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Detection of Genital Human Papillomavirus by Single-Tube Nested PCR and Type-Specific Oligonucleotide Hybridization

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Cervical cancer is, on a global scale, the second most common form of cancer in women. Development of cervical carcinoma is strongly associated with infection by certain types of human papillomavirus (HPV). To facilitate the detection and molecular typing of HPV in clinical samples, nested-PCR amplification systems were developed for regions of the E1 and L1 genes. The nested amplifications were performed in a single reaction tube, and shifting between inner and outer primer pairs was achieved by a two-phase amplification with different annealing temperatures. This method eliminates cross-contamination between samples during transfer from the first to the second amplification step. A set of type-specific oligonucleotide probes were designed for the E1 system and used to distinguish 19 genital HPV types. The sensitivities of our amplification systems compare favorably with that for the L1 system on the basis of the MY09-MY11 primer pair (M. M. Manos, Y. Ting, D. K. Wright, A. J. Lewis, T. R. Broker, and S. M. Wolinsky, Cancer Cells 7:209–214, 1989) and our systems can be used on materials such as HPV-infected cell lines, cytobrush samples, cancer biopsies, and recent as well as archival Papanicolaou (Pap) smears. The high sensitivity coupled with the effective elimination of contamination in the transfer between the two amplification steps of the nested PCR makes these systems suitable for research as well as clinical analyses.

Cervical cancer is, on a global scale, recognized as the second most common form of cancer in women. Since Papanicolaou (Pap) smear screening was introduced 50 years ago, the mortality rate for cervical cancer has been declining. Yet, worldwide, carcinoma of the cervix remains a leading cause of death from cancer in women. Epidemiological studies have shown human papillomavirus (HPV) infection to be the most important etiologic factor for the development of cancer of the cervix (4). HPV has a central etiologic role in CIN (cervical intraepithelial neoplasia) development, and other environmental risk factors, such as the number of sexual partners, the age at first intercourse, and parity, appear to influence through HPV infection (19). However, a small percentage of cervical neoplastic lesions do not appear to contain HPV. This absence of HPV could be due to the lack of sufficiently sensitive detection methods or the presence of unknown HPV types or other environmental factors acting independently of HPV. Genetic factors, such as mutations in the p53 and retinoblastoma tumor suppressor genes, have been suggested to contribute to the development of cervical carcinoma and have in some cases been detected in HPV-negative cervical carcinoma cell lines (18). In addition, genetic susceptibility to cervical carcinoma has recently been found to be associated with HLA class II *DQB1* and *DRB1* alleles (1, 2). In fact, HLA and HPV infection appear to be the most important risk factor for the development of cervical carcinoma.

In addition to the need for further studies of HPV cofactors, the natural history of HPV needs to be elucidated in more detail. Infection with HPV is common in young women, with some studies indicating a prevalence of up to 40% among sexually active young women (3, 9). Yet, most HPV carriers do not develop CIN, and among women who have developed HPV-positive cancer in situ, only some 12% will progress to invasive cancer (12, 13). Infection with particular high-risk HPV types, such as HPV type 16 (HPV16) and HPV18, is associated with a higher risk of developing high-grade cervical lesions than infection with low-risk types (14). The various genital HPV types also appear to differ in prevalence (27), natural course of infection, and ability to promote transition between developmental stages (23).

A necessary prerequisite for studies of the natural history of HPV is a sensitive detection system. The use of PCR for HPV detection provides several advantages over the classical Southern blotting technique (20). However, the commonly used PCR-based systems for detection of HPV in clinical samples all have disadvantages. For instance, the most common system for the L1 region (15) results in an amplification product that is too long (450 bp) to be compatible with efficient PCR on materials with limited DNA copy numbers. This size requirement can hamper an efficient amplification from materials such as formalin-fixed tissue sections and archival cervical smears. The use of a single degenerate primer pair may also limit the sensitivity, in particular for samples with degraded DNA. The nested-PCR systems described so far (7, 9), albeit more sensitive than systems employing single primer pairs, all suffer from the problem of being sensitive to cross-contamination of PCR products between reactions in the transfer from the first to the second amplification reaction, potentially resulting in false-positive reactions. To circumvent these limitations, we developed PCR systems targeting conserved sequences in the early (E1) and late (L1) regions of the HPV genome. To allow for the detection of the different genital HPV types, we designed degenerate consensus primer pairs located in the most conserved regions of these parts of the HPV genome. In the E1 open reading frame, a highly conserved nucleotide sequence, shared by all papillomaviruses (5), was identified and chosen as one priming site. The different HPV types were distinguished by hybridization to oligonucleotides specific for 19 different genital HPV types.

