Amplification of Human Papillomavirus DNA Sequences by Using Conserved Primers

LUCIE GREGOIRE,^{1,2} MAXIMILLIEN ARELLA,³ JOSE CAMPIONE-PICCARDO,^{1,3,4}
AND WAYNE D. LANCASTER^{5*}

Department of Microbiology and Immunology, University of Ottawa, Ottawa K1H 8M5,¹ Laboratory of Medicine, Ottawa Civic Hospital, Ottawa K1Y 4E9,² and Molecular Virology Section, Laboratory Center for Disease Control, Ottawa K1A 0L2,⁴ Ontario, and Centre de Recherches en Virologie, Institut Armand-Frappier, Laval H7N 4Z3, Quebec,³ Canada, and Department of Molecular Biology and Genetics, Center for Molecular Biology, Wayne State University School of Medicine, Detroit, Michigan 48201⁵

Received 28 June 1989/Accepted 16 August 1989

The polymerase chain reaction has potential for use in the detection of small amounts of human papillomavirus (HPV) viral nucleic acids present in clinical specimens. However, new HPV types for which no probes exist would remain undetected by using type-specific primers for the polymerase chain reaction before hybridization. Primers corresponding to highly conserved HPV sequences may be useful for detecting low amounts of known HPV DNA as well as new HPV types. Here we analyze a pair of primers derived from conserved sequences within the E1 open reading frame for HPV sequence amplification by using the polymerase chain reaction. The longest perfect homology among HPV sequences is a 12-mer within the first exon of E1M. A region of conserved amino acids coded by the E1 open reading frame allowed the detection of another highly conserved region about 850 base pairs downstream. Two 21-mers derived from these conserved regions were used to amplify sequences from all HPV DNAs used as templates. The amplified DNA was shown to be specific for HPV sequences within the E1 open reading frame. DNA from HPVs whose sequences were not available were amplified by using these two primers. HPV DNA sequences in clinical specimens could also be amplified with the primers.

Infection of the anogenital tract by human papillomavirus (HPV) is now recognized as a venereally transmitted disease associated with the pathogenesis of cancer and its precursor lesions. Of the more than 56 known HPV types, at least 21 infect the anogenital tract. These mucosotropic viruses are most frequently associated with benign condyloma or latent infections. However, the presence of HPV in premalignant lesions and invasive cancers, particularly of the cervix, may reflect the oncogenic potential of these viruses.

Certain virus types, namely, HPV type 16 (HPV-16) and HPV-18 and, to a lesser extent, HPV-31, -33, and -35, are found in a high proportion of invasive cervical cancers and their metastases (3, 4, 10, 13, 21–24). However, many HPVs infecting the anogenital tract, such as HPV-6 and -11, are found most commonly in benign condyloma and only rarely in invasive cancers (24). Thus, HPVs detected in the anogenital tract can be broadly classified as low-risk (HPV-6 and -11), intermediate-risk (HPV-31, -33, and -35), or high-risk (HPV-16 and -18) viruses based on their association with malignancy (24). In a recent study, HPV-18 was not detected in premalignant lesions but was found in 17% of invasive cervical cancers, suggesting that this virus may be associated with a rapid disease course (18). In addition, HPV-18 tended to be associated more frequently than HPV-16 with higher-grade tumors and metastases in younger patients (2).

For these reasons it would be advantageous to be able to identify and classify these viruses in clinical specimens. Since no immunological test is currently available for virus typing, molecular hybridization has routinely been used for the detection of virus nucleic acids in clinical specimens. Many of these methods are technically cumbersome or lack the required specificity or sensitivity. Given its high degree

of sensitivity and relative simplicity, the polymerase chain reaction (PCR) followed by hybridization provides a better alternative for the detection of HPV sequences. Virus type-specific primers have been used for amplification of HPV-11, -16, and -18 sequences (34, 35, 38), but the ponderous number of primers necessary to amplify DNA from every virus type would make PCR impractical for typing HPV in clinical specimens.

