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Comparison of L1 Consensus Primers with E6 Type Specific Primers for Detection of Human Papillomaviruses in Paraffin Sections of Cervical Neoplasia

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In our comparative study of L1 consensus primers with E6 type-specific primers for detection of human papillomavirus (HPVs) by polymerase chain reaction (PCR) in 35 cases of cervical neoplasia, the detection rate by E6 primers (54% ; 19/35) was significantly higher than that by L1 primers (25%; 9/35) ($p < 0.01$). And all specimens HPV-positive with L1 primers were also positive by E6 primers. HPV DNA could be amplified in 36% (9 of 25) of tissue by L1 consensus primers from which β-globin gene was amplified as compared with 64% (16 of 25) of tissue by E6 type-specific primers. With the L1 consensus primers, 8 cases were positive for HPV-16 and 1 case was positive for HPV-33. These results show that the L1 consensus primers have inferior sensitivity to the E6 type-specific primers for the detection of HPV by PCR. But the L1 consensus primers have great value in making simultaneous detection of various HPV types in a single tube reaction, thus they permit reduction of time and the economic burden of the experiment.

Key Words: *human papillomavirus (HPV), L1 consensus primers, E6 type-specific primers, polymerase chain reaction (PCR)*

INTRODUCTION

The genital human papillomaviruses (HPVs) comprise a group of over 20 viral subtypes and each HPV type is associated with distinct clinical manifestations (zur Hausen, 1989; Jenson & Howley, 1990). For example, HPV-6 and -11 have been associated with benign and low grade cervical intraepithelial neoplasias (CINs), while HPV-16 and -18, and to a lesser extent, HPV-31, -33, and -35 have been linked with high grade CINs and cervical carclinomas (Lorincz et al., 1987; Reid et al., 1987).

The HPV genome, which was molecularly cloned from a productive lesion and sequenced, consists of a double-stranded, circular DNA with 7, 946 base pairs in length. Analysis of the genome sequence indicates that the individual frames are classified as "early" (E) or "late" (L) in analog with other DNA viruses where genes are turned on according to a specific time schedule in the course of viral life cycle (Shah & Howley, 1990).

Newly developed, highly sensitive polymerase chain reaction (PCR) allowed in vitro enzymatic amplification of HPV DNA sequences in routinely processed pathological specimens (Shibata et al., 1988; Park et al., 1991a). We have already reported the results of HPV detection in cervical neoplastic tissue from Korean women using PCR amplification of 103 to 298 base pair regions of the E6 open reading frame (ORF) of the virus and emplogying type-specific primers (Park et al., 1991b).

Manos et al., (1989) have designed consensus oligonucleotide primer pairs to generate PCR products simultaneously from several types of genital HPVs, taking advantage of homologous interspersed regions of

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L1 ORF. The consensus primers were aimed to amplify a 450 base pair region of L1 ORF of many HPV types. The consensus primer pair allows amplification of the other known or unidentified genital HPVs in a single tube reaction and characterization of HPV types by type-specific oligonucleotide probes.

This study was attempted to detect and type HPVs in routinely processed, formalin-fixed, paraffin-embedded cervical neojplastic tissue from Korean women using *in vitro* amplification by the L1 consensus primers comparing with PCR results by the E6 type-specific primers.

MATERIALS AND METHODS

Tissue samples and processing

Tissue samples of 20 CINs, 5 microinvasive and 10 invasive squamous cell carcinomas were obtained at the Dysplasia Clinic, Department of Obstetrics and Gynecology, Catholic University Medical College, Seoul, Korea, from 1987 to 1989. Colposcope-directed biopsies and surgically excised specimens were fixed in neutral phosphate-buffered formalin immediately, and were processed for paraffin embedding by routine procedure. Pathologic diagnosis was made by standard criteria (Richardt, 1973; Hertig, 1979). Oligonucleotides were synthesized by the phosphoramidite method on a DNA synthesizer (Pharmacia, Sweden) and purified by electrophoresis on 20% polyacrylamide gels containing 7 M urea. Tissue sections were processed for PCR as described by Wright and Manos (1990). Briefly, each section was placed in a tube and extracted with octane to dissolve the paraffin and then extracted with absolute ethanol. The samples were resuspended in 100 μl of proteinase-K buffer (10mM Tris, 1% Laureth-12, 10 μg proteinase K) at 37°C for 12 to 16 hours. Proteinase-K was inactivated by boiling the specimens for 7 minutes, and 10 μl aliquot was used as a template for PCR.