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TABLE 1. Sequences and specificities of hybridization probes for the E1 region of the HPV genome

Oligonucleotide probe	Sequence	T_m (°C)
HPV6BE1B	B5′-TAAACTTACAAGACAG-3′	42
HPV11E1B	B5′-TAAACTTACAACACAG-3′	42
HPV16E1B	B5′-ATGTATAGAAAAACAAAGT-3′	46
HPV18E1B	B5′-TTTAAATAGTGGGCAGA-3′	46
HPV30E1B	B5′-TACAGACGTGGCCG-3′	46
HPV31E1B	B5′-ATGCATAGAAAATAACAG-3′	46
HPV33E1B	B5′-TAATAAAAATAAAGAATGCA-3′	46
HPV34E1B	B5′-GACGATAAGGGACAC-3′	46
HPV35E1B	B5′-TTGCATTGAAAATAAAAATA-3′	46
HPV39E1B	B5′-TTCATTAAATGTAAGCAG-3′	46
HPV40E1B	B5′-ACGGCTTGGCGGC-3′	46
HPV42E1B	B5′-ACGCTATGTCGGGG-3′	46
HPV45E1B	B5′-ATTAAATAGTGGGCACA-3′	46
HPV51E1B	B5′-CAAACGAGTCACAAGT-3′	46
HPV52E1B	B5′-GTGTAAATACAGAGTGT-3′	46
HPV53E1B	B5′-GATACAGAAGTGCCG-3′	46
HPV56E1B	B5′-TTTATCAGACCTACAAG-3′	46
HPV57E1B	B5′-CTCGCTAAACAGAAAG-3′	46
HPV58E1B	B5′-ATATAAAAATAAAGAATGC-3′	46

MATERIALS AND METHODS

DNA extraction from Pap smears. Pap smears were collected from healthy women visiting the women's clinic and student health at the Uppsala University Hospital, where Pap smears are taken routinely. The smears were fixed in alcohol, stained with the Papanicolaou stains (Orange G, hematoxylin, and EA polychrome solution consisting of light green sulfonated aryl methane dye, eosin, and Bismarck brown), and then covered with glass coverslips. For DNA extraction, the coverslips were removed by soaking the slides in xylene over a period of 2 to 3 days. Destaining was performed by immersing the slides in a series of graded alcohol solutions down to 50% ethanol. The cells were then scraped from the slides with a razor blade and transferred into a 1.5-ml Eppendorf microtube containing 200 μl of PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$). Subsequently, 20 μg of proteinase K was added, and the cells were digested at 60°C for 2 h. The digested samples were extracted three times with phenol-chloroform and one time with chloroform, and DNA was precipitated by adding 1/10 volume of sodium acetate and 2.5 volumes of ethanol. After 1 h at −70°C, the samples were centrifuged for 40 min at 14,000 rpm in a cold room. The DNA pellets were dried and dissolved in 50-μl portions of TE (10 mM Tris-HCl [pH 7.5], 0.1 mM EDTA). DNA from HPV-infected cell lines and cervical cytobrush samples was extracted with phenol-chloroform, precipitated by ethanol, and dissolved in TE, as described above.

Archival smears were obtained from the Department of Pathology, University of Uppsala, and the DNA was extracted by the protocol by Chua (7).

