by Saiki et al. (1985) because about 20% of samples that fail to amplify with the beta-globin primers are not inhibitory to PCR (results not shown; Shah et al., 1991). The conditions of amplification with HG1 and HG2 were: denaturation at 94^{o}C for 2 min, annealing and extension at 55^{o}C

for 2 min for 25 cycles with the last cycle having an extension step at 72°C for 7 min. However, when these samples were tested for HPV sequences by PCR using IU and IWDO only two of 26 showed amplification of HPV DNA that only could be detected by autoradiography.

To confirm the presence of HPV DNAs in these samples, the primers H1, H2 and H3 (Shibata et al., 1988) were used for HPV DNA amplification. In the original publication, in which Klenow polymerase was used, these primers were described as being specific for HPV 16 and HPV 18. However, it was demonstrated here that they were able to amplify cloned HPV DNA from type 6, 11, 16, 18, 31, 33, 35, 45 and 52 after 30 cycles using Taq polymerase when annealed at 37°C with an extension temperature of 55°C (Figure 15). One cycle consisted of denaturation at 94°C for 1.5 minutes, annealing at 37°C for 2 minutes followed by an increase of the temperature to 55°C over a period of 2 minutes. The expected 120 bp fragment was obtained for HPV types 11, 16, 18, 33, 35, 45 and 52. HPV 6 yielded two bands (1,300 bp and 600 bp) and HPV 11 gave an additional band at approximately 450 bp. HPV 31 amplified a fragment of 900 bp, HPV 33 gave several bands of different sizes and finally HPV 45, in addition to the expected 120 bp fragment, showed a band at 600 bp.

To detect HPV DNA amplified from the formalin-fixed specimens, hybridization was performed using HPV 16 and 18 H1, H2 and H3 amplimers as probes under nonstringent conditions (Tm-40°C). To type, dot blot hybridizations were done under

FIGURE 15. Amplification of HPV DNAs using type-specific primers. Denatured HPV types 6, 11, 16, 18, 31, 33, 35 45, and 52 were annealed with type-specific primers (Shibata et al. 1988) at 37°C, followed by 30 cycles of PCR. All DNAs successfully amplified to yield bands at approximately 120 bp with the exception of HPV type 6 which gave bands at 600 and 1300 bp. In addition to the 120 bp product HPV types 11 and 45 gave additional at 450 and 600 bp, respectively. HPV type 31 gave a single band at 900 bp. Lane C is phiX 174 RF Hae III-digested DNA as size marker.



stringent conditions (Tm-10°C) using amplified HPV 16 and 18 fragments as single probes. Sixty to 70% (11 to 13 samples) of these cervical carcinoma were expected to contain type 16 and 5 to 10% (1 or 2 samples) type 18 based on previous typing results. The hybridization results from these samples showed that 23 (88.5%) of the 26 cervical carcinomas were positive under nonstringent conditions. Under stringent conditions, 15 (57.7%) of the HPV DNA positive samples were positive for HPV 16 and 1 (3.8%) for HPV 18. Nonstringent hybridization showed that eight samples contained HPV DNA that failed to hybridize with HPV 16 or 18 and were not analyzed further.

3. Cervical scrapings

Two hundred cervical scrapings were obtained from patients visiting two family practices in Ann Arbor, Michigan, USA. Approximately two-thirds of the patients presented with complaints of vaginal odor, itching or swelling, the remainder were visiting the clinic for an annual gynecological examination. This population is considered to be at low risk for genital HPV infections. The characteristics of these samples are listed in Table 12.

These samples were collected in an attempt to: (i) correlate cytology with PCR/SB; (ii) compare the results of a commercially available HPV DNA detection system (ViraPap, Digene, Silver Spring, MD, USA) with PCR/SB; and (iii) evaluate the incidence of HPV infection in this population using PCR/SB.

At the time of sample collection, a Pap smear was

TABLE 12. Characteristics of cervical scrape samples.

total number of samples:	200
inhibition of PCR:	8
total samples tested:	192
patients with multiple samples:	11
total number of multiple samples:	26
total number of patients tested:	177

done and a cotton swab was used to harvest exfoliated cervical cells that were suspended in 1.0 ml of a commercial cell lysis solution supplied with the ViraPap kit. Pap smear results were reported from the Department of Pathology, University of Michigan Hospitals. DNA from 200 μl of the lysis solution was precipitated using ammonium acetate and isopropanol. The dried pellet was suspended in 60 to 100 μl of water. An aliquot was submitted to PCR using primers located within a moderately repetitive sequence of the human genome (HG1 and HG2) to ascertain whether inhibitors of PCR were present and that the concentration of cellular DNA was sufficient. Of the 200 samples, eight (4%) failed to support the amplification of human sequences; these samples were eliminated from the series.

Of the 192 samples tested for HPV DNA amplification with E1 consensus primers, 32 (16.7%) samples were positive for HPV DNA when hybridized under nonstringent conditions with HPV mixed probes 6/11, 16/18 and 31/33/35. Eleven patients had multiple samples taken; eight had two samples taken, two had three samples and one had four samples (Table 13). Of the eight patients with two samples each, four were negative at the time of the first sample and two of these patients became positive for the second sample. Four patients were positive at the first sample and three of these remained positive. Two patients were negative for both samples. The three patients positive for HPV for both samples had the same HPV type on each occasion. Of the two patients that had three samples taken; one was positive for all three samples and harbored HPV 18 and the other had all three

TABLE 13. Results of HPV typing by PCR/SB on patients with multiple cervical scrape samples.

	Sample number			
Patient no.	1	2	3	4
1	HPV 6/11	NEG	NEG	NEG
2	HPV 18	HPV 18	HPV 18	
3	NEG	NEG	NEG	
4	HPV 52	NEG		
5	HPV 16	HPV 16		
6	HPV 35	HPV 35		
7	HPV 18	HPV 18		
8	NEG	HPV 6/11		
9	NEG	HPV 18-related		
10	NEG	NEG		
11	NEG	NEG		

samples negative for HPV DNA. The one patient that had four samples taken was positive for HPV 6/11 on the first sample with the three remaining samples remaining negative. Four (36.4%) of these 11 patients had discordant results when samples were taken at different times. Sample to sample variation appears to be the most obvious explanation for this discordance; however, variation in virus shedding in relation with the menstrual cycle is another possibility which has been reported by other investigators (Chang-Claude et al., 1990). The reproducibility of the assay was not tested by collecting several samples from the same patient at one visit.

To evaluate the incidence of HPV infection in this population, as well as to compare the efficiency of the three different techniques used to detect HPV infection (Pap smear, ViraPap, PCR/SB), only the result obtained from the first patient sample was used. Cellular changes consistent with HPV infection by the Pap smear were reported in four of 177 cases. Only four samples were positive for HPV DNA using the ViraPap detection kit while 25 samples were found positive by PCR/SB (Table 14).

All four patient samples with abnormal Pap smears were found to contain HPV sequences by PCR/SB but were negative by ViraPap detection. Of the 173 normal Pap smears, 21 (12.1%) samples were positive by PCR/SB whereas only 4 (2.3%) were positive by ViraPap (Table 15).

