

Analysis by polymerase chain reaction of the physical state of human papillomavirus type 16 DNA in cervical preneoplastic and neoplastic lesions

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Integration of human papillomavirus (HPV) DNA into the host cell genome is believed to be essential for malignant progression. However unambiguous detection of the physical state of HPV is a difficult and time-consuming procedure. To resolve this issue a simple, rapid and highly sensitive technique of polymerase chain reaction (PCR) has been utilized for detecting the physical state of HPV-16 DNA. Investigations were carried out in 122 cervical specimens comprising the whole spectrum of cervical lesions starting from cervical dysplasia to invasive carcinoma including HPV-16-positive normal controls. A pair of oligonucleotide primers specific to the E2 open reading frame, which is often deleted or disrupted following HPV integration, was used for the study. Distinction between episomal and integrated forms of viral DNA was accomplished by detecting amplification of the E2-specific fragment (1139 bp) in the PCR product. The PCR results were compared with those obtained by the

conventional methods of Southern blotting, two-dimensional gel electrophoresis and chromosomal *in situ* hybridization; a high degree of agreement was observed between the methods. The findings indicate that although integrated forms of HPV-16 DNA were detected in more than 70% of cervical cancer specimens, integration was less frequent (23%) in severe dysplasia and carcinoma *in situ*. Only 2.5% of cases showed both episomal and integrated forms of HPV-16 DNA. The difference between episomal and integrated forms was statistically significant ($P < 0.01$). The absence of integration in about 30% of cancer cases suggests that integration of HPV may not be necessary for malignant progression and alternative mechanism(s) of malignant transformation may occur without HPV integration. The PCR test thus provides an effective complement to Southern blotting and two-dimensional gel electrophoresis for accurate detection of the integration of HPV DNA.

Introduction

Cancer of the uterine cervix is the most common malignant tumour in Indian women (Luthra *et al.*, 1987; WHO Bulletin, 1986; Murthy *et al.*, 1990). Infection with specific human papillomavirus (HPV) types has been considered to be the major aetiological factor for the development of this disease (Dürst *et al.*, 1983; zur Hausen, 1989b). In India as many as 98% of cervical carcinoma cases are found to harbour HPV DNA (Das *et al.*, 1992), a frequency not seen in other countries. The most prevalent type is HPV-16 while the frequency of HPV-18 and other types is either very low or zero (Pfister, 1987; Gissmann *et al.*, 1987; Das *et al.*, 1989, 1992). Since it is generally believed that integration of viral DNA into the host cell genome may be an essential prerequisite for malignant progression (Dürst *et al.*, 1985; Gissmann *et al.*, 1987; Schneider-Maunoury *et al.*, 1987; zur Hausen, 1989a), there have been several studies (Baker *et al.*, 1987; Boshart *et al.*, 1984; Dürst *et*

al., 1986; Gissmann *et al.*, 1984; Lehn *et al.*, 1985; McCance *et al.*, 1983; Riou *et al.*, 1985; Spence *et al.*, 1988) analysing the physical state of HPVs which may serve as a prognostic indicator for the preneoplastic lesions that are likely to progress to cancers. Furthermore it may offer clue(s) for understanding the mechanism(s) involved during the process of cervical carcinogenesis.

All these studies have utilized standard methodologies involving a number of Southern blots and two-dimensional (2D) gel electrophoresis, techniques which are cumbersome and time-consuming and require a large amount of biopsy DNA. Since biopsies are generally not available from preneoplastic or early neoplastic lesions and as the yield of DNA from cervical scrapes of normally small cervical dysplasias is often inadequate, we have designed a pair of oligonucleotide primers to detect the physical state of HPV by polymerase chain reaction (PCR).

It is known that integration of HPV usually disrupts or deletes the E1 or E2 open reading frames (ORFs)

Table 1. Specification of HPV-16-specific oligonucleotide sequences used as primers and human β -globin gene in PCR

Primer region	Nucleotide sequence	Nucleotide sequence localization	Amplimer length (bp)
E2-1	5'-AGG ACG AGG ACA AGG AAA A-3'	2734-2753	1139
E2-2	5'-GGA TGC AGT ATC AAG ATT TG-3'	3853-3872	
URR-1	5'-AAG GCC AAC TAA ATG TCA C-3'	7763-7781	217
URR-2	5'-CTG CTT TTA TAC TAA CCG G-3'	57-75	
E6-1	5'-ATG CAC CAA AAG AGA ACT GC-3'	83-102	477
E6-2	5'-TTA CAG CTG GGT TTC TCT AC-3'	540-559	
E7-1	5'-TGC ATG GAG ATA CAC CTA CA-3'	563-582	291
E7-2	5'-GGT TTC TGA GAA CAG ATG GGG-3'	834-854	
β -Globin-1	5'-GAA GAG CCA AGG ACA GGT AC-3'	-	268
β -Globin-2	5'-CAA CTT CAT CCA CGT TAC ACC-3'	-	

(Awady *et al.*, 1987; Choo *et al.*, 1987, 1988; Schwarz *et al.*, 1985), which could lead to over-expression of E6/E7 oncoproteins and subsequent malignant transformation (Choo *et al.*, 1988; Schwarz *et al.*, 1985). If this is true, there will be an absence of amplification of the E2 gene sequences in the PCR product following integration, or vice versa. Based on this hypothesis, a pair of oligonucleotide primers from the downstream E2 ORF of the HPV-16 genome has been synthesized and used for the present study to analyse the physical state of the virus in tumour DNA. The PCR results were compared to those obtained by Southern blotting, 2D gel electrophoresis and *in situ* hybridization to confirm the specificity and reliability of the PCR technique. We report that the PCR method offers easy detection of unintegrated viral DNA sequences and provides an effective complement to Southern blotting and 2D gel electrophoresis for accurate analysis of the physical state of HPV DNA.