PCR amplification. PCR amplification was performed in a 100-μl volume containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 0.5% Nonidet P-40–Tween 20, 2.5 U of *Taq* DNA polymerase (AmpliTaq; Perkin-Elmer), 25 pmol of each outer primer, 50 pmol of each inner primer, and 2 to 5 μl of sample DNA. Two drops of paraffin oil were added to overlay the reaction mixture (except when the 9600 PCR instrument was used). Amplification was performed in a Perkin-Elmer Cetus DNA TC1 or 9600 PCR instrument for a total of 45 cycles. The first 15 cycles consisted of 1 min at 94°C, 1 min at 45°C, and 1.5 min at 72°C. The annealing temperature was subsequently lowered to 30°C, and an additional 30 cycles were performed with the other parameters unchanged. Ten-microliter portions of the PCR products were loaded on 4% agarose gels (NuSieve; FMC BioProducts, Rockland, Maine) and electrophoresed, the gels were stained with ethidium bromide (10 mg/ml), and DNA was visualized by fluorescence under UV light.

Negative controls, with all the reaction components but void of DNA, and positive controls with DNA either from HPV plasmids (HPV16 or HPV18) or cell lines containing integrated HPV (SiHa [HPV16] or HeLa [HPV18]), were included in each experiment.

HPV typing by hybridization. From each amplified DNA sample that upon gel electrophoresis of 10 μl displayed a visible band, 2.5 μl of the PCR products was denatured in 50 μl of 0.4 M NaOH–25 mM EDTA and the mixture was applied to a nylon filter (BioDyne B membranes; Pall Corp.). The filter was successively hybridized to 19 HPV type-specific 5′-biotinylated oligonucleotide probes (Table 1). The probes were designed to have similar melting temperatures (T_ms) in order to allow for parallel hybridizations to multiple probes, either by using different membranes or by applying a probe mixture. Hybridization was carried out for 30 min at 42°C in 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]–0.5% sodium dodecyl sulfate (SDS), with the addition of 1 μl of streptavidin-POD conjugate (Boehringer-Mannheim Inc.) and 4 pmol of probe per 10 ml of solution. Filters were washed in 1× SSPE–0.1% SDS for 20 min in 50°C and the hybridization signal was detected by a bioluminescence assay (ECL; Amersham) and exposure onto Kodak Xomat-S X-ray film.

RESULTS

Design of amplification primers and probes. In order to detect regions with a high degree of conservation between the genital forms of HPV, 19 complete genome sequences were obtained from GenBank and aligned with a variety of alignment programs (Align and Megalign). The different HPV types showed enormous diversity, and successful alignments could be achieved only when the different open reading frames were aligned individually. Thus, separate E1 and L1 alignments were constructed. On the basis of these alignments, one region within the L1 open reading frame and one within the E1 open reading frame with a high degree of conservation between the genital types were identified (Fig. 1). These regions showed sufficient homology between HPV types to allow for the design of degenerate primers that are general to all genital HPV types. Also, the regions between these sites were sufficiently heterogeneous to allow for the identification of the different HPV types by hybridization to type-specific oligonucleotide probes.

Nested-PCR systems were developed. In these nested-PCR systems, the outer primer pair initiates the amplification reaction and the second primer pair, targeting sites internal to the first primer pair, in a second amplification step continues the reaction. In standard nested PCR in which the two primer pairs are used in two independent reactions, there is a possibility for cross-contaminating the reaction with amplification products from neighboring reactions when transferring materials from the reaction with the outer primer pair to that with the inner primer pair. We therefore designed a nested-PCR system in which all primers were present in the reaction from the first cycle and shifting between primer pairs is achieved by altering the annealing temperature from a high annealing temperature during the first 15 cycles to a low annealing temperature during the last 30 cycles. To allow for the differences in annealing temperature, the T_ms of inner and outer primer pairs were generally quite different. Also, different molar amounts of inner and outer primer pairs were used.

To optimize the efficiency of the amplification system, we set the size of the amplification products resulting from the inner primers to be shorter than 200 bp. Two nested-PCR systems were developed, one for the L1 region and one for the E1 region of the HPV genome. An outline of the amplification systems is shown in Fig. 1A. The final products span nucleotides 1112 to 1283 of the E1 open reading frame and nucleotides 6543 to 6775 of the L1 open reading frame, both relative to the numbering of the HPV16 genome (Fig. 1B and C).