Polymerase chain reaction

Details of the test described previously (Manos et al., 1989; Resnick et al., 1990; Park et al., 1991b, 1992). PCR was performed simultaneously for amplification of HPV sequences and human β-globin sequence. HPVs were amplified by consensus primers (Ting & Manos, 1990) which were designed to amplify an approximately 450 base pair region of L1 ORF of many HPV types (Table 1). The β-globin primers, which amplified a 268 base pair region of the gene, were used to evaluate the suitability of the DNA in the paraffin-embedded tissues (Saiki et al., 1985). Aliquots of processed specimens were added to a reaction mixture (total volume of 100 μl) containing 50 mM KCl, 6mM $MgCl_2$, 10 mM Tris (pH 8.3), 200 μM of each dNTPs, 100 μg/μl gelatin, 2.5 units Taq polymerase (Perkin-Elmer Cetus Instrument, Emeryville, CA), HPV L1 consensus primers at 0.5 μM and human β-globin primers at 0.05 μM and subjected to 40 cycles of amplification, consisting of 1 minute at 94°C, 1 minute at 55°C and 1 minute at 72°C PCR products were electrophoresed through 3% Nusieve and 1% agarose gel, transferred to nylon

Table 1. HPV L1 Consensus Oligonucleotide Primers and Probes for PCR

L1 consensus primers		
MY 09	CGTCCMARRGGAWACTGATC	
MY 11	GCMCAGGGWCATAAYAATGG	
L1 HPV specific probes		
MY 12	CATCCGTAACTACATCTTCCA	(HPV 6)
MY 13	TCTGTGTCTAAATCTGCTACA	(HPV 11)
MY 14	CATACACCTCCAGCACCTAA	(HPV 16)
WD 74	GGATGCTGCACCGGCTGA	(HPV 18)
WD 126	CCAAAAGCCCAAGGAAGATC	(HPV 31a)
WD 128	TTGCAAACAGTGATAGTGCACG	(HPV 31b)
MY 16	CACACAAGTAACTAGTGACAG	(HPV 33)
L1 consensus probes		
MY 1019	CTGTGGTAGATACCACWCGCAGTAC	
MY 18	CTGTTGTTGATACTAACGCAGTAC	

(M denotes A Plus C; R denotes A plus G;
W denotes A plus T; Y denotes C plus T)

filters, and identified by hybridization and autoradiography with, ^{32}P-labeled oligonucleotide type-specific HPV, consensus HPV and β-globin probes. The digesting patterns by specific restriction enzymes of 450 base pair sized amplified products from each HPV L1 are shown in Figure 1. It was possible to distinguish