Methods

Study population and specimens. A total of 21 carcinoma *in situ* (CIS) and 59 specimens of different grades of dysplasias including 10 normal controls, which were diagnosed histo- or cytopathologically (see Table 1) and found positive for HPV-16 DNA, were used for the present study. Also, 42 HPV-16-positive invasive carcinoma specimens, which had been analysed earlier for the physical state of HPV by combined restriction digestion and 2D gel electrophoresis, were also employed for the PCR test.

DNA extraction. The tissue biopsies collected in culture medium (Dulbecco's MEM) were immediately divided into three parts. One part was used for histology and a second part for chromosome preparation. The rest was kept frozen in a deep freezer (at -70°C) and was later used for DNA extraction. The detailed DNA extraction method is described elsewhere (Das *et al.*, 1992). Briefly, following homogenization in 2 ml of 10 mM-Tris-HCl and 1 mM-EDTA (pH 8.0), the tissue was suspended in equal volumes of 3% SDS/Sarkosyl and digested with 100 $\mu\text{g}/\text{ml}$ proteinase K (Boehringer-Mannheim) for 1 to 2 h at 37°C . DNA was extracted with phenol-chloroform-isoamyl alcohol three times. DNA was precipitated with 3 M-sodium acetate (pH 5.0) and absolute ethanol. Cervical scrapes from dysplasia cases

and normal controls were collected in PBS and DNA was extracted according to a newly developed method (Gopalkrishna *et al.*, 1992) employed routinely in our laboratory. The purity and concentration of DNA was determined in ethidium bromide-stained mini-gels.

Restriction enzyme digestion and gel electrophoresis. Ten μg of total cellular DNA from each sample was digested first with *Pst*I to detect HPV-16-positive cases with the authentic *Pst*I cleavage pattern. To detect shifting of off-sized bands, 10 to 15 μg biopsy DNA was digested with no-cut (*Hind*III), single-cut (*Bam*HI) and multicut (*Pst*I) enzymes for HPV-16, and was electrophoresed in a 1% agarose gel at 30 V for 16 to 20 h.

2D gel electrophoresis. To differentiate between linear and circular extrachromosomal HPV DNA molecules, 2D gel electrophoresis was done using undigested/sheared DNA or DNA incubated with a no-cut enzyme, *Hind*III-digested tumour DNA. Following separation in a 0.4% agarose gel in the first dimension, DNA was again electrophoresed in the second dimension in 0.8% agarose gels (Dürst *et al.*, 1985; Wettstein & Stevens, 1982).

Southern blot and hybridization. After electrophoresis the gels were stained in ethidium bromide and photographed under a short-wave u.v. transilluminator. Gel-separated tumour DNA was transferred on to Gene-Screen Plus membranes (DuPont) by the method of Southern (1975) with certain modifications. Following prehybridization at 42°C for 1 to 2 h, the filters were hybridized with a ^{32}P -labelled HPV-16 probe (Dürst, 1987). Prototype HPV-16 DNA was made vector-free using gel electrophoresis, purified on a DEAE membrane and was nick-translated to a specific activity of 1×10^8 to 2×10^8 c.p.m./ μg DNA. Hybridization was performed under stringent conditions ($T_m - 20^{\circ}\text{C}$). The hybridization mix contained 50% formamide, $5 \times \text{SSC}$, 0.02% Denhardt's solution, 0.25 $\mu\text{g}/\text{ml}$ tRNA and 50 mM- Na_3PO_4 and incubation was at 42°C for 16 to 20 h. One genome equivalent of HPV-16 DNA (10 pg) was used as a positive control marker to check the sensitivity of hybridization.

Dot blot hybridization. Six to eight μg of DNA extracted from cervical scrape cell samples was denatured at 65°C for 30 min and then chilled immediately on ice and blotted onto a nitrocellulose membrane using a dot blot apparatus (Minifold I; Schleicher & Schüll). After either baking at 80°C for 1 h or cross-linking under u.v. light, the membrane was hybridized under stringent conditions ($T_m - 20^{\circ}\text{C}$) using ^{32}P -labelled HPV probes.

PCR. For detection of integration, two specific oligoprimers from the downstream E2 ORF of the HPV-16 genome between nucleotides 2734 and 3872 were used. In addition, oligonucleotide primers from the upstream regulatory region (URR) and E6/E7 ORFs of HPV-16 and a

pair of primers from the human β -globin gene were used to check HPV positivity as well as the purity of tumour DNA for the PCR test. The primers were synthesized in an Applied Biosystems DNA Synthesizer (Model 381A, Applied Biosystems) and were purified in NAP-10 columns (Pharmacia). The primer sequences are presented in Table 1.