Of the 4 samples positive by ViraPap, three of these were also positive by PCR/SB. On the other hand, of the 173 samples negative by ViraPap, PCR/SB showed 22 samples positive

TABLE 14. Comparison of Pap smear, ViraPap, and PCR/SB for detection of HPV in cervical scrapes from 177 women.

Method	No. Positive	% Positive
Abnormal Papanicolaou smear	4	2.3
ViraPap	4	2.3
PCR/SB	25	14.1
All three methods	27	15.3

TABLE 15. Comparison of PCR/SB and ViraPap to cytology for detection of HPV sequences.

	PCR/SB		ViraPap	
Cytology	+	-	+	-
Abnormal (n=4)	4	0	0	4
Normal (n=173)	21	152	4	169

TABLE 16. Comparison of PCR/SB to ViraPap for detection of HPV sequences in samples of cervical scrapes from 177 women.

		PCR/SB +	PCR/SB -	Totals
ViraPap	+	3	1	4
	-	22	151	173
	Totals	25	152	177

for HPV sequences (Table 16). In the one ViraPap positive PCR/SB negative sample, the reason for the discordance is not readily evident. However, this could represent a false positive result because of the subjective nature of the ViraPap test in which the signal strength from a known negative is compared to the signal strength of a HPV DNA low copy number positive control.

Overall, 14.1% of the samples demonstrated the presence of HPV sequences using PCR/SB where only 2.3% of the samples were positive in the ViraPap detection kit. In this series of specimens PCR/SB had a detection rate six times that of ViraPap.

The question arises about the significance of these HPV DNA positive samples from patients with an apparently normal Pap smear. Of the 25 women positive by PCR/SB, 21 consented to have colposcopically directed biopsies of any suspicious area of the cervix. The histopathology was read at the Department of Pathology, University of Michigan Hospitals. Five of the 21 women (24%) had normal biopsies reported. Of the 16 remaining patients, the results of the biopsy were either cervicitis in five (24%), condylomatous changes in eight (38%) or cervical intraepithelial neoplasia in three (14%) (Table 17). The results indicate that 52% of the patients biopsied had histopathological evidence for HPV infection suggesting that HPV DNA positive by PCR/SB may be a disease indicator.

Three of the four patients that had normal Pap smears and positive for HPV DNA by ViraPap were also colposcopically examined. One patient had a normal biopsy and

TABLE 17. Comparison of colposcopic-directed biopsy diagnosis to PCR/SB HPV typing in women with normal cytology.

HPV Type	No. samples positive	No. patients with colposcopy	Biopsy diagnosis* N	CRV	CONDY	CIN
6/11	4	4	2		2	
16	3	3	1	1	1	
16-related	2	2		1	1	
18	4	2		2		
18-related	2	1		1		
31	1	1	1			
35	3	2			2	
52	2	2			1	1
unknown	4	4	1		1	2
Total	25	21	5	5	8	3
% of total		100	24	24	38	14

*N=normal, CRV=cervicitis, CONDY=condyloma, CIN=cervical intraepithelial neoplasia

the other two showed either cervicitis or condylomatous changes. This series of patients is too small to draw any conclusions but the trend appears to indicate that a positive ViraPap may also be an indicator of disease but at a significantly lower sensitivity than PCR/SB.

In summary, the cervical scraping samples in what is considered a low risk population for HPV infection, 2.3% had abnormal cytology and 2.3% had HPV DNA detected using the ViraPap detection kit; however, the ViraPap positive samples did not correlate with abnormal cytology. PCR followed by Southern blot hybridization detected HPV DNA in 14.1% of the patients including those samples reported with abnormal cytology. Of the 173 normal Pap smears, HPV DNA sequences were detected in 21 (12.1%) of samples by PCR/SB. This is in agreement with previous results using the conventional technique of Southern blot hybridization in a normal population (Toon et al., 1986; Lorincz et al., 1987c; Fuchs et al., 1988). The advantage of PCR/SB is that very limited amounts of clinical material can be used to screen for HPV DNA. Since the clinical material used in this study was taken from the same swab used for the Pap smear, the amount of DNA extracted from the sample would have been too small for conventional Southern blot analysis.

4. Recurrent infections

a. Detection of HPV types

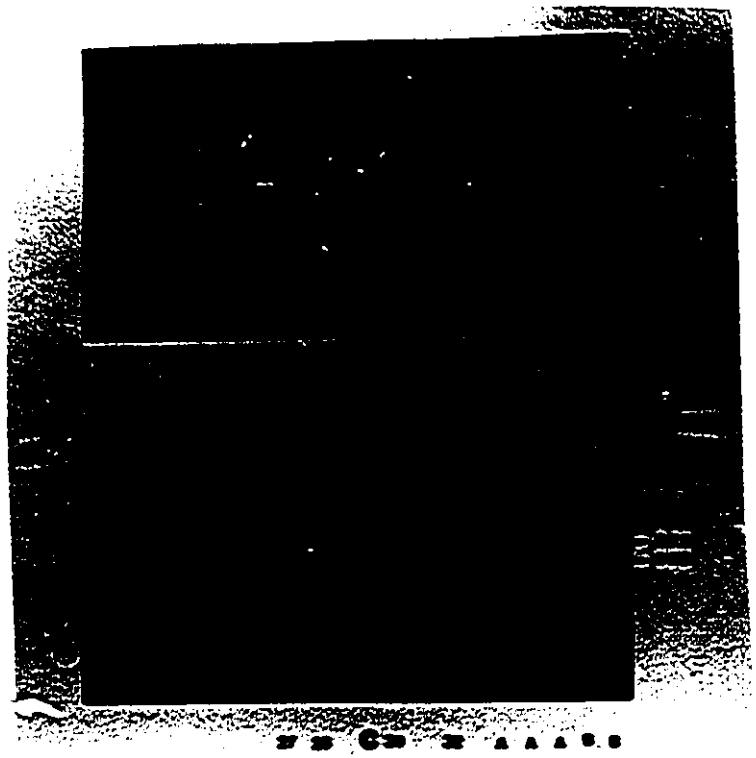
Thirty-three women with a history of recurrent

HPV-associated lesions of the anogenital tract were seen at a referral clinic in Southfield, Michigan, USA. At the time of the gynecological examination, their Pap smear was normal. These patients were tested by PCR/SB to determine whether a different spectrum of virus types was present in this population of women at higher risk for HPV infection than the family practice population.

After the Pap smear was taken, exfoliated cervical cells were harvested using a fresh cotton swab and the cells deposited in phosphate buffered saline (PBS). A crude extraction of cellular DNA was prepared by lysing the cells in 200 μl of PCR buffer, without BSA and containing 100 μg/ml of proteinase K which was inactivated by heating the sample to 95^{o}C for 10 minutes. An aliquot was assayed by PCR using the human genomic primers HG1 and HG2 to verify the quality of the DNA. One sample failed to amplify sequences using HG1 and HG2 and was eliminated. Of the 32 remaining samples submitted to PCR using HPV E1 consensus primers, 17 (53.1%) were considered negative for HPV DNA since no bands could be seen on ethidium bromide-stained gels (Figure 16) and no signal was observed after hybridization using nonstringent conditions (Tm-40^{o}C) with IU and IWDO generated amplimer probes 6/11, 16/18, and 31/33/52.