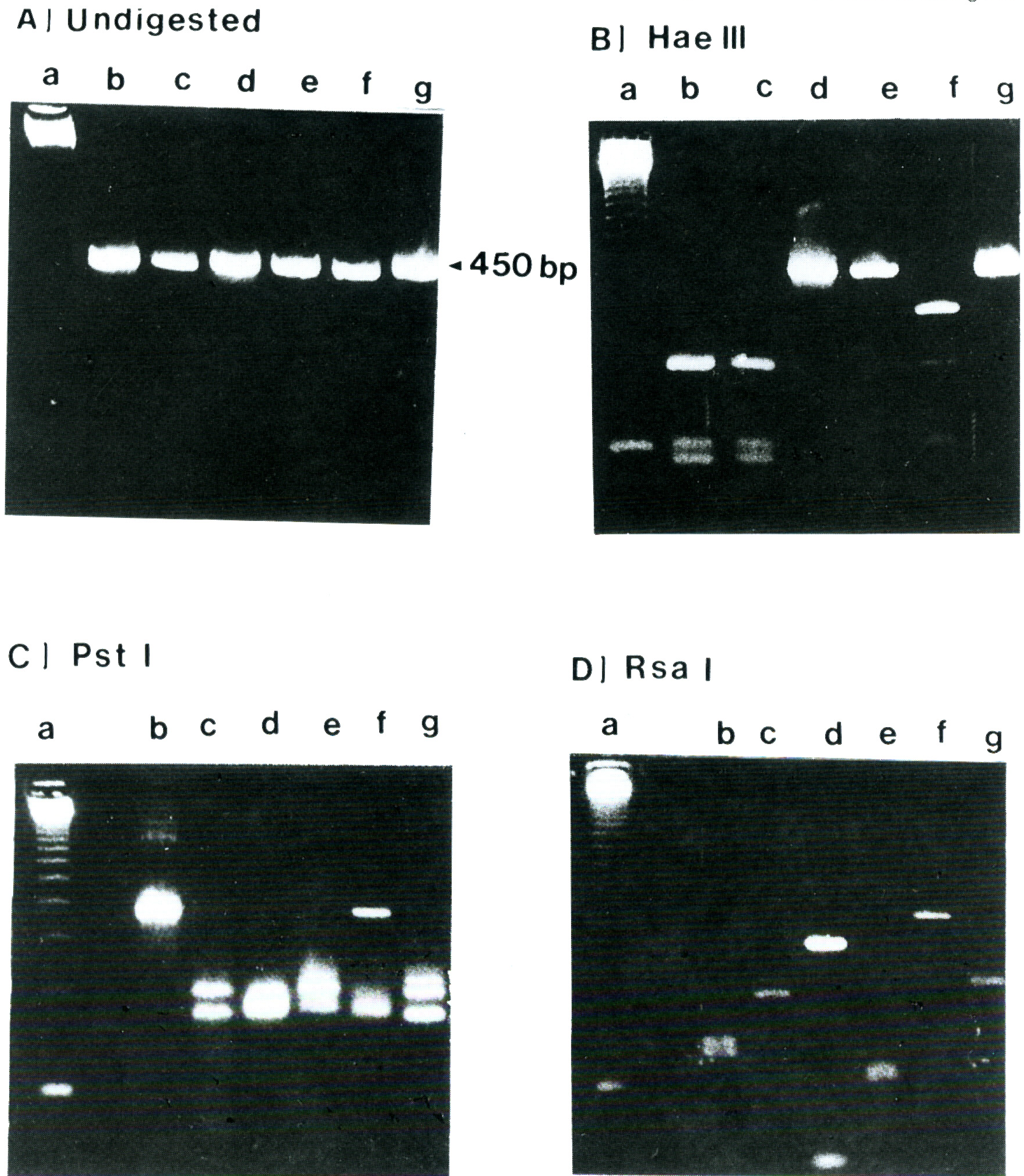

Fig. 1. PCR products after electrophoresis and ethidium bromide staining. 1pg of each HPV DNA was amplified a common part of the L1 open reading frame of HPVs. A) undigested 450 based pair sized PCR products of HPV-6, -11, -16, -18, -31, and -33. B) Hae III digested PCR products. C) Pst I digested PCR products. D) Rsa I digested PCR products. lane a: 123 base pair sized marker, lane b; amplified product of 1 pg HPV-6, lane c; HPV-11, lane d; HPV-16, lane e; HPV-18, lane f; HPV-31, and lane g; HPV-33

Table 2. Correlation between β-globin and HPV DNA Amplification by PCR Using E6 Type-specific Primers and L1 Consensus Primers in 35 Paraffin Sections of Cervical Neoplastic Lesions

β-globin gene amplification	Number of tissues	Number of positive HPV DNA (%)	
		E6 primers	L1 primers
Positive	25	16 (64)	9 (36)
Negative	10	3 (30)	0 (0)
Total	35	19 (54)	9 (26)

the HPV types by the different sized DNA bands in polaroid picture after gel electrophoresis and ethidium staining.

To evaluate the potential inhibitory amplifying effect of fixatives, the proteinase-K digested DNAs from deparaffinized sections were purified by phenol-chloroform extraction, precipitated by ethanol (Sambrook et al., 1989) and amplified in parallel with unpurified proteinase-K digested DNA. As positive controls for HPVs, 1 pg of each HPV plasmid (HPV -6, -11, -16, -18, -31, and -33) and SiHa (for HPV-16) and HeLa (for HPV-18) cell lines were employed. And these PCR products from positive HPV plasmid controls were digested with specific restriction enzymes (Pst I, Rsa I, and Hae III; Boerhinger Mannheim, Germany) to differentiate the HPV types by the digestion patterns of the reaction products. The identity of amplified products was confirmed in hybridization experiments with HPV oligonucleotide probes spanning the internal portion of the amplified sequences.

RESULTS

The 268 base pair sized human β-globin amplification of phenol-chloroform extracted, digested samples (lane A) is similar with the unpurified samples (land B) that have been amplified immediately after digestion (Fig. 2). There was no significant improvement in amplification by further purification procedure.

To assess the integrity and the relative quantity of DNA in the archival tissue sections, we simultaneously amplified a region of the human β-globin gene and HPV in a single tube reaction. Amplification of β-globin gene was achieved in 25 of the 35 paraffin sections (Table 2).