PCR amplification (Saiki *et al.*, 1988) was carried out using about 50 to 100 ng of purified tumour DNA in 50 or 100 μ l reaction mix containing 1 μ M of each primer, 50 mM-KCl, 10 mM-Tris-HCl pH 8.3, 2.5 mM-MgCl₂, 75 μ g/ml BSA, 200 μ M of each dNTP and 1 to 2 units of *Taq* DNA polymerase (Cetus Corporation). The reaction mix was overlaid with 50 μ l mineral oil in order to avoid evaporation. Amplification was done for 35 to 40 cycles in a Cetus DNA Thermal Cycler (Perkin-Elmer Cetus). The first cycle was 94 °C for 5 min (denaturing), 55 °C for 1 min (annealing) and 72 °C for 2 min (extension) followed by 38 cycles each at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min. The last cycle was as above except that the extension period at 72 °C was extended up to 7 min. After amplification, 15 to 20 μ l of the mix was electrophoresed in a 3% Nusieve-agarose gel with ethidium bromide and photographed under a u.v. transilluminator.

Chromosome preparation from tumour biopsy specimens and in situ hybridization. To confirm integration of HPV DNA into host cell chromosomes, attempts were made to visualize integration sites in tumour chromosomes by *in situ* hybridization. A part of the biopsy specimen collected in tissue culture medium (DMEM) was immediately processed for either direct preparation or primary culturing for chromosome preparation.

Chromosome preparation. The free tumour cells along with minced tumour tissue were incubated at 37 °C in 5 ml medium supplemented with serum and colcemid (0.02 μ l/ml) for 5 to 6 h. Following centrifugation the cell pellet was given a hypotonic treatment for 15 to 20 min in 0.75 M-KCl and the cells were fixed in methanol:acetic acid (3:1) fixative and washed three or four times in fresh fixative. Air-dried chromosome slides were prepared by making a final cell suspension in 0.5 ml freshly prepared chilled fixative.

Alternatively, a part of the tumour tissue was denatured by trypsin or collagenase treatment, washed in PBS and cultured in DMEM supplemented with 10% foetal calf serum (FCS) and antibiotics. Within 7 to 10 days when the culture was almost confluent, it was harvested for chromosome preparation following prolonged colcemid treatment.

In situ hybridization. Chromosome *in situ* hybridization was carried out using a method used for single copy gene localization (Neel *et al.*, 1982). A vector-purified 8 kb insert of HPV-16 was nick-translated to a specific activity of 1×10^8 or 2×10^8 c.p.m./ μ g DNA using three ³H-labelled nucleotides (dTTP, dCTP and dATP, Amersham). Following proteinase K and RNase treatment of slides, the chromosomal DNA was denatured in 70% formamide in $2 \times$ SSC for 3 min. Hybridization was performed for 48 h at 42 °C in a moist chamber. Following washing and drying, the slides were coated with Kodak NTB II or III nuclear track emulsion (Eastman Kodak) in the dark, and were stored in the dark at 4 °C under moisture-free conditions for 5 to 6 weeks. The slides were developed in D-19b developer, stained in 5% Giemsa in phosphate buffer pH 6.8 and observed under a Zeiss photomicroscope (Germany) using oil immersion objectives.

Results

Presence of HPV-16 genome in all invasive cervical carcinoma, CIS and dysplasia cases

All invasive cervical cancer biopsy specimens diagnosed histopathologically were initially screened using the ³²P-

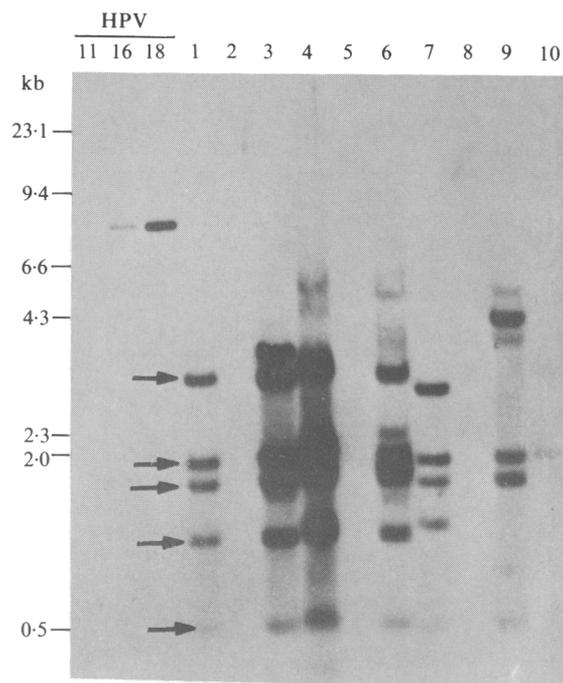


Fig. 1. Southern blot hybridization analysis of HPV-16 DNA sequences in cervical cancer biopsy specimens. Ten micrograms of cellular DNA was digested with *Pst*I, electrophoresed in a 1% agarose gel and hybridized with a ³²P-labelled HPV-16 + 18 DNA probe under stringent conditions ($T_m - 20$ °C). Results are shown for 10 specimens (lanes 1 to 10) of which seven were positive (lanes 1, 3, 4, 6, 7, 9 and 10) for HPV-16. The characteristic *Pst*I cleavage pattern of HPV-16 is shown by arrows and the fragment sizes are indicated in kilobases. The first two lanes served as positive control markers having 10 and 50 μ g of HPV-16 and -18 DNA respectively.