Twelve samples showed amplified products at the expected size of about 850 bp on ethidium bromide-stained gels. Three of these showed a double band which could indicate a double infection based on the slightly different fragment sizes seen for the various HPV types amplified by the E1 consensus primers

FIGURE 16. Amplification of HPV sequences in samples from 32 patients with recurrent HPV-associated cervical lesions using consensus primers IU-IWDO. Total cellular DNAs were denatured and submitted to PCR using IU-IWDO primers. Twelve samples showed amplified products at the expected size of about 850 bp, three samples have doublets suggesting double infections. Lanes A are cell line 293 DNA after amplification indicating the ethidium bromide staining of a negative control. Lanes B are negative reagent controls. Lane C is phiX 174 RF Hae III-digested DNA as size marker.



(Figure 16). Hybridization under nonstringent conditions with mixed probes 6/11, 16/18, and 31/33/52 gave a positive signal on autoradiography for the 12 samples. In addition, two other samples, R1 and R9, that failed to produce visible bands on ethidium bromide-stained gels were also positive by autoradiography. However, two samples (R17 and R19) that produced amplified products visualized on stained gels did not hybridize with any probe. Under stringent conditions (Tm-10^{o}C), six (18.8%) samples could be typed. One sample contained HPV type 6/11, four had HPV type 18, one sample which showed two closely spaced bands of about 850 bp on ethidium bromide staining was positive for HPV 6/11 and HPV 31. For the remaining six samples, the autoradiographic signal produced under stringent conditions of hybridization was too weak compared to the intensity of the amplified product found on ethidium bromide-stained gel. This suggested that the amplified viral sequences were not homologous to the probes used and may represent new virus types.

Additional single probes consisting of HPV 30, 35, and 45 were used under standard conditions of hybridization (Tm-25^{o}C). After autoradiography the blots were rewashed under stringent (Tm-10^{o}C) conditions and reexposed to determine whether the intensity of the signal changed. Two samples (R9 and R12) were found positive for HPV 30 and two (R13 and R17) for HPV 35. Two samples (R11 and R27) were typed as HPV 35-related since with probe HPV 35 they showed a strong signal at Tm-25^{o}C but at Tm-10^{o}C the signal was greatly reduced. These samples gave the same positive signal at Tm-25^{o}C when the amplified HPV DNA from

TABLE 18. HPV typing by PCR/SB in cervical scrapes of women with recurrent cervical HPV infections using mixed or single HPV DNA probes under stringent hybridization conditions.

		Mixed probes			Single probes					
Patient No.	EtBr stain	6/11	16/18	31/33 52	30	35	45	R13	R15	HPV type
R1	-	3+*	-	-	-	-	-	-	-	6/11
R3	+	-	-	-	+	-	-	-	2+	New ?**
R7	+	-	4+	-	-	-	-	-	-	18
R9	-	-	-	-	2+	-	-	-	-	30
R11	db***	-	-	-	-	+	-	2+	-	35-related
R12	-	-	-	-	2+	-	-	-	+	30
R13	+	-	-	-	-	2+	-	4+	-	35
R15	+	-	-	-	+	+	-	-	4+	New ?
R17	+	-	-	-	-	3+	-	4+	-	35
R18	db	4+	-	4+	-	-	-	-	-	6/11, 31
R19	+	-	-	-	-	-	-	-	-	New ?
R27	+	-	-	-	-	+	-	+	-	35-related
R28	+	-	4+	-	-	-	-	-	-	18
R30	+	-	4+	-	-	-	-	-	-	18
R32	db	-	4+	-	-	-	-	-	-	18

*+ to 4+ represents weak to strong hybridization signal.

**Potential new HPV type.

***Indicates two bands seen on ethidium bromide-stained gel.

one of the samples carrying HPV 35 (R13) was used as a probe (Table 18).

Three samples, R3, R15 and R19, that showed a 850 bp fragment on ethidium bromide-stained gels did not hybridize strongly with any probe. R3 and R15 gave a weak signal against HPV 30 and R3 cross-hybridized to the R15 amplified product. R19 did not hybridize with any probe. These results suggested that three putative new HPV types were present in this series of 32 patients with recurrent anogenital infections. Sample R15 was selected to be further investigated because of its abundant yield of amplified product seen on ethidium bromide-stained gels.

To summarize, of the 32 samples from this high risk population, 17 (53.1%) failed to show an amplified product either by ethidium staining or hybridization and were considered negative for HPV sequences. Fourteen (43.8%) of the samples showed amplified products that were detected by hybridization under nonstringent conditions and one (3.1%) sample showed an amplified fragment at the expected size on ethidium bromide-stained gels but failed to hybridize to any of the probes used. Three samples showed two bands of amplified product at approximately 850 bp but two virus types were identified in only one sample after hybridization. The remaining two samples may also represent a double infection where only one of the types has been identified with the panel of probes used here.

Surprisingly, no sample contained HPV 16. Also not expected was the presence of HPV 18 in four (26.7%) of the 15

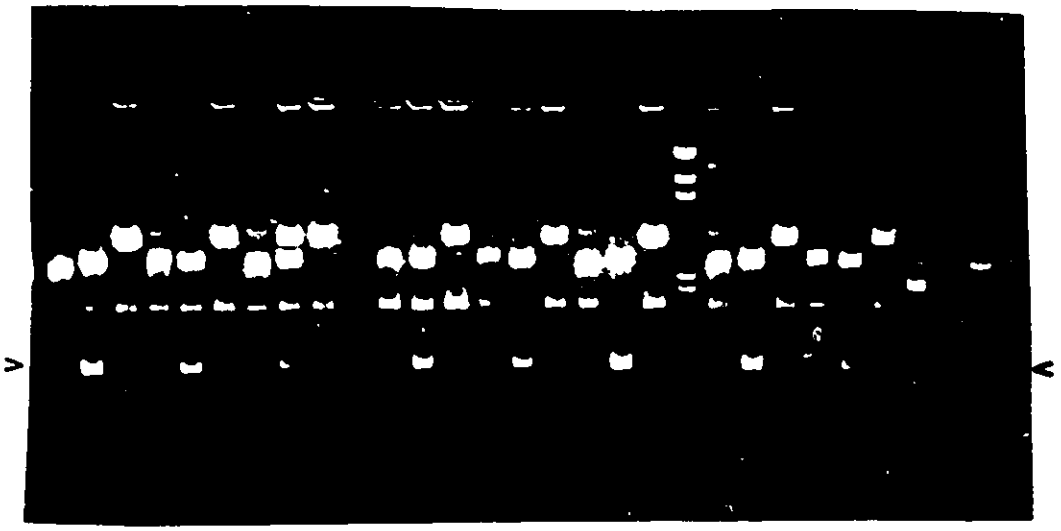
positive samples since in other studies HPV 18 has been detected in about 5% of CIN lesions (Schneider et al. 1985, Lorincz et al. 1987c). Seven (46.7%) samples were positive for HPV types in the intermediate risk group. Three (20%) samples produced the expected 850 bp amplified fragment. Two of these samples cross-hybridized weakly with HPV 30 and the third contained HPV DNA sequences since an 850 bp fragment was generated by PCR. However, the HPV DNA sequences in this sample failed to hybridize to any HPV DNA probe used.