Evaluation of amplification primers. The reaction conditions were optimized by changing the annealing temperature in the interval from 30 to 60°C and by altering the cycle number at which the phase change is initiated (i.e., the shift from using outer to inner primer pairs) in the interval from 10 to 30 cycles. The highest amplification sensitivity and efficiency was obtained by an initial annealing temperature of 45°C for 15 cycles, followed by a shift to an annealing temperature of 30°C for an additional 30 cycles.

Optimization of the PCR was initially performed by testing the 5′ and 3′ primers in all possible combinations, using reconstruction experiments with cloned HPV16, -18, -31, and -33 genomes. The sensitivities of the amplification systems were tested by using either cloned HPV alone, cloned HPV mixed with 1 μg of human genomic DNA or DNA from cell lines

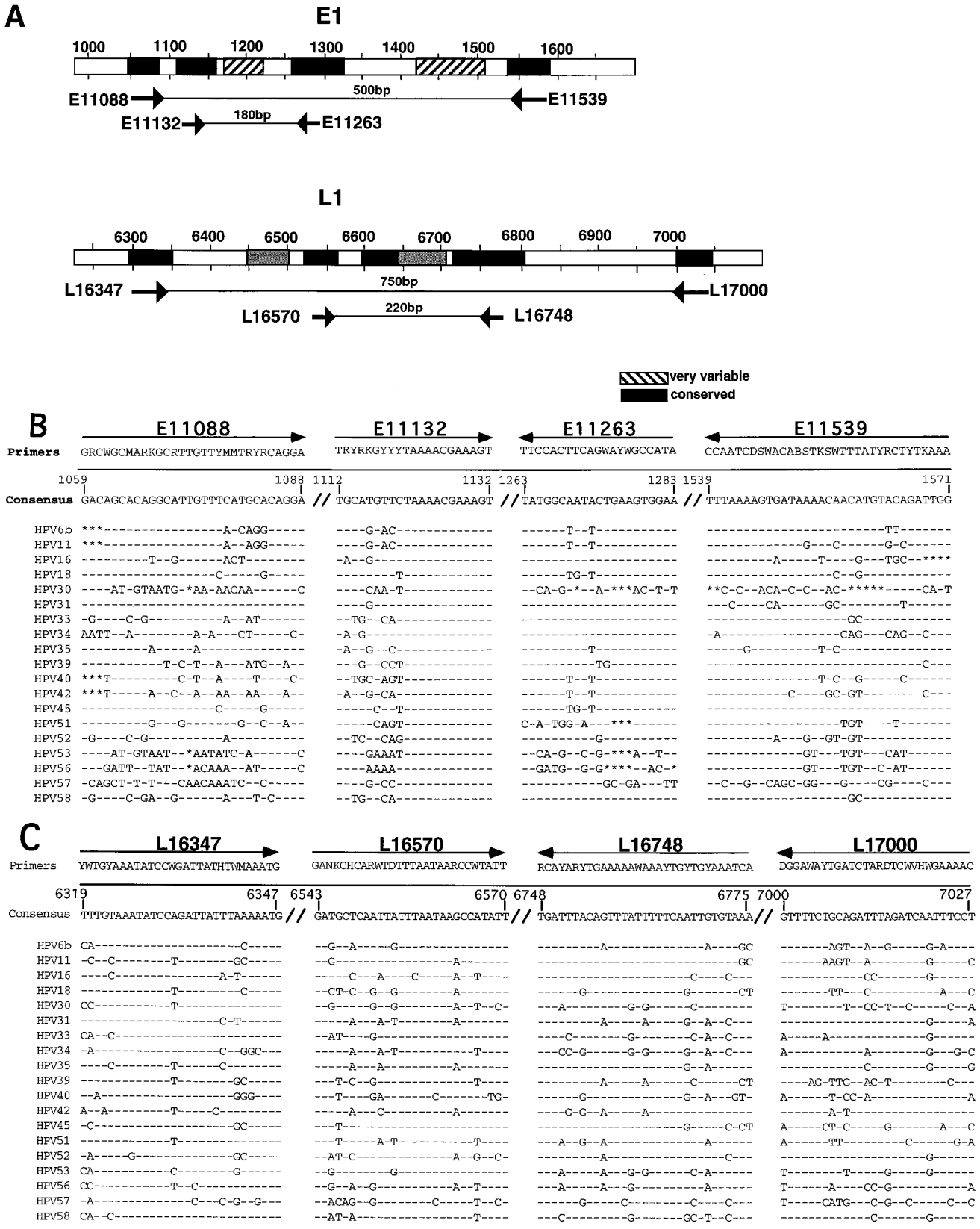

FIG. 1. (A) Outline of the amplification systems for the E1 and L1 open reading frames. The arrows mark the locations of the outer (big arrows) and inner (smaller arrows) primer pairs. The numbering is relative to the HPV16 genome. (B and C) Alignment of the E1 (B) and L1 (C) open reading frames, showing the positions of the primers, with the arrows showing the 5′-to-3′ direction. The primers are denoted by the number of the 3′ nucleotide of the HPV16 genome. The top line shows the degenerate primer sequence (S = G or C; R = A or G; N = A, C, G, or T; Y = T or C; W = T or A; H = A, T, or C; M = A or C; K = G or T; D = T, G, or A). Deletions are indicated by asterisks, and nucleotides identical to those in the consensus sequence are indicated by dashes.

TABLE 2. Sensitivities of amplification systems[a]