labelled vector-free 8 kb insert of HPV-16. *Pst*I, a multi-cut enzyme for HPV-16, was used to detect authentic restriction fragments of the viral genome. Forty-two HPV-16-positive specimens which showed the characteristic *Pst*I cleavage pattern, i.e. 2.8, 1.9, 1.6, 1.0 and 0.5 kb bands, and had at least five to 10 copies of the viral genome were selected for the analysis of the physical state. The autoradiogram presented in Fig. 1 shows the *Pst*I-specific fragments (arrows) of HPV-16. There are cases (lanes 3 and 4) which showed a very high copy number of HPV-16 including certain genomic rearrangements and the presence of off-sized fragments. All these HPV-16-positive specimens were employed for physical state analysis by four different molecular techniques. Since the amounts of DNA extracted from cervical scrapes of dysplasia and control cases were insufficient, these were confirmed by dot blot hybridization and the physical state was analysed by PCR only. All 122 HPV-16-positive samples were found to show amplification of both HPV-16-specific primers as well as the β -globin gene in PCR (See Fig. 2).

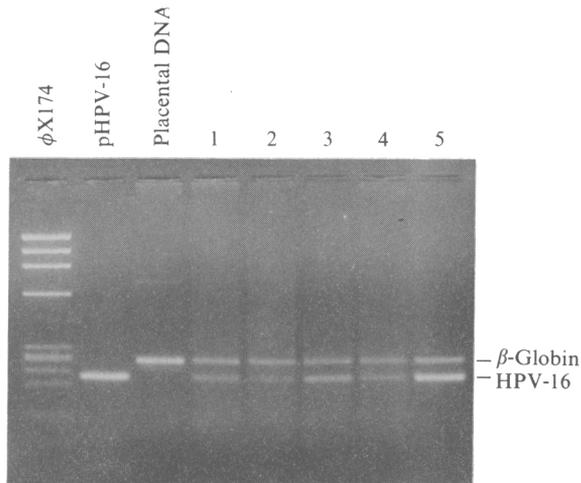


Fig. 2. Amplification of oligonucleotide primers from the URR region of HPV-16 along with human β -globin gene as an experimental control. First three lanes are *Hae*III-digested ϕ X174 DNA size markers, HPV-16 plasmid DNA showing 217 bp amplification and human placental DNA showing 268 bp β -globin gene amplification respectively. Lanes 1 to 5 are cervical carcinoma DNA samples showing amplification of HPV-16 as well as β -globin gene primers in the same reaction mix.

The majority of tumour DNA showed shifts of off-sized fragments after digestion with various enzymes

In order to determine the physical form of viral DNA, 10 to 15 μ g of tumour DNA was digested with a no-cut enzyme for HPV-16 (*Hind*III), a single-cut enzyme (*Bam*HI) and a multicut enzyme (*Pst*I). These digested tumour DNAs together with undigested sheared DNA samples and one genome equivalent (10 pg) of vector-free HPV-16 DNA were electrophoresed on a 1% agarose gel. Southern blot hybridization was performed with an HPV-16 DNA probe. Fig. 3 is a representative autoradiograph showing results for two specimens (a, b). For specimen (a) the lanes UD and NC show three forms of high M_r DNA and also, as expected, lanes SC and MC show the single 8 kb band and five *Pst*I fragments respectively. Clearly, there is an absence of off-sized fragments and this is indicative of the episomal DNA form. In contrast, in specimen (b), there were a number of off-sized fragments (thin arrows) along with authentic *Bam*HI and *Pst*I fragments (thick arrows) and a single high M_r DNA band in lanes UD and NC. This suggests that HPV DNA was in an integrated state and is indicative of a tandem integration of HPV-16. Of 42 specimens, 29 (69%) showed integrated forms, and in 11 (26%) cases HPV DNA was found to be exclusively episomal. Only two cases (4.76%) revealed the presence of both episomal and integrated forms; in these we did not observe either off-sized bands or E2 amplification (see Tables 2 and 3).

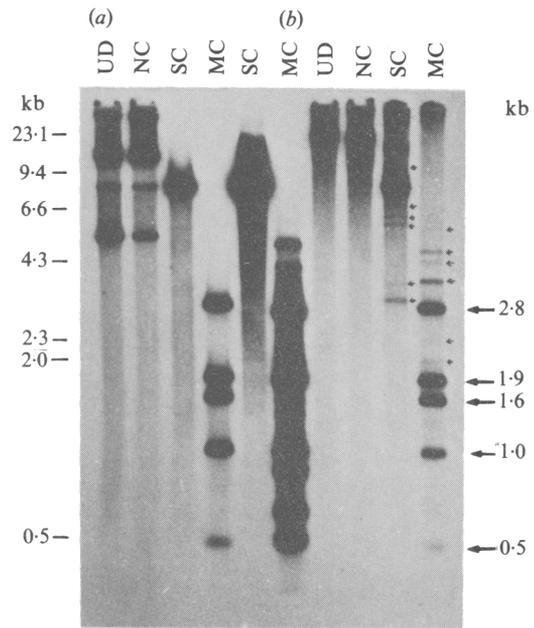


Fig. 3. Southern blot hybridization of two cancer biopsy DNA following combined digestion with no-cut (NC), *Hind*III, single-cut (SC), *Bam*HI, and multicut (MC), *Pst*I, in addition to lanes of undigested (UD) DNA. In the episomal form (a) it shows characteristic *Bam*HI and *Pst*I cleavage patterns along with three forms of high M_r DNA in UD and NC but no indication of additional off-sized fragments. In the integrated form (b) there is a shift in light off-sized fragments (thin arrows) in lanes SC and MC along with authentic *Pst*I fragments (thick arrows) suggesting integration of head-to-tail tandem repeats at more than one site.