Because of the abundance of product found after amplification of sample R15, this DNA was selected to be further investigated as a putative new HPV type by cloning of the amplified products and partial sequencing of the clone. In an attempt to determine whether the DNA, termed R15, in this sample represented a new HPV type, a comparison was made between the nucleotide sequence of R15 and the available nucleotide sequence of the E1 ORF of other HPVs. Also, the R15 sequence was translated and the amino acid sequence analyzed for the presence of consensus motifs found in the E1 ORF of other HPVs.

b. Cloning of R15

Several attempts were made to clone amplified fragments from sample R15. Amplified fragments carrying at their 5′ ends Klenow-repaired Bam HI restriction sites, and ligated as blunt ends, was the only approach which yielded colonies containing the appropriate recombinant plasmids. These positive colonies were expanded and the purified constructs digested with

FIGURE 17. Restriction enzyme digestion of plasmid DNAs obtained from transformants positive by colony hybridization against probe R15. Colonies positive by hybridization against probe R15 were expanded and cleaved with BamHI to release the 850 bp amplified products (indicated by an arrow) and EcoRI to linearize the constructs. Lane U represents uncut plasmid, lane B is BamHI digestion and lane E is EcoRI digestion. Lane C is lambda Hind III-digested DNA as size marker.



U B E U B E U B E U B E U B E U B E C U B E U B E U E

Bam HI (Figure 17) to release the 850 bp insert. This fragment cross-hybridized under nonstringent conditions (Tm-40°C) to HPV types 6, 11, 16, 18, 31, 33, 35 45, 51 and 52, weakly with type 37 and not at all with type 38.

c. Sequencing of R15

Sequencing of the insert was done using the dideoxynucleotide chain termination method (Sanger et al., 1977). IU was selected as the sequencing primer in order to obtain the 5' end of the E1 ORF in the sense orientation. Two hundred and fifty bases were read from the reaction and the nucleotide sequence is shown in Figure 18.

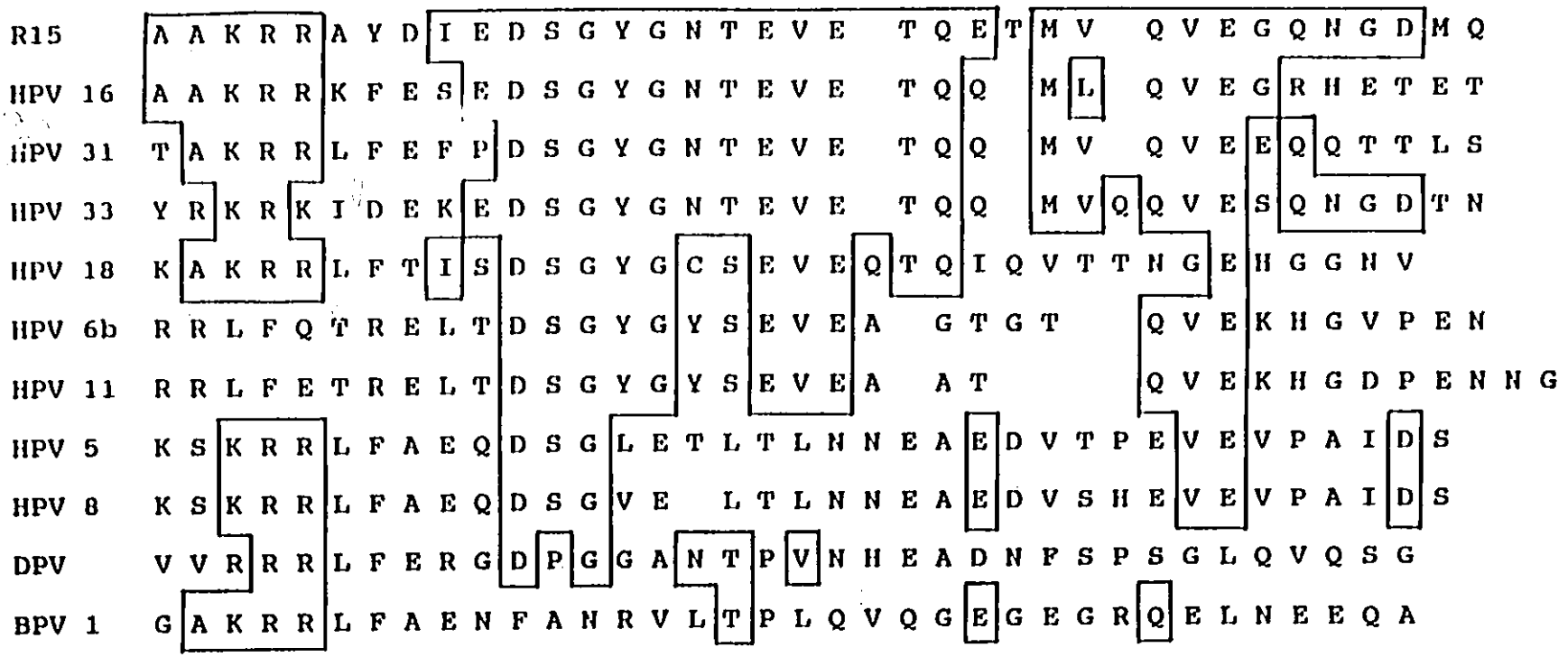
Pair-wise comparisons of the partial sequence of R15 were conducted against HPV sequences available in GenBank version 72. No perfect match was found, however, the closest related HPV was type 16 with 143/250 nt showing homology, followed by HPV types 33, 35 and 31 with 140, 140 and 136 matches, respectively. HPV types 6 and 11 were more distant with 122 and 120 corresponding matches. Very little homology was found with HPVs of cutaneous origin (HPV types 1, 5, 8 and 47 with 91, 104, 103 and 103 nt matches, respectively). These results are summarized in Table 20.

The nucleotide sequence of R15 was translated into the corresponding amino acid sequence and compared to the E1 products of HPV types 16 and 33 since these viruses have the closest homology. One region of homology was detected with the sequence KRxxxxxEDSGYGNTEVETQxxxxQVE. This sequence was compared

FIGURE 18. Nucleotide sequence of cloned DNA obtained from amplification or sample R15 with IU and IWDO consensus primers. The sequence represents the coding strand.

```
          10          20          30          40          50
  AGGTAATAGT  AATGGTATAG  AAAACCAAGC  ATGTACAGCC  GCAAAACGCA

          60          70          80          90
100
  GAGCATACGA  CATAGAAGAC  AGCGGATATG  GCAATACTGA  AGTGGAAACT

          110         120         130         140
150
  CAAGAGACAA  TGGTGCAGGT  AGAGGGGCAA  AATGGCGATA  TGCAGTGCAG

          160         170         180         190
200
  TAGTCAGTGT  AGTACGGGGG  CAAGTGATAC  TGGAGAACAG  ATGTGTAATA

          210         220         230         240
250
  GCATACGGAA  AGTAATAGTA  GTCAGAACAA  AGCATGCCAT  TGCAACTGTG
```

TABLE 19. ORF E1 amino acid motif in mucosal and cutaneous HPVs and animal papillomaviruses.*



*The regions of homology are indicated in the boxed areas. Spaces were introduced in the amino acid sequences for purposes of alignment.