From early studies it was demonstrated that papillomaviruses share amino acid sequences in the major capsid protein (reviewed in reference 1) and that the DNAs of diverse members of the virus genus cross-hybridize, indicating homologous sequences (20). Analysis of the sequences of a number of human and animal papillomaviruses has revealed conserved sequences in the noncoding region as well as the E1, E6, E7, and L1 open reading frames (ORFs). Use of primers derived from sequences shared by different virus types for PCR amplification of papillomavirus DNA sequences could be of value for the identification of viral sequences in clinical specimens.

We utilized two consensus sequence primers from the 5' half of the E1 ORF that anneal to a wide variety of human and animal papillomavirus DNAs. These universal primers were used for amplification of HPV-6, -11, -16, -18, and -33 DNA. In addition, we amplified the genomes of both human and animal cutaneous and mucosal viral DNAs whose sequences are unknown. Viral genomes in clinical specimens can also be amplified with these universal primers.

MATERIALS AND METHODS

Primers. Primers were synthesized from consensus sequences detected by a computer homology search of HPV genomes (6–8, 31, 32). The primers used in this study were

* Corresponding author.

TABLE 1. Nucleotide positions for the primary annealing sites of the E1 ORF universal primers^a

Virus	Nucleotide position of annealing site	
	IU	IWDO
HPV-1a	1019	1798
HPV-5	1180	1929
HPV-6b	1066	1932
HPV-8	1164	1910
HPV-11	1066	1932
HPV-16	1111	1962
HPV-18	1167	2032
HPV-33	1122	1955

^a IU anneals to the noncoding strand, and IWDO anneals to the coding strand.

derived from the E1 ORF. Primers, termed IU and IWDO, consisted of 21-mers with the following sequences:

IU 5'-TII(AG)I(AG)II(CT)TAAAACGAAAGT-3'
IWDO 5'-(AG)TC(AG)(AT)AIGCCCA(CT)TGIACCAT-3'

Inosine residues were used in place of four-base permutations to facilitate stabilization during annealing (27). The locations of these primers on sequenced HPV genomes are given in Table 1. Primers and probes described by Saiki et al. (29) for β -globin were used for PCR with total cellular DNA.

PCR. PCR for amplification of DNA sequences was carried out as described by Saiki et al. (30), with minor modifications. Briefly, amplification reactions were performed in a volume of 100 μ l in 0.5-ml microcentrifuge tubes in 10 mM Tris hydrochloride (pH 8.3)–1.5 mM MgCl₂–50 mM KCl with the deoxyribonucleotides at final concentrations of 200 μ M each and primers at 1 μ M. HPV DNAs at a concentration of 1 ng in 100 μ l of amplification mixture were denatured at 95°C for 5 min before the addition of 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus) (5, 11). The amplification mixture was overlaid with 100 μ l of mineral oil, and the amplifications were carried out in a DNA thermal cycler (Perkin-Elmer Cetus). DNAs were annealed at 37°C for 2 min with a rise in temperature to 55°C over a period of 90 s. The first extension was at 55°C for 1 min, followed by a rise in temperature to 72°C in 60 s with a 3-min extension period. DNAs were denatured at 94°C, and the steps were repeated for an additional 24 cycles unless specified otherwise.

DNA preparation. HPV types whose sequences are 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 (9), HPV-11 (15), and HPV-16 (10) were released by *Bam*HI cleavage. HPV-18 sequences (4) were released by digestion with *Eco*RI and HPV-33 DNA (3) by digestion with *Bgl*II. Virus DNAs whose sequences have not been published were released from flanking vector sequences by cleavage at the unique cloning site. HPV-2b (17), HPV-31 (21), and HPV-52 (36) 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 (17), bovine papillomavirus type 7 (BPV-7) (R. Olson and W. D. Lancaster, unpublished data), and the two HPV-35 fragments (23) from their vectors.

Total cellular DNA from clinical samples was purified by lysing cells in 0.6% sodium dodecyl sulfate–0.01 M EDTA containing 100 μ g of proteinase K per ml and incubated overnight at 37°C. Proteins were removed by two extractions

FIG. 1. Amplification of HPV DNAs with universal 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). *Hind*III-digested lambda DNA was used for size markers. Lane C contained amplified lambda DNA (500 bp).

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 H₂O, and digested with *Bam*HI and *Hind*III (19). Approximately 0.01 to 0.1 μ g of DNA was used in the amplification reaction.