2D gel electrophoresis: majority of single track linear DNA molecules

2D gel electrophoresis was carried out using undigested or *Hind*III-digested tumour DNA (*Hind*III does not cut in HPV-16 DNA) to reveal linear or circular extrachromosomal DNA molecules of HPV. The integration of HPV DNA is indicated by a single track of linear DNA molecules in the second dimension of an 0.8% agarose gel following separation in 0.4% agarose in the first dimension. Representative Southern blot hybridization pictures presented in Fig. 4(a, b) and 5(a, b) show the 2D gel analysis of the same two samples depicted in Fig. 3(a) and (b) respectively. Fig. 4(a, b) with a track of circular DNA molecules is indicative of the episomal form, detected in 13 samples (31%). Some linearized DNA is due to degradation. All 42 specimens analysed by 2D gel electrophoresis showed perfect correlation with the results obtained by the multiple enzyme digestion assay. All the 29 integrated samples (69%) with off-sized fragments revealed a single track of linear DNA molecules (Table 2). This confirmed the integrated state of HPV-16 DNA in a majority of invasive cervical cancers.

Table 2. Physical state of HPV-16 DNA in precancerous and cancerous lesions of the uterine cervix and in normal samples

Histo-cytological grade	No. of cases analysed (n = 122)	Episomal form (%)	Integrated form (%)	Episomal + integrated form (%)
Normal	10	10 (100)	0	0
Mild dysplasia	22	22 (100)	0	0
Moderate dysplasia	17	17 (100)	0	0
Severe dysplasia	10	9 (90)	1 (10)	0
Carcinoma <i>in situ</i>	21	15 (71.43)	5 (23.8)	1 (4.76)
Invasive carcinoma	42	11 (26.19)	29 (69.05)	2 (4.76)

Table 3. Analysis of physical state of HPV-16 DNA in invasive cervical cancers by different molecular methods

Molecular method employed	Total no. of cases studied	Episomal form (%)	Integrated form (%)	Episomal + integrated forms (%)
Combined restriction enzyme digestion assay	42	13 (30.95)	29 (69.05)	0
2D gel electrophoresis	42	13 (30.95)	29 (69.05)	0
E2 ORF-specific primer-directed amplification in PCR	42	11 (26.19)	29 (69.05)	2 (4.76)
Chromosomal <i>in situ</i> hybridization	3	0	3 (100)	0

Absence of PCR amplification of E2 ORF primers in the majority of cervical cancer specimens

It is generally accepted that because of integration the E2 ORF region of the HPV genome is deleted or disrupted (Awady *et al.*, 1987; Choo *et al.*, 1987, 1988; Schwarz *et al.*, 1985). If this is true, there will be no amplification of the E2 sequences in PCR. In the present study, out of 42 invasive carcinoma samples only 11 showed amplification and the others showed no amplification of the expected 1139 bp fragment (see Fig. 6a). In two samples off-size fragments and linear DNA molecules could not be detected, suggesting an episomal configuration. However, as these samples also failed to show E2 amplification, suggestive of integration, they may contain episomal as well as integrated forms (Table 3). Using PCR, 73.8% (31/42) were found to be in integrated forms whereas only 26.2% (11/42) were in the episomal form (Tables 2 and 3). Fig. 6(a) shows the results from eight carcinoma samples; only three (lanes 4, 6 and 7) were found to contain integrated HPV DNA, as revealed by the absence of amplification.

The majority of precancerous lesions show amplification of the E2 ORF sequences

Since the amount of DNA extracted from cervical scrapes of dysplasia and CIS cases was not sufficient to

carry out Southern blotting and 2D gel electrophoresis, these were analysed by PCR only. Of 49 dysplasia cases, consisting of 22 mild, 17 moderate and 10 severe dysplasias, all showed E2 amplification except one (2.04%); an absence of amplification was recorded in five cases (23.8%) of 21 CIS specimens analysed. Only one case (4.76) revealed HPV in both the episomal and integrated states (see Table 2). Fig. 6(b) shows the results for eight representative CIS samples of which only one (lane 1) showed the absence of E2 amplification, hence integration. The last two lanes (lanes 9 and 10) in the lower panel are control male and female lymphocyte DNA. All the 10 normal controls showed the presence of extrachromosomal DNA as scored by the appearance of a 1139 bp E2-specific band in PCR.