TABLE 20. Nucleotide sequence homology between the R15 sequence and the E1 ORF sequences of sequenced HPVs. The partial nucleotide sequence of R15 (250 nt) was compared to the available sequences of HPV E1 ORFs contained in GenBank version 72 using a pair-wise matching program.

HPV Type	% Homology to R15	Total No. of Matches
1a	36.4	91
5	41.6	104
6b	48.8	122
8	41.2	103
11	48.0	120
16	57.2	143
18	42.4	106
31	54.4	136
33	56.0	140
35	56.0	140
47	41.2	103
58	53.2	133

to HPVs whose sequences have been determined (Table 19). The results showed that HPVs from the anogenital tract had a higher degree of homology to the sequence than HPVs from cutaneous sources or animal viruses. The E1 ORF of all anogenital HPV types show a consensus motif DSGYGxxEVE. In addition, HPV 6 and 11 share the sequence QVE which is downstream of the 10 amino acid motif. HPV 18 does not code for this sequence but additional homology to the HPV 16 and 33 and R15 sequences was noted downstream of the motif increasing the homology to DSGYGxxEVExTQ. In addition, HPV 16, 18, 31 and R15 have four conserved amino acids (AKRR) five amino acids upstream of the motif.

V. DISCUSSION

The need to identify HPV sequences in clinical material is evidenced by the close association of a sub-group of viruses with anogenital tract lesions. Furthermore, these viruses have been subdivided into low, intermediate and high risk types based on their association with malignancy. The ability to discern individual genotypes is important for epidemiological studies and may be of prognostic value. The available technique for their detection, molecular hybridization, has certain limitations that makes the utility of the method less than optimal. Major problems with molecular hybridization are sensitivity and the time necessary to identify by type the large number of different virus types found in clinical samples.

This work was undertaken in an attempt to make the identification of HPV in clinical samples more sensitive and accurate. The technique of amplification of HPV sequences using PCR prior to hybridization was chosen because of its exquisite sensitivity and the potential to identify new HPV types as well as delineate currently identified virus types. The objectives of this work were to: (i) identify regions of DNA sequence homology in the genomes of HPVs whose sequences were known at the time this study was initiated in order to evaluate their utility for the design of HPV genus-specific PCR primers; (ii) optimize the conditions for the use of genus-specific primers for PCR; (iii) demonstrate the utility of genus-specific primers for amplification of HPV DNA in clinical samples from normal and

diseased anogenital tissues.

A. Primer Design

From computer assisted pair-wise comparisons at the nucleotide level on five anogenital HPV genomic sequences, homologous sequences were identified in ORFs E6, E1 and L1. A prerequisite in the selection of primers was that the segment of DNA to be amplified must be maintained in the event there is integration of viral DNA into host chromosomes. The HPV genome is frequently interrupted between ORFs E1 and E2, rearranged and viral sequences downstream of the integration site deleted in cervical cancers and carcinoma *in situ* (Wettstein, 1990). The value of the detection strategy would be compromised if virus in high grade premalignant lesions and cervical cancers could not be amplified. For this reason homologous sequences detected in the L1 ORF were not examined further. Comparison of sequences in the E6 and E1 ORFs showed that the longest string of perfect homology with 12 nucleotides in length was in the E1 ORF. This sequence was termed UNI.

At the time this study was initiated, the Klenow fragment of *E. coli* DNA polymerase I was to be used. To ensure efficient amplification with this polymerase, a distance limitation was placed on the location of the second primer of about 1,000 bp. To locate potential sequence homologies for the second primer, a pair-wise comparison at the amino acid level was done on the translated E1 ORF for all HPVs whose sequences were available at the time. A stretch of seven amino acids were identified with

the sequence Met-Val-Gln-Trp-Ala-Tyr-Asp located approximately 850 nt downstream of UNI that was conserved in the viral genomes tested. Since the string had two amino acids with unique codons (Met and Trp), the sequence was selected as a potential second primer site. The nucleotide sequence of this amino acid string was termed WDO.

Although UNI has perfect homology among the HPV genomes tested, a 12 nt sequence would not be expected to anneal in a specific fashion. Therefore, the 5′ end of UNI was extended to 21 nt. The 5′ extension of 9 nt showed only partial homology. To increase stability and specificity of annealing, the final oligonucleotide, termed IU, was synthesized to contain either base at two base degeneracies and inosine at the sites of four base degeneracies. This limited the total number of different molecules during the synthesis of IU to eight. Because inosine can form stable hydrogen bonds with any base and the perfect homology at the 3′ end of the oligonucleotide, it was expected that this primer would exhibit specific annealing at the appropriate target site. The 3′ end is important because of the requirement for DNA polymerase to have an annealed 3′ base to act as the starting point on the template strand. In order to have the 3′ ends of the primers facing each other, it was necessary to use the complement of WDO (termed IWDO). This was fortuitous since the 3′ end of IWDO would begin with the unique Met codon thus providing the appropriately annealed nucleotide start site for DNA polymerase. As with IU, IWDO contained either base at two base degeneracies and inosine at four base degeneracies.

This configuration resulted in the synthesis of 16 different molecules for IWDO. The presence of the internal unique Trp codon would aid in stabilization of the primer during annealing. IU was expected to anneal to the non-coding strand and IWDO to the coding strand.

The two primers were assayed by PCR for their ability to amplify the appropriate segment of DNA from cloned HPV 6, 11, 16, 18 and 33 DNAs. Each reaction yielded the expected fragment size of about 850 bp in length. A selection of restriction endonuclease digestions confirmed that the amplification products originated from the E1 ORF. Furthermore, when the amplified fragments were used as probes against Pst I-digested HPV DNA, they hybridized only to the viral DNA fragments carrying the E1 ORF. These results confirmed the specificity of the primers and the fidelity of the E1 ORF amplified product. To assure that the primers were capable of amplifying a wide range of HPV DNAs, additional virus clones whose sequences were unknown were submitted to PCR; all produced a band at the expected size of 850 bp on ethidium bromide-stained gels. This demonstrated that IU and IWDO had a broad range of specificity for HPV DNA. Furthermore, the ability of the primers to amplify HPV DNAs from both cutaneous and mucosal sources and papillomavirus DNA from animal viruses demonstrated the potential to amplify new HPV types when present in clinical samples.