In our study, 35 specimens of cervical neoplasias were tested separately with E6 primers and L1 primers for HPV amplification. Detection rate by E6 primers (in 54% of the specimens; 19/35) was significantly higher than that by L1 primers (in 26% of the specimens; 9/35), (McNemar's test, $X^2=7.0$, $p<0.01$). All specimens HPV-positive with L1 primers were also positive with E6 primers (Table 3). By L1 consensus primers, HPV DNA could be amplified in 36% (9 of 25) of tissue from which β-globin gene was amplified as compared with 64% (16 of 25) of tissue by E6 type-specific primers.

The 450 base pair sized PCR products, which were shown after gel electrophoresis and ethidium bromide staining, were identified by Southern blot analysis with ^{32}P-labeled HPV type-specific oligonucleotide probe (Fig. 3). Eight cases were positive for HPV-16 (2 cases of CIN II, 2 cases of CIN III, 2 cases of microinvasive cancer, and 2 invasive cancers) and one case was positive for HPV 33 (CIN III) with the L1 consensus

Table 3. HPV DNA Amplification in Fixed Cervical Neoplastic Tissues with E6 Type-specific Primers and L1 Consensus Primers

E6 primers	L1 primers	No. of specimens
+	+	9
−	+	0
+	−	10
−	−	16
	Total	35

Table 4. HPV Types and Severity of Lesions in the Neoplastic Tissue from the Uterine Cervix of Korean Women by PCR Using L1 Consensus Primers.

Diagnosis	Number of HPV positive	Distribution of HPV type					
		6	11	16	18	31	33
CIN* I (n=5)	0	0	0	0	0	0	0
CIN II (n=5)	2	0	0	2	0	0	0
CIN III (n=10)	3	0	0	2	0	0	1
Microinvasion (n=5)	2	0	0	2	0	0	0
Invasive cancer (n=10)	2	0	0	2	0	0	0
Total 35	9	0	0	8	0	0	1