In situ detection of HPV-16 DNA integration in tumour chromosomes

In a preliminary attempt, chromosomes from only three invasive cancer biopsy specimens which were also analysed by other methods could be prepared by a direct method for visualization of integration sites on chromosomes. Mostly polyploid cells were observed with chromosome numbers varying from 60 to 70 with a mode of 66 chromosomes. Various structural abnormalities were also observed. Fig. 7(a, b) shows the *in situ*

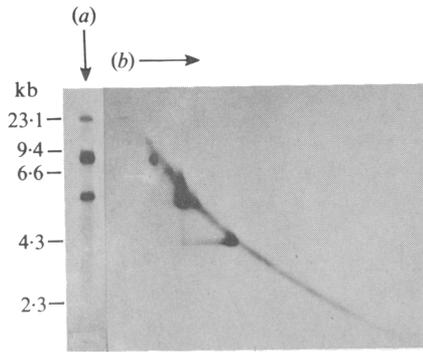


Fig. 4. Two-dimensional gel electrophoresis of HPV-16-positive DNA (10 µg) from cervical cancer biopsy showing episomal state of viral genome. (a) Undigested/sheared DNA shows viral DNA in three forms in the first dimension (separated in a 0.4% agarose gel). Form I is supercoiled virion DNA, form II nicked circular and form III linear DNA. (b) One of the first dimension lanes was separated again in an 0.8% agarose gel in the second dimension by rotating the gel lane through 90° and casting a new gel; a track of circular DNA molecules was detected. Indication of a light linearized DNA track is due to degradation/nicking of DNA during preparation.

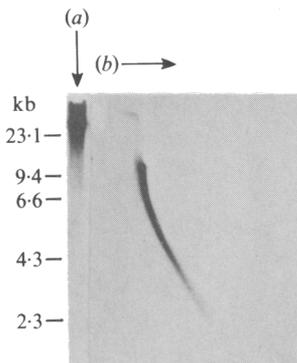


Fig. 5. Two-dimensional gel electrophoresis of HPV-positive biopsy DNA (10 µg) showing integrated state of viral DNA. (a) First dimension (0.4% gel), undigested/sheared DNA showing a single band of high M_r (>23.1 kb) viral DNA; (b) second dimension (0.8%), one track of linear DNA molecules indicating integrated state of HPV DNA within the host genome.

hybridization and integration sites (arrows) of HPV-16 DNA in tumour chromosomes. There are three integration sites in Fig. 7(a) whereas 7(b) shows four integration sites. It appears from the grain concentration that the HPV copy number may vary from about five (Fig. 7b) to 50 (Fig. 7a). In view of their poor morphology and inadequate number of metaphases, no attempts were made to band and identify the chromosomes. The three specimens in which *in situ* localization could be done had already been found to have integrated forms of HPV DNA by three other molecular approaches. HPV DNA in the same specimen (Fig. 7a) can be seen integrated in Fig. 3(b), 5(a, b) and 6(a), lane 4.

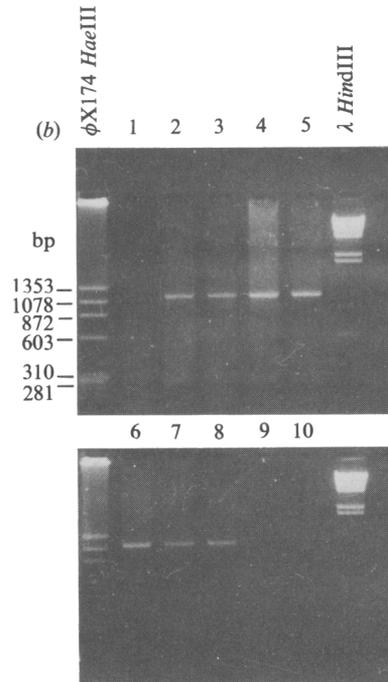
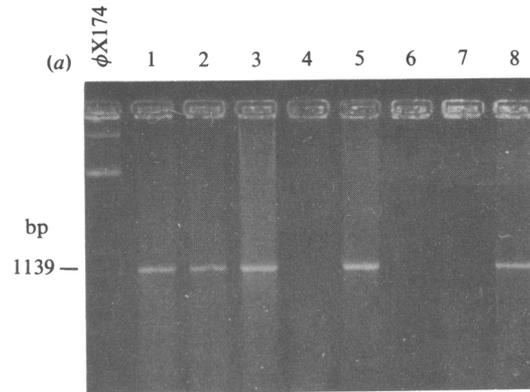


Fig. 6. Amplification of HPV-16 E2 ORF-specific oligoprimers, E2-1 and E2-2, in (a) invasive carcinoma and (b) CIS DNA by PCR. Twenty µl PCR products show amplification of E2 sequences between nucleotides 2734 and 3872, i.e. 1139 bp, in 3% NuSieve agarose gel electrophoresis, ethidium bromide staining and u.v. irradiation. (a) Carcinoma DNA samples showing absence of E2 amplification in three lanes (4, 6 and 7) out of eight cases. (b) Lanes 2 to 8 (2 to 5 in upper and 6 to 8 in lower panel) show E2 amplification indicating episomal state of HPV; the absence of amplification in lane 1 is indicative of integrated viral DNA. Lanes 9 and 10 in the lower panel contain lymphocyte DNA from normal male and female controls respectively.