B. Optimization of the Amplification Reaction with Consensus Primers for Use with Clinical Samples

The attractiveness of PCR is its conceptual simplicity; however, several variables may affect the efficiency of the reaction. For example, primer design, annealing temperature, the concentrations of enzyme and Mg^{2+}, extension times, sample DNA, as well as other factors, should be taken into consideration when attempting PCR (Saiki, 1989).

Here, the buffer capacity and pH was assayed in the presence of 1 ng of cloned HPV 16 DNA with Mg^{2+} concentrations varying from 0.5 to 3.0 mM. Neither the buffering capacity (10 mM Tris-HCl versus 67 mM Tris-HCl) nor pH values tested (pH 8.3 versus pH 8.8) had an obvious effect on the amplification reaction. Both buffers appeared to yield comparable amounts of amplified products on ethidium bromide-stained gels at the same Mg^{2+} concentrations. However, as reported by others (Saiki et al., 1989), the production of amplified fragments was dependent on the concentration of Mg^{2+}. At Mg^{2+} concentrations between 0.5 and 1.0 mM, no product could be detected by ethidium staining of agarose gels. At a concentration of 1.5 mM Mg^{2+} and greater, amplification products were obtained but additional faint bands were observed on ethidium bromide-stained gels at concentration of 4.0 mM or greater. In general, low Mg^{2+} levels will reduce the yield of amplified product whereas excess Mg^{2+} can produce nonspecific amplification (Saiki, 1989). When the evaluation of the concentration of magnesium was done in presence of 1 μg of cellular DNA, which is more representative of reactions performed with DNA isolated from clinical samples, with 1.5 to 3.0 mM Mg^{2+}, no bands other than the expected fragment were seen on ethidium

bromide-stained gels.

The amount of cellular DNA present in an amplification reaction may alter its efficiency. Excessive concentrations of cellular DNA (10 μg or greater per reaction), completely eliminated the ability to detect product on ethidium bromide-stained gels. The inhibition of amplification may be due to binding of Mg^{2+} by cellular DNA depleting free ions necessary for activation of Taq DNA polymerase. Alternatively, high cellular DNA concentrations may nonspecifically bind Taq DNA polymerase such that little if any enzyme is available for the amplification reaction. The efficiency of amplification depends on primer concentration (Innis and Gelfand, 1990), presumably for similar reasons cited above. However, no effect on the reaction was seen between 0.2 μM to 1 μM of primer.

The annealing of oligonucleotide to its target sequence is dependent on the annealing temperature (Td) which is a factor of length and G+C content (Mason and Williams, 1985). Thus, the temperature of annealing of primers influences the yield of amplified product. Using the formula derived by Mason and Williams (1985) for calculation of Td, it was estimated that the optimal Td for IU and IWDO would be 46°C. At this temperature of annealing as few as 10^3 target molecules yielded sufficient product to be detected by ethidium bromide staining. The sensitivity was increased 10 to 50-fold when the amplified products were blotted and hybridized to specific probes. However, at the annealing temperature of 46°C, the primers bound to alternative target sites as evidenced by single primer

amplification and the production of bands not of the expected size. To increase the specificity of the reaction, the annealing temperature was raised to 52°C. As expected, the efficiency of the reaction decreased and a minimum of 10^5 target molecules were required to yield an ethidium bromide detectable product. However, the specificity of primer annealing was increased since the additional bands seen at 46°C were not detectable at the higher annealing temperature.

It is clear that at lower annealing temperatures the degree of mismatch between primer and alternative target sequences was tolerated. Once annealed, the oligonucleotides are stabilized through extension by Taq DNA polymerase since the enzyme has residual polymerization activity at temperatures as low as 25°C (Gelfand, 1989).

Although additional bands were observed on ethidium bromide-stained gels as a result of mispriming at the annealing temperature of 46°C, one objective of this work was to be able to detect HPV in a variety of clinical samples. Therefore, it would be most desirable to have the highest degree of sensitivity because of small sample size in many instances as well as detection of the low concentration of viral sequences associated with latent infections. Because of these criteria, the annealing temperature of 46°C was selected for the amplification of HPV sequences even though aberrant bands were observed with some of the virus DNAs. Since target sequences were to be detected by hybridization using specific probes rather than ethidium bromide staining, the possibility of false positives would be minimized

while maintaining a high level of sensitivity.

One factor that would increase the utility of the assay is the speed at which an amplification reaction can be performed. In an attempt to reduce the time for amplification reactions the extension time and temperature were examined. The extension time in the original assays was 3 minutes at 72°C. In light of the size of the amplified fragment and the fact that Taq polymerase can synthesize >2,000 bases per minute at 72°C and about 1,400 bases per minute at 55°C (Gelfand, 1989), the extension time was dropped from 3 minutes at 72°C to 1 minute at 55°C. At the shorter extension time and reduced temperature, no difference in the yield of amplified product was observed on ethidium bromide-stained gels. This modification resulted in shorter cycling times and accelerated access to amplified product.

Evaluation of the amount Taq polymerase per reaction indicated that lower concentrations of enzyme (0.5 to 1.0 U) resulted in lower efficiency of amplification. Amplification reactions were most efficient, as determined by ethidium bromide staining, when the enzyme concentration was between 2.0 and 2.5 U per reaction. At higher concentrations of enzyme (up to 3.5 U) the amount of product did not increase. Higher concentrations of enzyme were not assayed with the consensus primers since similar experiments have shown no value in increasing enzyme concentrations beyond the observed optimum (Saiki, 1989).

The above studies for optimization of PCR using consensus primers were designed to yield the most product from clinical samples in the shortest period of time. Thus, for PCR using the

consensus primers IU and IWDO for detection of HPV DNA sequences in clinical samples, the reaction consisted of: 67 mM Tris-HCl, pH 8.8, 2.0 mM $MgCl_2$, 31 mM KCl, 2.0 μg/ml of BSA, with 200 μM of each deoxynucleotide, 1.0 μM of each primer, 2.0 U of Taq DNA polymerase in a total volume of 100 μl. The total cellular DNA concentration should not exceed 5 μg/reaction. All reactions were submitted to 30 cycles of amplification where one cycle consisted of 2 minutes of denaturation of the template at 94°C, annealing of the primers at 46°C for 2 minutes followed by an increase of the temperature to 55°C for 1 minute with an extension time of 1 min at 55°C.

C. Evaluation of the Utility of Consensus Primers for PCR in Identification of HPV Sequences in Clinical Samples

1. Fresh biopsies

The PCR using E1 consensus primers was evaluated for its ability to amplify and accurately identify HPV sequences in clinical samples previously typed by the conventional technique of Southern blot hybridization (Lorincz et al., 1987c; Lorincz et al., 1992). Overall, of the 120 anogenital samples tested, 109 (86.6%) samples gave concordant results for both techniques (83 were positive and 26 were negative). Based on histopathology, the samples could be placed into one of three groups: normal tissue (8 samples), benign and dysplastic lesions (88 samples) and carcinomas (30 samples). Based on this grouping, 7 of 8 normal samples (88%), 80 of 88 benign and dysplastic lesions

(91%) and 22 of 30 carcinomas (73%) were concordanc for both techniques.