DNA sample	Sensitivity (no. of copies) of amplification system	
	L1 region	E1 region
HPV16 plasmid	100	1
HPV18 plasmid	1	1
HPV31 plasmid	100	10*
HPV33 plasmid	1	10*
SiHa cells	100	10
HeLa cells	10	10

[a] All experiments, except those indicated with asterisks, were performed in a background of genomic DNA.

SiHa (containing about 1 copy of HPV16 genome per cell) and HeLa (containing about 10 copies of HPV18 genome per cell). The amplification systems were able to detect as few as 10 to 100 copies in a background of 10^5 copies of heterologous DNA (Table 2). Using nested PCR, the sensitivity of the amplification increased from 10^8 to about 10^2 copies of starting DNA (Fig. 2). Additional testing of the E1 nested-PCR system with one primer acting as both outer and inner 3′ primer showed that the sensitivity was comparable to that with four primers. Therefore, we subsequently used only the three primers E11132, E11263, and E11539 in experiments with the E1 amplification system.

The E1 and L1 amplification systems appeared to have similar amplification efficiencies (Table 2, Fig. 3). By using the cell lines with integrated HPV, we also compared the efficiencies of our amplification systems with the efficiency of amplification with the MY09-MY11 primer pair (15). Our systems performed as well as the MY09-MY11 system on these types of

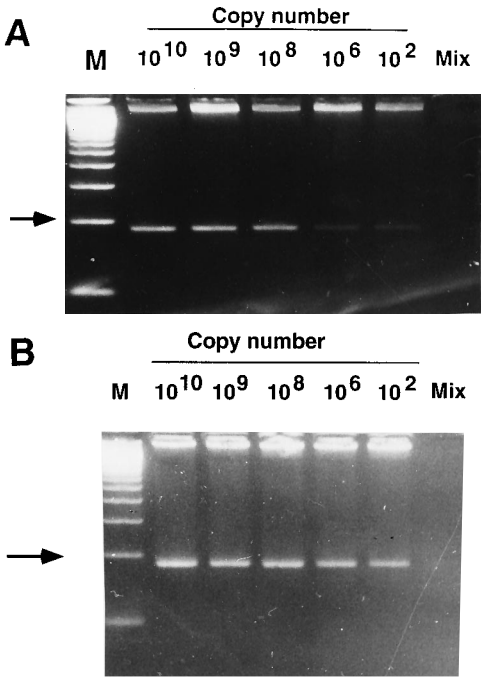

FIG. 2. Comparison of the sensitivity of the E1 amplification system from HPV18 plasmid in a mixture with 1 μg of genomic DNA, using a single primer pair (A) and nested PCR (B). The positions of the product resulting from the amplification are indicated by the arrows. Lane M, 100-bp ladder used as size standard; Mix, amplification reaction components void of DNA.