Discussion

Observation of *Pst*I-specific restriction fragments for HPV-16 on Southern blotting is generally taken to be a reliable method for detection of the viral genome in cervical lesions. As shown in Fig. 1 we have detected the characteristic *Pst*I cleavage pattern of HPV-16 in all HPV-16-positive cervical cancer specimens and this is in

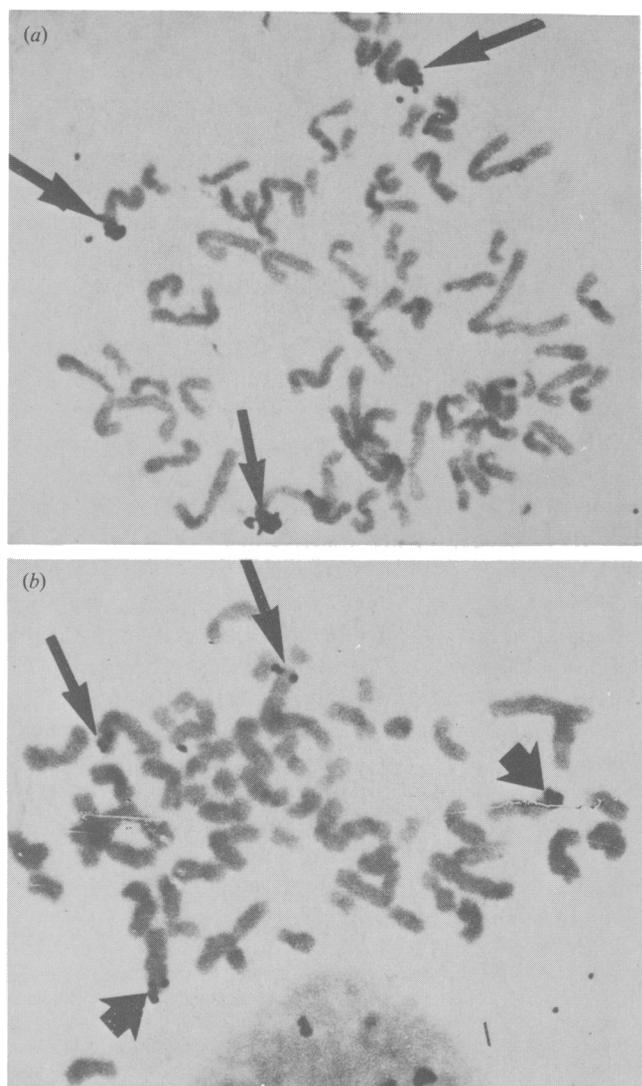


Fig. 7. Directly prepared metaphase chromosomes from invasive cancer biopsy specimens showing HPV-16 integration sites involving three chromosomes (a) or four chromosomes (b) (arrows). Chromosome slides were hybridized with ^3H -labelled dTTP, dCTP and dATP under stringent conditions and coated with Kodak NTB II nuclear track emulsion.

agreement with earlier reports (Yoshikawa *et al.*, 1985; Scholl *et al.*, 1985; Lancaster *et al.*, 1986; Meanwell *et al.*, 1987). However some samples showed deletions or certain genomic rearrangements leading to an atypical pattern which also may be indicative of integration of HPV-16 DNA into the host cell genome (Lehn *et al.*, 1985; Millan *et al.*, 1986; Choo *et al.*, 1987; Takebe *et al.*, 1987; Wilczynski *et al.*, 1988). In almost all cases, two *Pst*I fragments, i.e. 1.9 and 1.6 kb, representing the E6/E7 and L1/L2 regions respectively, are found to be maintained (Fig. 1). This indicates that the presence of these specific viral sequences are essential for maintenance of a malignant phenotype.

It has been reported that HPVs such as types 6 and 11 detected in benign lesions and warts are often present in an episomal form (McCance *et al.*, 1983; Gissmann *et al.*, 1984) whereas HPV types 16 and 18 associated with invasive cervical cancers and CIS are usually integrated into the host genome (Dürst *et al.*, 1985; Schwarz *et al.*, 1985; Lehn *et al.*, 1985; Riou *et al.*, 1985; zur Hausen, 1989b). These two oncogenic HPVs have also been shown to be in an integrated form in a majority of established cell lines derived from cervical carcinomas (Boshart *et al.*, 1984; Yee *et al.*, 1985; Tsunokawa *et al.*, 1986; Mincheva *et al.*, 1987; Baker *et al.*, 1987; Spence *et al.*, 1988). It is, therefore, suggested that integration of viral DNA into the host genome may be an essential prerequisite for malignant transformation. In the present study, as many as four different molecular techniques were employed (see Table 3) and the results indicate about 70% integration of HPV-16 DNA in cervical cancer specimens. The basis of our interpretation was as follows. (i) Combined digestion with no-cut (*Hind*III), single-cut (*Bam*HI) and multi-cut (*Pst*I) enzymes and comparison of band patterns with that of undigested DNA, irrespective of copy number and minor rearrangements of HPV DNA (Fig. 3a, b), showed off-sized bands. The detection of bands larger than 23.1 kb (see Fig. 3b) in lanes UD and SC was indicative of integration. In addition the presence and/or shifting in the size of off-sized fragments in lanes SC and MC in Fig. 3(b) together with the authentic *Bam*HI and *Pst*I cleavage pattern respectively, suggests integration of head-to-tail tandem repeats at more than one site (Dürst *et al.*, 1983; Seedorf *et al.*, 1985). All the off-sized fragments detected cannot be due to minor rearrangements only. (ii) These results were confirmed by 2D gel electrophoresis in which linear and circular forms of viral DNA can be differentiated (see Fig. 4a, b and 5a, b). The detection of a single track of linear HPV DNA molecules rather than the circular DNA track that is characteristic of the episomal form is considered to be due to an integrated state of viral DNA. (iii) There was no PCR amplification of specific oligoprimers from the downstream E2 ORF (see Fig. 6a, b), since viral integration leads to disruption or deletion of E1 and E2 ORFs and perhaps deregulation of expression of other early genes (Schwarz *et al.*, 1985; Lehn *et al.*, 1985; Choo *et al.*, 1987; Baker *et al.*, 1987; Cripe *et al.*, 1987; Shirasawa *et al.*, 1988; Takebe *et al.*, 1987; Wilczynski *et al.*, 1988). (iv) Finally, we used *in situ* localization and visualization of HPV-16 DNA integration sites in tumour chromosomes (see Fig. 7a, b).