Labeling HPV DNA. PCR products from recombinant HPV DNA were electrophoresed in low-melting-point agarose gels. Ethidium bromide-stained 850-base-pair (bp) fragments were excised and labeled with [α -³²P]dATP by the random primer technique by using Klenow polymerase directly within the low-melting-point agarose (12). Specific activities of about 10⁸ cpm/ μ g of DNA were routinely obtained with this method. The β -globin probe was labeled with [γ -³²P]ATP by the T4 kinase exchange reaction (25).

RESULTS

Based on the G+C content and the size of the universal primers IWDO and IU, the calculated temperatures of dissociation (T_d) were 58 and 48°C, respectively (16). Annealing of these primers was carried out at 11°C below the T_d of IU. In preliminary studies, the amplification reactions failed to yield a detectable product when carried out at the optimal temperature of extension by *Taq* polymerase. Presumably one or both primers melted from the template before extension was initiated. To maintain annealing of primers to the template, we first slowly increased the reaction temperature from 37 to 55°C for the first period of polymerization (1 min), followed by a second period of extension at 72°C for 3 min. All HPV DNAs amplified under these conditions showed the expected 850-bp fragment. In addition to the expected band, HPV-18 consistently showed the presence of a fragment about 550 bp in length (Fig. 1).

To determine the source of this additional fragment, PCR was run with only one of the two primers. When IWDO was used as the primer, HPV-6 and -18 DNAs showed fragments of about 850 and 550 bp, respectively. No amplification was observed with HPV-11, -16, or -33 DNAs when IWDO was used as a single primer. When IU was used as the primer, a very faint band at about 850 bp was detected only for HPV-33 (Fig. 2). The extra fragments for HPV-6 and -33 observed after single-primer amplification were not detected in dual-primer reactions because of comigration with the

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

expected 850-bp fragment. A computer search of the viral sequences revealed potential alternative annealing sites at 69% homology with IWDO only for HPV-6 and -18. This degree of mismatch would be tolerated under the conditions of primer annealing (37°C). The annealing (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 localized, 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 nucleotides downstream on the negative strand. For HPV-6, only one set of additional alternative annealing sites was detected. One site was located at position 3006 on the positive strand, and the other was located 859 bp downstream on the negative strand. For HPV-33 the target site for IU was 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 at position 976 on the positive strand and 845 bp downstream in the correct orientation on the negative strand.

To eliminate any alternative primer-binding sites, the temperature of annealing was increased. The PCR was repeated for HPV-6, -18, and -33 with only one primer, and the annealing temperature was increased from 37 to 46°C. HPV-6 and -33 did not show amplification at this temperature, but a 550-bp band was still present for HPV-18. When the temperature of annealing was increased from 46 to 52°C, HPV-18 failed to amplify with a single primer. At an annealing temperature of 52°C, no extension time was included at that temperature, but rather a slow increase in temperature from 52 to 72°C over a period of 90 s followed by extension completed the cycle. By increasing the temperature to 52°C, either we prevented the annealing of IU or IWDO to secondary sites demonstrated by the disappearance of these additional bands in HPV-6, -18, and -33 (Fig. 3) or the annealing was drastically reduced. Amplification with these two primers was successful even though annealing was carried out at a temperature 4°C higher than the lowest T_d for IU.

No differences were noted in the intensity of ethidium bromide-stained fragments generated at 46°C versus 52°C

FIG. 3. Effect of temperature of annealing on amplification of HPV DNA with universal 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. *Hind*III-digested lambda DNA was used for size markers.

annealing temperatures. However, serial dilution of the HPV-6 and -16 templates indicated difference in the amount of DNA amplified of about 2 orders of magnitude with limiting amounts of template (Fig. 4). At 46°C (40 cycles) we could easily detect 0.01 pg of amplified fragment, whereas only 1.0 pg was detectable at 52°C (40 cycles). Hybridization revealed that we could detect the product of amplification from as little as 0.001 fg (1 to 10 molecules) of HPV-6 DNA at 46°C and 1 pg (2×10^5 molecules) at 52°C.

Amplified fragments obtained after annealing at 52°C were labeled and hybridized to *Pst*I digests of the HPV DNAs under standard (25°C) conditions (Fig. 5). In each instance the PCR product of each HPV hybridized only to its respective template. However, HPV-6 and -11 share a high degree of homology within this region and cross-hybridize. Furthermore, only the fragment containing the target sites for the universal primers hybridized to the PCR product. Thus, the reaction conditions described above produced a PCR product that was specific for virus type as well as the predicted region of viral DNA.