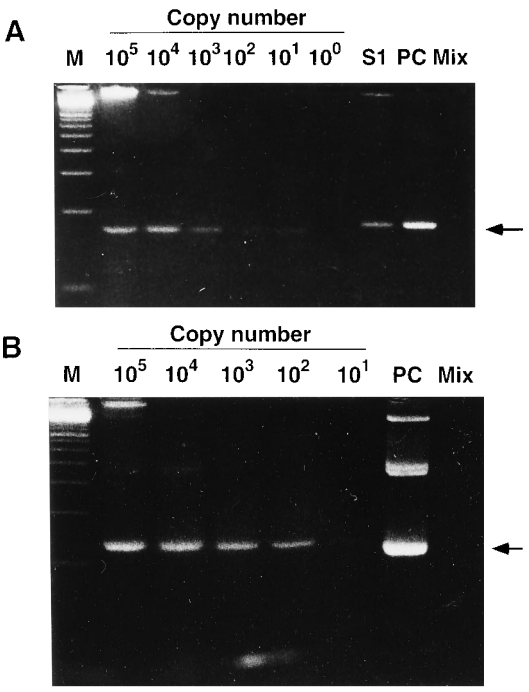

FIG. 3. Sensitivities of the E1 (A) and L1 (B) amplification systems. (A) E1 amplification system on genomic DNA of the SiHa cell line (1 copy of HPV16 per cell). (B) L1 amplification system on DNA of the HeLa cell line (10 copies of HPV18 per cell). Lanes: M, 100-bp ladder used as size standard; S1, amplification from a cervical smear; PC, positive control; Mix, amplification reaction components void of DNA. The positions of the products resulting from the amplification with the inner primers are indicated by the arrows.

samples (data not shown). Because of the relative ease by which HPV can be amplified from these high-molecular-weight DNA samples, the lack of substantial differences between systems should not be taken to reflect their relative performance when analyzing clinical samples with smaller amounts of DNA.

To study the abilities of the degenerate amplification primers in directing synthesis from different HPV types, different viral types were amplified. The results indicate some variation in the sensitivity of detecting different HPV types. In particular, HPV31 and HPV33 appeared to amplify somewhat less efficiently than HPV16 and HPV18 (Table 2). These differences were not predictable on the basis of the nucleotide sequences. However, the variation in amplification efficiency between HPV types is limited, given the very high overall sensitivities of the systems. Also, the results of typing cancer biopsies (see below) showed that these HPV types, as well as others, could be efficiently amplified from clinical samples.

Typing of amplification products. Oligonucleotide probes for the detection and typing of the PCR products from E1 amplification were designed to detect most of the known genital HPV types (Table 1). The length of the probes was set to result in similar T_ms to allow identical hybridization and washing conditions. The specificity of hybridization was determined by using amplification products from HPV plasmids and cell lines. An example of the typing results for a subset of the probes for control and clinical samples (cervical cancer biopsies) is shown in Fig. 4.

Comparison with other amplification systems. We compared the amplification efficiency and consistency in HPV typing of our E1 nested-PCR system with those of the commonly used primer pair MY09-MY11 (15), designed to amplify a