*CIN: cervical intrapithelial neoplasm

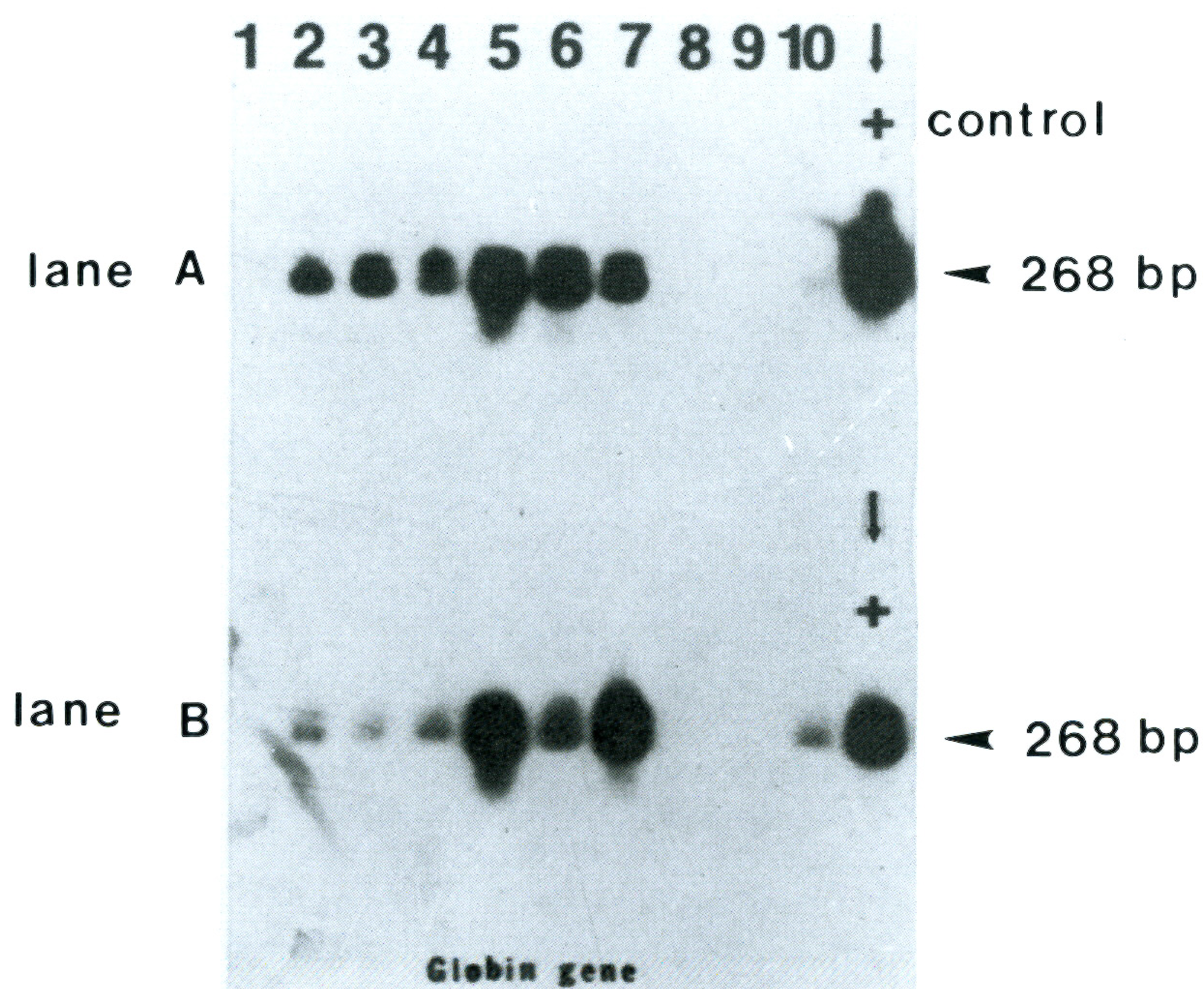

Fig. 2. Effects of tissue DNA on β-globin amplification. Following amplification, PCR products were hybridized with ^{32}P-labeled β-globin probe in a Southern hyridization format. Lower panel contains PCR products from unpurified proteinase-K digests. Upper panel contains PCR products from DNAs which were purified by phenol chloroform extraction of the proteinase-K digests. Positive control: 10^5 copies of human placental DNA.

primers. But HPV types -6, -11, -18, and -31 were not detected in any of the specimens (Table 4). The samples that failed to give β-globin amplification were also negative for HPV amplification with L1 consensus primers.

DISCUSSION

In situ DNA hybridization and immunoperoxidase techinques can utilize a small quantity of cells of paraffin-embedded, fixed tissue, but they lack sufficient sensitivity to detect the low number of HPV genomes and antigens usually present in cervical cancers (Gupta et al., 1986). But PCR is an extremely useful method of amplifying specific HPV DNA sequences from trace amounts of DNA in 5-10 μm paraffin sections of fixed tissue (Shibata et al., 1988).

In our previous study (Park et al., 1991b), we modified Shibata's E6 type-specific primer sequences (Shibata et al., 1988) to contain 50% guanine plus cytosine content to provide optimal annealing conditions. Manos et al. (1989) and Resnick et al. (1990) have developed a system for PCR amplification and subsequent typing of genital HPVs. The approach relies on the fact that each unique HPV type shares interspersed regions of considerable DNA sequence homology. Using consensus and type-specific oligonucleotide probes in hybridization analysis, they were able to detect as few as 10 copies of HPVs. The L1 primers system provided amplification of a 450 base pair sized region from a broad spectrum of HPV types, such as HPV -6, -11, -16, -18, -31, -33 and unidentified HPV types. By using gel electrophoresis and ethidium bromide staining, each type could be distinguished from the others by cutting the amplified DNA of each HPV with different restriction enzymes, generating restriction fragments that are specific for each type. For conclusive results, radiolabeled oligonucleotide probes were necessary for hybridization to allow definite identification of the viral types.

We used HPV plasmids and HPV containing cervical cancer cell lines as positive controls for HPV types, β-globin as an internal control for evaluation of DNA integrity in archival tissue sections, PCR mixtures without HPV and cellular DNA as negative controls.