For normal tissues and benign and dysplastic lesions, all samples that were positive for HPV DNA by Southern blot hybridization were also found positive by PCR followed by Southern blot hybridization (PCR/SB). However, additional samples were found to harbor HPV DNA when assayed by PCR/SB. This difference in detection of HPV sequences can be explained by the relative sensitivities of the two techniques. For Southern blot hybridization it has been estimated that about 10^5 copies of target are required for a positive signal (Lancaster and Norrild, 1987) whereas in the work presented here the level of sensitivity for PCR/SB is between 20 and 100 copies of target.

Of the 30 carcinoma samples, only 22 (73%) were found positive for HPV sequences by both techniques. Six of the remaining eight samples were positive for HPV DNA only by Southern blot and two were positive only by PCR/SB. The two PCR/SB positive samples in this discordant group can be explained by the increased sensitivity of PCR/SB over Southern blot alone.

A subset of the six samples that were negative by PCR/SB using E1 consensus primers were investigated further in an attempt to discern the discrepancy with the Southern blot results. Four of these six samples were subjected to PCR/SB using three different sets of primers for the amplification reaction. One set of primers were located in the E6 ORF as previously described by Shibata et al. (1988). Two additional primer sets consisted of either IWDO-LG2 or IU-LG5. LG2 and LG5

were derived from a second region of conserved sequences in the E1 ORF that lies midway between IU and IWDO. Three of the four samples were positive for HPV DNA after PCR/SB using primers from the E6 ORF confirming the presence of viral sequences. Two of these three samples were also positive by PCR/SB for the primer combination IWDO-LG2 but negative in reactions using the combination IU-LG5. These results suggest that the IU and/or LG5 target sequences were not available for primer annealing. These results are surprising since both the IU and LG5 target sequences are located upstream of the IWDO annealing site. The IWDO primer annealing site is located at the 3' end of the E1 ORF making it more distant from the E6 ORF than the IU annealing site. Therefore, the IU and/or LG5 annealing sites are either absent due to deletion or rearrangement of the viral genome, or are mutated to the extent that one or the other can no longer allow for primer annealing. The third sample that was positive for HPV DNA using the E6 primers was negative by PCR/SB using both combinations of consensus primers from the E1 ORF. This suggests that the region of the E1 ORF encompassing the annealing sites for both IU and IWDO were deleted. The sample that was negative for HPV DNA by the E6 primers confirmed the negative results obtained with the E1 consensus primers IU and IWDO.

Surprisingly, the primer combinations IWDO-LG2 and IU-LG5 both gave amplified products from this sample. These contradictory results are difficult to reconcile. The possibility that contamination could account for the positive results with IU-LG5 and IWDO-LG2 cannot be eliminated. However,

the negative controls remained negative throughout the study. In addition, the likelihood that two different reactions (IU-LG5 and IWDO-LG2) performed on two different days would yield a positive result due to contamination seems remote. The inability of the E6 primers to amplify HPV sequences in this sample is also surprising since the sample was reported to contain HPV 16 by Southern blot analysis. The hypothesis that continued expression of E6 and E7 are required to maintain the phenotype of HPV transformed cells (Schenider-Gadicke and Schwarz, 1986; Smotkin and Wettstein, 1986; Seedorf et al., 1987) would predict that the E6 primers would amplify this region of HPV 16 DNA in this sample. Since the E6 primers have a narrower range of virus types they can amplify, it is conceivable that the sample contained a virus DNA sufficiently distinct from HPV 16 that the E6 primers were unable to anneal. However, this fails to explain the discrepancy of the consensus primers. It is possible that gross rearrangements to the viral genome resulted in abnormal juxtaposition of the IU and IWDO annealing sites but somehow retained the appropriate IU-LG5 and IWDO-LG2 annealing sites. This problem can only be resolved by detailed sequence analysis of the products of the IU-LG5 and IWDO-LG2 amplification reactions.

The inability of the E1 consensus primers to consistently identify HPV sequences in carcinomas detracts from their utility. However, in the practical sense, dependence on the sensitivity of PCR/SB may not be necessary for carcinomas since, in general, the majority of biopsies contain diseased

tissue. The amount of DNA representing uninvolved tissue is relatively low in these samples and there would be sufficient concentration of viral DNA so as not to present a sensitivity problem for HPV detection and typing by Southern blot. However, the disparity in the results of HPV typing by Southern blot and PCR/SB in those samples that had intact target sites for IU and IWDO suggests that rearrangements to the viral genome in carcinomas may lead to aberrant restriction patterns in Southern blots that could result in misinterpretation of the results.

The HPV types reported after Southern blot hybridization were compared to the results after PCR/SB. Of the 83 samples positive by both techniques, the virus type could only be confirmed in 72 samples by Southern blot. Typing was based on signal strength after stringent hybridization and analysis of the Pst I restriction cleavage pattern of the virus DNA. Although 11 samples showed a positive hybridization signal under standard hybridization conditions (Tm-25^{o}C), the Pst I cleavage patterns were aberrant. This was attributed to integration of the viral genome into host sequences (Lorincz et al., 1987; Lorincz et al., 1992). Of the 72 samples in which the HPV type was determined by Southern blot, 63 (87.5%) were identified as the same type by PCR/SB. For the nine discordant samples one was reported by Southern blot as HPV 6 but was found to be HPV 18 by PCR/SB, three were HPV 16 or 16-related by Southern blot but two were typed as HPV 18 and one as HPV 31 by PCR/SB and finally, four samples reported as HPV 31 or HPV 31-related by Southern blot were typed by PCR/SB as HPV 6/11, HPV 16 and two as HPV 35.

These results can be explained in several ways. The most plausible explanation would be the presence of mixed infections in which there was preferential amplification of sequences from one virus type over that of another virus type. Also, cross-hybridization among different HPV types under standard conditions of hybridization such as that observed between HPV 16 and HPV 31 (deVilliers, 1989) could explain some of the typing discrepancies. This, as well as virus variants that exhibit aberrant cleavage patterns due to the loss or gain of a restriction site in the viral genome could lead to misinterpretation of signal on Southern blot autoradiograms. These potential difficulties with interpretation of Southern blot data may be reflected by the possible geographical distribution of HPV variants. For example, when typing results were compared with samples obtained from women from the Washignton, DC, USA area, 91.2% of the samples were condordant; however, only 73% of the samples from women from Lima, Peru were concordant.