To evaluate the utility of the primers as HPV universal primers, we attempted amplification of a variety of human and animal papillomavirus DNAs whose sequences are not available. At a temperature of annealing of 52°C, amplification of HPV-2, -4, -31, -35, and -52 and COPV DNAs produced fragments about 850 bp in length (Fig. 6). On ethidium bromide staining, no amplification product was detectable for BPV-7 DNA. Although equal amounts (1 ng)

FIG. 4. Effect of temperature on the efficiency of amplification of HPV DNA with universal primers. HPV-6 DNA was denatured and allowed to anneal to the universal primers at 46 or 52°C, followed by 40 cycles of PCR.

FIG. 5. Specificity of amplified E1 ORF HPV DNA with universal primers. 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 *Pst*I. (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. *Hind*III-digested lambda DNA was used for markers. The marker in panel A is for the gel in which HPV-6, -11, -16, and -18 were run, whereas the marker in panel B is for HPV-33, which was run in a different gel.

of viral DNA were used for amplification, HPV-2 produced a detectable but fainter signal, suggesting that one or both primers did not efficiently anneal to the template.

DNA from clinical samples, previously typed by Southern blot hybridization, were selected to be amplified with these

FIG. 6. Utility of universal primers for amplification of different papillomavirus DNAs. DNAs from HPV-2, -4, -31, -35, and -52, BPV-7, and COPV were denatured and annealed to IU and IWDO at 52°C, followed by 25 cycles of PCR. All DNAs were successfully amplified, with the exception of BPV-7. HPV-2 produced a weak band (arrow). Two different preparations of HPV-31 and -52 were used (A and B). HPV-35 S and L (L contains the E1 ORF) represent the 3.75- and 4.1-kb fragments, respectively, of the HPV-35 genome (22). HPV-33 was used as the positive control. *Hind*III-digested lambda DNA was used for size markers.

TABLE 2. Comparison of Southern blot HPV typing before and after PCR with universal primers on DNA obtained from clinical specimens and human cell lines

DNA source	HPV type	
	Before PCR	After PCR
Penile condyloma	6	6
Anal condyloma	6	6
Conjunctival papilloma	11	11
Cervical carcinoma	16	16
Cervical carcinoma	16	16
CIN ^a I	16	16
Cervical carcinoma	18	18
Cervical carcinoma	18	18
Cervical carcinoma	31	31
CIN II	31	31
CIN I	31	31
Cervical carcinoma	Negative	31
Normal vulva	Negative	31
Normal cervix	Negative	Negative
CaSki cells	16	16
HeLa cells	18	18
293 cells	Negative	Negative

^a CIN, Cervical intraepithelial neoplasia.

universal primers. After double digestion with *Bam*HI and *Hind*III, the samples were subjected to 25 cycles of amplification in the presence of IU and IWDO as well as β -globin primers at an annealing temperature of 46°C. Samples (15 μ l) of the amplification mixture were electrophoresed through 0.8% agarose gels, transferred to nylon membranes, and hybridized to labeled amplified fragments of HPV-6, -11, -16, -18, or -31 as well as β -globin oligomer under standard (25°C) conditions. For negative controls, three samples previously negative by Southern blot and 1 μ g DNA from the human cell line 293 were included.

All samples hybridized to the β -globin probe, indicating that sufficient cellular DNA was present in the sample and that the amplification reaction was not inhibited. The HPV hybridization results on the amplified DNA correlated with those of previous Southern blots (Table 2). Two of the previously negative samples by Southern blotting were faintly positive for HPV-31. These samples represented biopsy specimens from a normal vulva and a squamous cell carcinoma of the cervix. The positive normal vulva could be the result of latent infection, and the squamous cell cervical cancer could have been negative by Southern blotting because of low virus DNA concentration. Alternatively, these two samples could have been contaminated during the numerous manipulations involved in DNA extraction, restriction enzyme digestion, and PCR. This seems unlikely, however, since the other negative control (normal cervix) and DNA from human cell line 293 remained negative. Furthermore, in no instance did we detect HPV DNA from two different types in any sample.