To our knowlege of molecular hybridization tech-

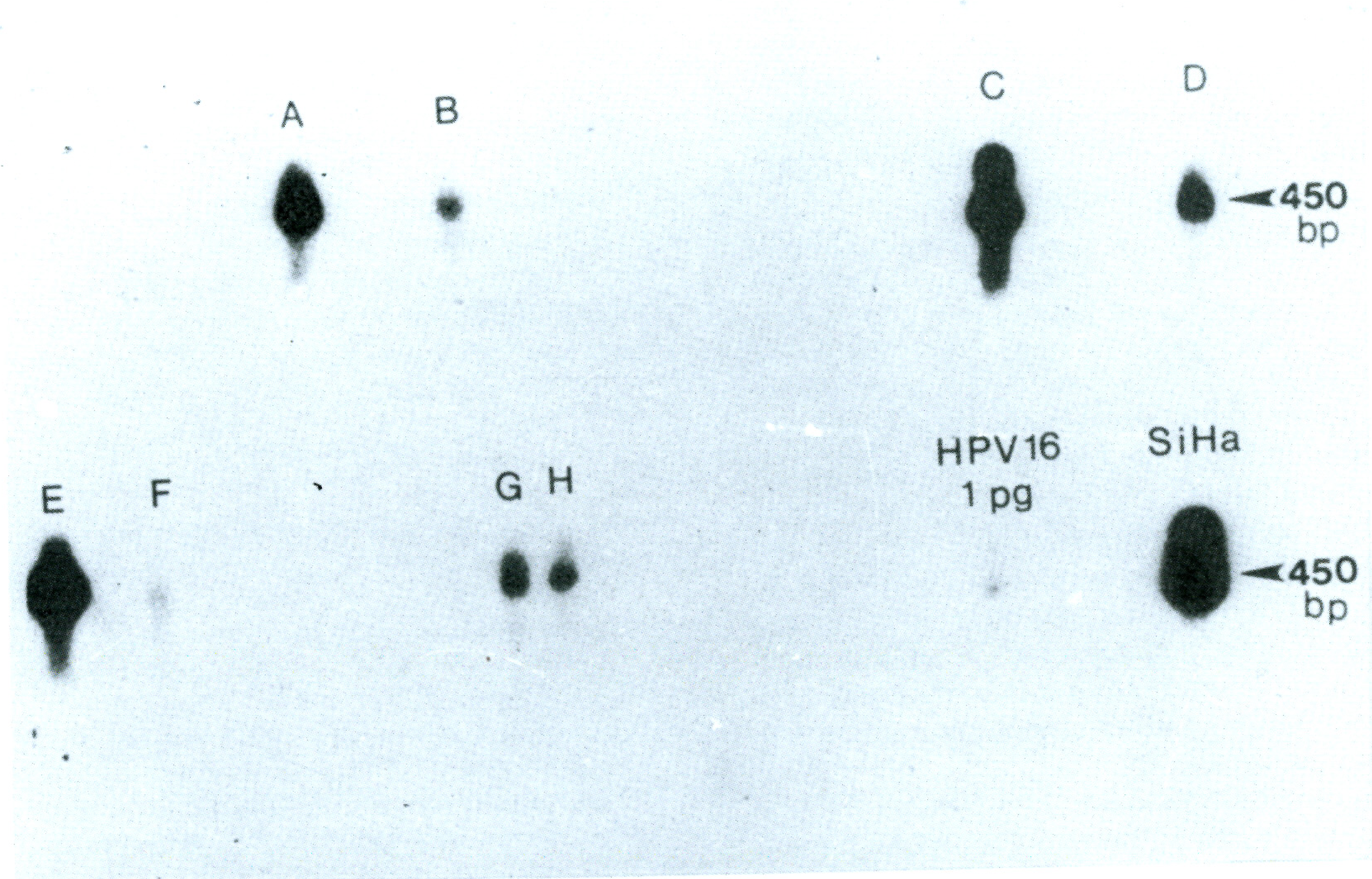

Fig. 3. Hybridization of Southern blot with HPV-16 oligonucleotide probe. Positive amplifications of HPV-16 from the paraffin sections of cervical neoplasia were noted at the size of 450 base pairs (A-H). Positive controls: PCR products of SiHa cells and 1 pg of HPV 16 plasmid.

niques for detection of HPV subtypes, the PCR by E6 type-specific primers was applicable only for the amplification of specific target HPV DNA with a known nucleotide sequence. In contrast, PCR based on the use of L1 consensus primers enabled the possible detection of unknown types of HPVs (Manos et al., 1989), although we couldn't detect the unidentified types of HPVs.

In this study we tried to compare the sensitivity and efficiency of L1 consensus primers and E6 type-specific primers *in vitro* amplification for detecting HPV DNA. By using the E6 type specific primers, HPV was found in 19 (54%) cases. HPV types -6, -18, and -33 were detected only in 6 (30%) cases of preneoplastic lesions, but the HPV-16 was found in 8 (40%) premalignant lesions and in 7 (47%) invasive carcinomas. These finding suggested that HPV-16 was the predominant and important viral subtype in invasive cervical cancer. With the L1 consensus primers, only 8 cases were positive for HPV-16 (4 cases of premalignant lesions; 4 cases of invasive carcinomas) and 1 case was positive for HPV-33 (CIN III).