There was a high degree of agreement between the observations obtained by these four different techniques, which overall demonstrate that integrated forms of HPV-16 DNA are present in a majority (69%) of cervical cancer specimens (Table 2). This is in accordance with

the findings of several authors (Dürst *et al.*, 1985, Dürst, 1987; Lehn *et al.*, 1985; Riou *et al.*, 1985; zur Hausen, 1989*b*; Cullen *et al.*, 1991) but is not in conformity with the recent report of Matsukura *et al.* (1989) who showed that the frequency of integration of HPV-16 DNA in cervical carcinoma is only 30% compared to the 70% that we and others have observed.

Unambiguous detection of the physical state of HPV DNA in tumour DNA specimens is a difficult and cumbersome procedure. Generally, it poses problems when both episomal as well as integrated forms are present in a specimen, or a small sequence of HPV DNA is integrated. Thus there have been several conflicting reports (Cullen *et al.*, 1991; DiLuca *et al.*, 1986; Lehn *et al.*, 1988; Matsukura *et al.*, 1989). To resolve this issue, the present study was initiated to develop a convenient and sensitive method for detection of HPV DNA integration. Because of its high sensitivity, the recently developed PCR (Saiki *et al.*, 1988) is rapidly becoming the preferred method for various diagnostic purposes. The present PCR results compare well with those obtained by standard procedures. In tumour DNA specimens, the frequent lack of amplification of E2 ORF sequences indicates that the E2 region is preferentially disrupted or deleted due to integration of HPV. The results for 42 carcinoma samples which were analysed by both Southern blotting and 2D gel methods are largely comparable with those obtained by the present PCR method. This was further confirmed by visualization of HPV DNA integration sites in tumour chromosomes prepared directly from the same tissue specimens (Fig. 7*a, b*). The PCR method thus facilitates the detection of a low level of HPV integration which may not be detected by conventional methods. It allows processing of a large number of samples at a time and requires a small amount of tumour DNA. However, to rule out the possibility of integration outside the 1139 bp region or at the E1 ORF region, additional sets of primers from these regions may have to be used for further confirmation. Alternatively, amplification of virus-cell junctions could be most appropriate, although it would be difficult to know the 'fusion sequences' of the junctions for every case without cloning and sequencing. Nevertheless, since PCR results are in good agreement with the results obtained by standard procedures, it can be reliably used for rapid analysis of the physical state of oncogenic HPV-16. This is most useful in screening pre-neoplastic or early neoplastic lesions from which biopsies are generally not available and the yield of DNA from scraped cervical cells is often inadequate.

Although there are reports that about 50% of integration events occur in preneoplastic lesions, particularly those of CIN III cases (DiLuca *et al.*, 1986; Lehn *et al.*, 1988), we could detect only 19% ($n = 31$)

integration in these cases (severe dysplasia and CIS cases) (see Table 2). This is in sharp contrast to the recent report of Cullen *et al.* (1991) who could detect only 3% integration in these cases. Observation of such a low frequency of integration may be due to either geographic variation or non-detection of small integrated sequences by conventional methods. The PCR test, therefore, can provide an effective complement to Southern blotting or 2D gel electrophoresis for an accurate detection of the physical state of HPV DNA.

Although it has been suggested that integration of HPV DNA into the host cell genome is essential for malignant progression (Dürst *et al.*, 1985; Gissmann *et al.*, 1987; Schneider-Maunoury *et al.*, 1987; zur Hausen, 1989*a, b*) it seems unlikely that integration alone is sufficient to account for malignant proliferation. This is not only because of the absence of integration in about 30% of carcinomas as observed by us and others (Cullen *et al.*, 1991) but is also supported by evidence from cell hybrids produced by fusion of HeLa cells and normal human fibroblasts or keratinocytes (Stanbridge, 1976). These hybrids contained HPV-18 in an integrated form but the malignant phenotype was not expressed (Schwarz *et al.*, 1987; Stanbridge *et al.*, 1982). Therefore a detailed understanding of the expression of HPV transforming genes, E6/E7, along with other growth regulatory cellular genes following infection by HPV may throw light on the mechanisms involved during the malignant progression of cervical lesions.

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