2. Formalin-fixed, paraffin-embedded tissues

PCR/SB analysis of the 26 formalin-fixed, paraffin-embedded tissues revealed that only two (7.7%) of the samples yielded a 850 bp band. However, the amplified fragment was only detectable after the hybridization step. These results were unexpected. To confirm the presence of HPV DNA sequences in the series of samples, they were submitted to PCR/SB using the E6 primers described by Shibata et al. (1988) that generate amplification products approximately 120 nt in length. These

primers confirmed the presence of HPV sequences in 23 of the 26 specimens. In addition, the samples did not inhibit Taq DNA polymerase since amplification reactions with primers located in moderately repetitive sequences of the human genome 350 nt in length were positive. However, the reaction was not as efficient as expected since the amplified products from these primers could only be detected by hybridization. The lack of amplification using the E1 consensus primers may be due to the absence of target sites for one or both of the primers. However, formalin-fixation of tissues has been shown to break nucleic acids and limits the amplification fragment size in PCR (Greer et al., 1991). The HPV E1 consensus primers IU and IWDO which are situated 850 bp apart were considerably less efficient in the amplification reaction than primers 350 bp apart which in turn were less efficient than primers 120 bp apart. This shows that the average DNA fragment size in the formalin-fixed specimens tested here was significantly lower than 850 bp. Therefore, the utility of HPV E1 consensus primers would be limited in retrospective studies using formalin-fixed, paraffin-embedded tissues.

3. Cervical scrapings

Exfoliated cervical cells were tested for the presence of HPV DNA using the E1 consensus primers. Two populations of patients were studied. One population consisted of women seen for their yearly gynecological exam at family practice clinics were considered at low risk based on the low

rate of abnormal Pap smears traditionally seen in this population (Spriggs, 1981). The second group of women were considered at high risk for HPV infection since they had recurrent HPV infections but were cytologically negative at the time of sampling.

a. Low risk population. This population was tested for evidence of HPV infection by three methods: (i) cytology; (ii) commercial ViraPap detection kit; (iii) PCR/SB using E1 consensus primers. Comparison of the three techniques showed that 14.1% of the population was positive for HPV infection by PCR/SB which was six times greater than either cytology (2.3%) or ViraPap (2.3%) alone. ViraPap failed to detect HPV DNA in any of the samples that were reported as abnormal cytology. On the other hand, PCR/SB detected HPV DNA sequences in the four samples reported positive by cytology as well as three of the four samples tested positive the by ViraPap detection kit. These disparate results suggest that either PCR/SB or ViraPap is highly inaccurate in detecting HPV DNA in exfoliated cervical cells.

Of the 25 women with evidence of HPV infection by PCR/SB, 21 consented to colposcopic examination and biopsy. The histopathology revealed that 11 of the women had evidence for HPV infection with the diagnosis of condyloma or intraepithelial neoplasia. Clearly, PCR/SB was able to identify patients with histopathologically confirmed HPV-induced disease. Assuming PCR/SB does not have an unrecognized problem with false positives, it is a more sensitive technique for detection of HPV

DNA than either Southern blot or Pap smears. This observation is not surprising and has been made by other investigators (Burmer et al., 1990; Gravitt et al., 1991). However, correlation of colposcopic and histopathologic findings suggests that PCR/SB positivity may be an indicator of HPV-induced cervical disease and not necessarily evidence for latent infection. This study is flawed, however, by the lack of colposcopic data for women that were PCR/SB negative and the relatively small sample size. If more complete studies in the future confirm these preliminary results, PCR/SB may be a valuable adjunct for identifying women with active HPV infections.

b. High risk population. Analysis of cervical scrapings from women diagnosed with recurrent infections showed that almost half of them harbored HPV sequences based on the presence of an amplified 850 bp fragment on ethidium bromide-stained gels and Southern blot hybridization. Three samples showed a band on ethidium bromide-stained gels at the expected 850 bp size which only hybridized weakly under stringent conditions with the probes used in this work. In addition, other samples showed double bands on ethidium bromide staining where only one virus type was identified. These results suggest that a number of these samples contain potentially new HPV types.

D. Verification of the Utility of IU and IWDO to Amplify Sequences from Novel HPVs

One sample from the high risk population group of

samples, R15, where no specific signal could be seen after hybridization under stringent conditions (Tm-10°C) was further investigated. Hybridization of the cloned R15 sequence under nonstringent conditions (Tm-40°C) showed cross-hybridization to DNA from HPV types 6, 11, 16, 18, 31, 33, 35, 37, 38, 45, 51 and 52 but not cellular DNA. These results suggest that R15 represents HPV sequences. However, under stringent conditions of hybridization, no signal was seen with any of the HPV DNA clones thus indicating that the viral sequences were not closely related to a representation of known anogenital HPVs.

Pair-wise comparisons of the partial sequence of R15 to HPV sequences available in GenBank version 72 showed that no perfect match was found; however, the highest degree of homology was with HPV 16 (143 matches of 250), followed by HPV types 33, 35 and 31. HPV types 6 and 11 were more distantly related with HPVs of cutaneous origin showing even less homology. This close homology with anogenital HPV sequences, especially HPV 16, 31, 33 and 35, which belong to the high and intermediate risk virus groups, indicate that R15 may be a new type based on the cervical origin of the viral DNA. These intermediate and high risk group viruses are more associated with recurrent and progressing lesions than members of the low risk virus group. Furthermore, this high degree of homology reinforces the probability that the R15 sequence is of papillomavirus origin and not human cellular sequences amplified in a nonspecific manner. In addition, the lack of perfect match with known HPVs indicates that this sequence is clearly distinct from previously sequenced viruses.

Unfortunately, a partial HPV genome is not sufficient for type designation of a putative new virus (E.-M. de Villiers, personal communication). Cloning of the R15 viral genome could not be done from the sample obtained due to the low yield of DNA in the cervical scrape. Additional samples from the same patient could not be obtained.

Translation of the R15 nucleotide sequence revealed a conserved amino acid motif, EDSGYGNTEVETQ, present in the sequences of only intermediate and high risk viruses, with the exception of HPV type 18. This finding is in agreement with the phylogenetic tree developed based on the HPV nucleotide sequences (Campione-Picarrdo et al., 1991). In this study, the amino acid string showing homology was KR(R)xxxx(E)DSGYGNTEVETQxxM(V)QVE where (R) in HPV 33 is replaced by (K), the (E) in HPV 31 by (P) and the (V) in HPV 16 by (L). This homology at the amino acid level being limited to anogential HPVs, and more specifically, to the intermediate and high risk groups, suggests that the sequence may play a role in tissue specificity (mucosal versus cutaneous) or the type of lesion (benign versus neoplasitic).
Identification of the anogenital tract HPV E1 amino acid motif DSGYGNTEVE in the R15 sequence further establishes the HPV origin of this DNA. The presence of this motif in R15 suggests that this viral sequence is from a virus that could be a member of the intermediate or high risk group.

The R15 sequence was used as a probe against a series of clinical specimens that were either negative for HPV sequences or contained known and unknown HPV types. The hybridization results

were as expected with R15 hybridizing under nonstringent conditions only to samples containing HPV sequences. However, under stringent conditions of hybridization, two samples strongly hybridized to R15. These two samples represented mixed infection since they were originally identified as containing either HPV 18 or HPV 33. The frequency of R15 in the population of samples was 2.7%. Although R15 is relatively rare in the population studied, other virus types, most notably HPV 52, is also rare in samples obtained from the USA (Shimoda et al., 1988; Lorincz et al., 1992). However, HPV 52 is frequently encountered in samples from Japan (Yajima et al., 1988). This suggests that R15, like HPV 52, may have differences in geographic distribution.

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