In the PCR results by using type-specific primers for the detection of HPVs in cervical scrapes and biopsies (Brule et al, 1989), HPV positivity was 58%, 67%, and 90% for mild, severe dysplasias, and carcinomas of the cervix uteri, respectively. Their HPV detectability in purified DNA from the fresh specimens of dysplasia and carcinoma much higher than the HPV amplification rates of our old formalin-fixed, paraffin-embedded tissues. While the ability to analyze archival tissues by PCR significantly extends the scope of investigation, there are considerable variabilities in the amplification obtained by PCR from fixed tissues (Wright & Manos, 1990; Park et al., 1991c). Factors known to influence the sensitivity of DNA amplification from fixed tissues include the type of fixative used, time elapsed between removal of tissue and fixation, duration of fixation, nuclease level in the tissue, method used to extract the DNA from the fixed in buffered formaline was found to be suitable for amplification, but tissue fixed in Bouin's, Hollander's, and Hartmann's solutions was known to be quite unsuitable for amplification (Park et al., 1991c).

The inhibitory effects of certain fixatives in amplification may be overcome by further purification (phenol-chloroform extraction and ethanol precipitation after the protease digestion) of the tissue extracts.

The DNAs in buffered formalin-fixed, paraffin-embedded tissue that were isolated by the detergent/protease method in this experiment worked out as well as phenol-chloroform purified samples did, and the sensitivity of detection of PCR-amplified products was sufficient for our purpose.

Typing results with the E6 type-specific and L1 consensus schemes were in complete agreement in 25 specimens examined (9 were positive and 16 were negative by both sets). The remaining 10 specimens, which were positive by HPV E6 primers, failed to produce HPV L1 amplification. And HPVs were detected in 36% of 25 globin amplified tissue samples by L1 consensus primers and in 64% of globin-positive tissue by E6 type-specific primers. It was of interest that HPV DNA could be amplified by E6 type-specific primers in 3 cases which were negative for β-globin amplification, although the samples that gave negative β-globin amplification were also negative for HPV amplification by L1 consensus primers. Several factors may account for the identification of HPVs in β-globin negative specimens. Contamination of specimens with the target DNA would give a false positive result. The superior sensitivity of E6 type-specific primers over L1 consensus primers might lead to disadvantages, as trace amounts of contaminations could be detected by E6 primers, resulting in false positive results. The amount of HPV DNA in the specimens may be higher than the copy numbers of β-globin DNA. Episomal HPV DNA may be more accessible for amplification than celular β-globin DNA. But in the progression of cervical neoplasia the sequences of L1 genes are known to be disrupted as a consequence of HPV integration, whereas the E6 genes are generally intact and expressed in cervical tumors.

Although the number of specimens in this study is too small, we found HPV DNA in each of the cervical tissue samples by both primer sets. The amplification of the E6 region may allow to easy detection of such L1 region deleted integrated virus (Durst et al., 1989; Wadanabe et al., 1989). PCR made it possible to amplify β-globin sequences from ancient DNA (Paabo et al., 1989). The size of DNA fragments in archival tissue is quite variable as a consequence of different fixation methods. The smaller sized amplified products from the type-specific E6 primer system are more likely to detect HPV sequences in fixed tissue compared with 450 base pair sized products from the L1 consensus primer system.

In this study of Korean cases of dysplasia and cancer of the cervix, eight of nine HPV-positive cases by L1 consensus primers were positive for HPV-16 and HPV-16 was found across the entire spectrum of histologic lesions from CIN to invasive carcinoma. These findings suggested that HPV-16 associated with cervical dysplasia could be the precursor of cervical cancer.

In conclusion, we suggest that the L1 consensus PCR set which made possible the simultaneous detection of various viral types is efficient for epidemiological studies aimed at elucidating the oncogenic potential of HPV and ascertaining the prevalence of HPV in fixed, paraffin-embedded tissue.

REFERENCE

Brule AJC, Claas ECJ, Maine M, Melchers WJG, Helmerhorst T, Quint WGV, Lindeman J, Meijer JLM, Walboomers JMM: *Use of anticontamination primers in the polymerase reaction for the detection of human papillomavirus genotypes in cervical scapes and biopsies. J Med Virol 29:20-27, 1989.*

Durst M, Dzarlieva-Pertrusevzka P, Boukamp P, Fusenig NE, Gissmann L: *Molecular and cytogenetic analysis of immortalized human primary keratinocytes obtained after transfection with human papillomavirus type 16 DNA. Oncogene 1:251-256, 1989.*

Gupta J, Schneider A, Shah KV: *Detection of papilloma antigen and DNA in cells and tissue. In: Peto R, zur Hausen H eds. Viral etiology of cervical cancer. p. 247-251, New York, Cold Spring Harbor Laboratory Press, 1986.*

Hertig AT: *Early concepts dysplasia and carcinoma in situ (a backward glance at a forward process): a brief historical review. Obstet. Gynecol. Surv. 34:795-803, 1979.*