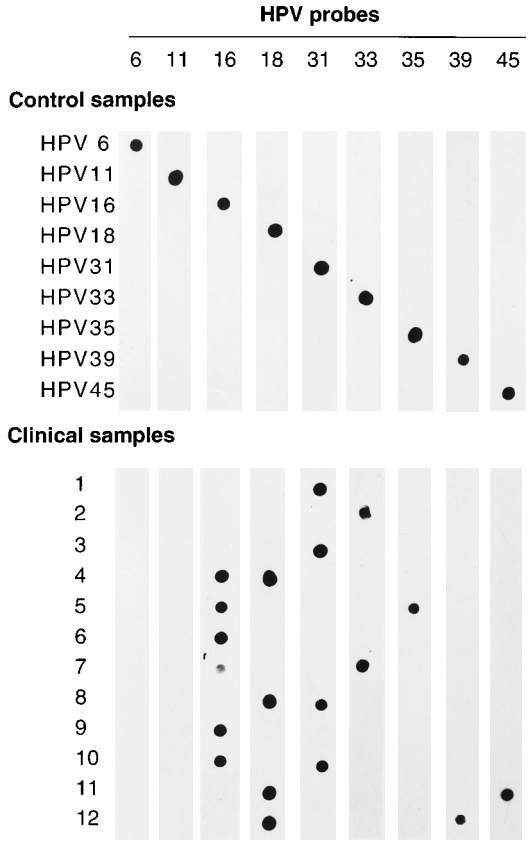

FIG. 4. Results of HPV typing by hybridization with type-specific oligonucleotide probes for nine different genital HPV types. Amplifications from HPV plasmids (Control samples) and from cervical cancer biopsies (Clinical samples) are shown.

region of the L1 open reading frame. The DNAs from 111 cervical cancer biopsies, previously analyzed by using MY09-MY11 and typed by using oligonucleotide probes for HPV16, -18, -31, and -33 (1), were amplified with the E1 primers, and the amplification products hybridized to the 19 type-specific oligonucleotide probes. Our system detected HPV in 67% (74 of 111) of the samples, while 58% (65 of 111) of the samples were found to be positive by using MY09-MY11 (Table 3). Our amplification system thus appears to be more sensitive, even with cancer biopsy material, which is usually considerably easier to analyze than cervical smears. Also, our amplification products, when visualized on ethidium bromide-stained gels, appeared to contain less nonspecific background DNA, indicative of a higher specificity for the primers, as expected for a nested amplification system (results not shown).

The typing results obtained by our E1 and the MY09-MY11 systems showed a high degree of agreement (Table 3). Since the amplification products obtained with MY09-MY11 were hybridized to only a few type-specific probes, comparison between the systems has to be restricted to HPV16, -18, -31, and -33. For three of these HPV types, the number of positive samples differs between the amplification systems. Most conspicuous is the difference for HPV16, for which our E1 system detected seven additional positive samples. Also, the lower number of HPV-negative samples and the higher number of samples carrying a mixture of HPV types indicate a higher sensitivity for our E1 system.

Analyses of cervical smears. DNA extracted from 50 Pap smears collected from a group of healthy young women attending gynecological control were analyzed by using the E1 amplification system. HPV was detected in 27% of the samples. These results show that the E1 system is able to amplify HPV in clinical samples, such as recently prepared Pap smears.

To evaluate the utility of our system for HPV detection in archival smears, we analyzed a set of smears collected from 1971 on. DNA from 20 archival smears, diagnosed as CIN grades II and III, or carcinoma in situ, was extracted by the protocol of Chua and Hjerpe (7). The smears were PCR amplified by using the E1 system, and the products hybridized to the different HPV probes. HPV was detected in 15 of the 20 samples (Table 4). Amplification products from two samples did not hybridize to any of the type-specific probes, indicating the presence of two additional HPV types. These products are currently being characterized by DNA sequencing. Two samples contained mixed infections of either HPV31 and HPV45 or HPV16 and HPV18. These results show that the E1 system is able to detect HPV in a high proportion of archival smears.

TABLE 3. Comparison of the amplification and typing of cervical cancer biopsies with L1 and E1 region primers

HPV type or result	No. of samples	
	L1 region[a]	E1 region[b]
HPV6	–[c]	0
HPV11	–	0
HPV16	41	48
HPV18	8	7
HPV30	–	0
HPV31	11	11
HPV33	10	8
HPV34	–	0
HPV35	–	5
HPV39	–	2
HPV40	–	0
HPV42	–	0
HPV45	–	3
HPV51	–	0
HPV52	–	0
HPV53	–	0
HPV56	–	0
HPV57	–	0
HPV58	–	0
HPV positive	65	74
HPV negative	46	37
HPV mixed	5	9

[a] Amplification with primer pair MY09-MY11, according to reference 15.
[b] Amplification with E1 primers 350L,547R,847R described in this article.
[c] –, not typed.

DISCUSSION

The strong association found between infection with HPV and the development of cervical neoplasia has been established in numerous case control studies (16). Still, many issues concerning the natural history of HPV remain to be solved. A prerequisite for studying the presence and variation of HPV in cervical lesions is a sensitive and high-resolution HPV detection system. The inherent sensitivity of PCR makes this method suitable for the analysis of even the most demanding clinical samples.

Several PCR systems have been designed to amplify a multitude of HPV types (10, 15, 21, 22, 26). In addition, systems for type-specific amplification are available (24, 25). These systems

TABLE 4. Results of the amplification and typing of archival smears by using the E1 system

Sample	Cytological status[a]	HPV type
1	CIN II	+[b]
2	CIN II	HPV31, HPV45
3	CIN II	−
4	CIN II	HPV33
5	CIN II	HPV18
6	CIN II	−
7	CIN II	−
8	CIN II	HPV31
9	CIS	HPV16
10	CIN III	+[b]
11	CIN II	HPV16
12	CIN II	HPV39
13	CIN II	HPV31
14	CIN III	−
15	CIN II	−
16	CIN II	HPV16
17	CIN III	HPV16, HPV18
18	CIN III	HPV16
19	CIN III	HPV31
20	CIN II	HPV31

[a] CIN II or III, CIN grade II or III, respectively; CIS, cancer in situ.
[b] Amplification products of expected size obtained, but no hybridization to any of the genital HPV types tested for.

have been shown to be able to detect HPV in a variety of clinical materials. However, none of these systems fulfil all the requirements for an ideal PCR system with regard to sensitivity, contamination safety, and HPV type generality. In this study, we have described two new amplification systems for genital HPV, designed to overcome some of the difficulties presented by the previous systems.

An important factor contributing to the efficiency of the PCR is the length of the product. In many samples, such as archival smears, the amount of intact HPV genome is very small. Since the amplification efficiency of a genomic fragment is inversely related to the length of the region targeted by PCR, we designed the final amplification products of our systems to be as short as 180 to 250 bp. This length is only 40 to 50% of the size of the MY09-MY11 system and similar to the size for the GP5 and GP6 system (9, 15). The sensitivity as well as the specificity of the amplification reaction can also be dramatically enhanced by using several overlapping primer pairs in a set of consecutive reactions. This procedure has been used for the MY09-MY11 and other HPV amplification systems (9). The high contamination risk of such systems that cannot be controlled by standard anticontamination systems, such as the use of dUTP and uracil *N*-glycosylase treatment, makes these approaches highly susceptible to cross-contamination between samples. A single-tube nested-PCR system effectively eliminates the risk of such contamination. Our system thus fulfils a necessary prerequisite for a method suitable for clinical use. Studies to determine the sensitivities of our systems relative to those of methods employing regular nested PCR are in progress.

After amplification, we carried out HPV typing by hybridization to a set of 19 different type-specific probes, designed to have similar hybridization kinetics. The hybridization system used is sensitive and covers almost the whole spectrum of genital HPV types known at this time. To save time using the present detection format (membrane hybridization), some of the most uncommon probes can be mixed into one or a few hybridization reactions, reducing the number of parallel hybridizations. For initial screening of amplification products, a pool of high- and low-risk HPV-type probes could be used. For clinical use, a microtiter plate-based format for the typing of the E1 amplification products would be preferential and is currently being developed.

Our typing system appears to be well suited for studies of HPV in cervical smears. One of the main limitations of PCR-based analysis of Pap smears appears to be the method for DNA extraction. Attempts to analyze archival smears by PCR have been made with only moderate success (6, 11, 17). However, more recent experiences, employing altered extraction protocols, have shown more promising results (7, 8).

In conclusion, the results indicate that our amplification and typing systems for HPV will be extremely useful for the analysis of very limited amounts of DNA, such as those present in archival cervical smears. In Sweden, a large collection of Pap smear material is available and retrospective epidemiological studies of this collection will provide a unique opportunity to study the natural history of HPV over a period of 15 to 20 years.

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