Jenson AB, Lancaster WD: *Association of human papillomaviruses with benign, premalignant, and malignant anogenital lesions. In: Pfister H ed. Human papillomaviruses and human cancer. p. 11-43, Boca Radon, CRC Press, 1990.*

Lorincz AT, Temple GF, Kurman RJ, Jenson AB, Lancaster WD: *Oncogenic association of specific human papillomavirus types with cervical neoplasia. J Natl Cancer Inst 79:671-677, 1987.*

Manos MM, Ting Y, Wright DK, Lewis AJ, Broker TR, Wolinsky SM: *Use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. In: Furth M, Graves M eds. Molecular Diagnostics of Human Cancer: Cancer Cells. vol 7, p. 209-214, New York, Cold Spring Harbor Laboratory Press, 1989.*

Paabo S, Higushi RG, Wilson AC: *Ancient DNA and the polymerase chain reaction. The emerging field of molecular archaeology. J Biol Chem 264:9709-9712, 1989.*

Park JS, Jones RW, McLean MR, Currie JL, Woodruff JD, Shah KV, Kurman RJ: *Possible etiologic heterogeneity of vulvar intraepithelial neoplasia: A correlation of pathologic characteristics with human papillomavirus detection by in situ hybridization and polymerase chain*

reaction. Cancer 67:1599-1607. 1991a

Park JS, Namkoong SE, Lee HY, Kim SJ, Daniel RW, Shah KV: *Detection of human papillomavirus genotypes in cervical neoplasia from Korean women using polymerase Polymerase chain reaction. Gynecol Oncol 41:129-134, 1991b.*

Park JS, Leake JF, Sharma BK, Toki T, Kessis TD, Ambros RA, Shah KV: *Variabilty in β-globin and HPV DNA amplification by PCR from fixed tissues. Mod Pathol 4: 667, 1991c.*

Reid R, Greenberg M, Jenson AB, Husain M, Willett J, Daoud Y, Temple G, Stanhope CR, Sherman AI, Phibbs GD, Lorincz, AT: *Sexually transmitted papillomaviral infections. I. The anatomic distribution and pathologic grade of neoplastic lesions associated with different viral types. Am J Obstet Gynecol 156:212-222: 1987.*

Resnick RM, Cornelissen MTE, Wright DK, Eichinger GH, fox Hs, Schgget JT, Manos MM: *Detection and typing of human papillomavirus in archival cervical cancer specimens by DNA amplification with consensus primers. J Natl Cancer Inst 82:1477-1484, 1991.*

Richardt RM: *Cervical intraepithelial neoplasia. In: Sommers SC ed. Pathology Annual. p. 301-306, New York, Appleton-Centry-Crofts, 1973.*

Sambrook J, Fritsch EF, Maniatis T: *Isolation of high-molecular-wegith DNA from mammalian cells. In: Molecular Cloning: a laboratory manual. 2nd ed. p 14, New York, Cold Spring Harbor Laboratory Press, 1989.*

Saiki RK, Scharf S, Faloona FA, Mullis KB, Horn GT, Erlich HA, Arnheim N: *Enzymatic amplification of a β-globin sequence and restriction site analysis for diagnosis of sickle cell anemia. Science 230:1350-1354, 1985.*

Shah KV, Howley PM: *Papillomaviruses. In: Fields BN, Knipe DM, eds. Virology. p. 1651-1675, New York, Raven Press, 1990.*

Shibata DK, Arnheim N, Martin WJ: *Detection of human papillomavirus in paraffin-embedded tissue using the polymerase chain reaction. J Exp Med 167:225-230, 1989.*

Ting Y, Manos MM: *Detection and typing of genital human papillomaviruses. In: Innis MA, Gelford DM, Sninsky JJ, White TJ, eds. PCR Protocols: a guide to methods and applications, p. 356-367, San Diego, Academic Press, 1990.*

Wadanabe S, Kanda T, Yoshige K: *Human papillomavirus type 16 transformation of primary human embryonic fibroblasts requires expression of open reading frames E6 and E7. J Virol 63:965-969, 1989.*

Wright DK, Manos MM: *Sample preparation from paraffin-embedded tissues. In: Innis MA, Gelford DM, Sninsky JJ, White TJ, eds. PCR Protocols: a guide to methods and application, p. 153-158, San Diego, Academic Press. 1990.*

Zur Hausen H: *Papillomaviruses in anogenital cancer as a model to understanding the role of viruses in human cancers. Cancer Res 49:4677-4681, 1989.*