DISCUSSION

We have demonstrated that consensus sequences derived from the E1 ORF can be used as universal primers for amplification of HPVs associated with genital tract infections. Because of the large number of HPVs associated with these infections, it would be difficult to use all of the primers necessary for amplification of viral sequences in the PCR. Universal primers, on the other hand, obviate the need for type-specific primers in the PCR.

Because these primers show varied degrees of homology with the templates, we initially employed an annealing temperature based on the lowest possible T_d . Amplification at this temperature resulted in unexpected bands that were eliminated by raising the annealing temperature. However, this temperature increase resulted in a twofold order reduction in efficiency of amplification. This could be accounted for by the degeneration of the primers, in which only a small proportion of the primers formed thermostable duplexes with the template. Consensus primers derived from the L1 ORF have been described for HPV DNA amplification (26). However, because of degenerate positions, a mix of primers with varied sequences was required. To eliminate the need for a large number of specific primers in this study, inosine was used in the place of any four-base redundancy to increase the thermal stability of the primers. This has the additional advantage of avoiding the reduction in the concentration of the hybridizing primers when there are degenerate positions.

The universal primers used in this study for amplification of HPV-6, -11, -16, -18, and -33 DNA were derived from previously published sequences. However, we were also able to amplify a number of unsequenced HPV DNAs regardless of their tissue preference. Since the universal primers are based on sequences highly conserved among the tested HPVs, they are likely to represent conserved sequences characteristic of the virus genus. Therefore, the universal primers described here could be useful for the detection of as yet uncharacterized virus types. They would also be useful for the confirmation of HPV sequences in lesions not normally associated with HPV infection. Even though we were able to amplify COPV DNA, the broad utility of these primers may be limited to HPVs. The inability to amplify BPV-7 DNA supports this conclusion.

Since benign and premalignant lesions contain episomal HPV DNA sequences, PCR with these universal primers would be useful as a general screening tool for detection of viral sequences in clinical specimens. Amplified fragments could then be typed with specific oligonucleotide probes. Based on the analysis of cervical cancer cell lines, it is generally felt that integration of the HPV genome is a common event during the development of the invasive cervical cancers (28). Integration of viral sequences during malignant transformation could result in modification of the viral genome with preferential retention of the noncoding region and the E6 and E7 ORFs. Analysis of cervical cancer cell lines has revealed that HPV integration is within the E1 or E2 ORF, resulting in deletion of a portion or all of the region (33). However, analysis of cervical cancers has indicated that HPV genomes are present as multiple-copy episomes in about 60% of cancers (14, 37). Thus, the primers described here should be of value for the determination of HPV type in cervical cancers. Recently, it was shown that approximately 30% of histologically normal lymph nodes from patients with cervical cancer contained HPV sequences detectable by Southern blotting (14). Because of the sensitivity of PCR and the utility of universal primers, it should be possible to detect HPV sequences in a high percentage of covert metastases in women undergoing surgical treatment for cervical cancer.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants CA32603 and CA32638 from the National Institutes of Health.

We thank Joan Holsinger for excellent technical assistance.

LITERATURE CITED

1. Baker, C. C. 1987. Sequence analysis of papillomavirus genomes, p. 321-385. In P. M. Howley and N. P. Salzman (ed.), *The papovaviridae*, vol. 2. Plenum Publishing Corp., New York.
2. Barnes, W., G. Delgado, R. J. Kurman, E. S. Petrilli, D. M. Smith, S. Ahmed, A. T. Lorincz, G. F. Temple, A. B. Jenson, and W. D. Lancaster. 1988. Possible prognostic significance of human papillomavirus type in cervical cancer. *Gynecol. Oncol.* 29:267-273.
3. Beaudenon, S., D. Kremsdorf, O. Croissant, S. Jablonska, and G. Orth. 1986. A novel type of human papillomavirus associated with genital neoplasias. *Nature (London)* 321:246-249.
4. Boshart, M., L. Gissmann, H. Ikenburg, A. Kleinheinz, W. Scheurlen, and H. zur Hausen. 1984. A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. *EMBO J.* 3:1151-1157.
5. Chien, A., D. B. Edgar, and J. N. Trela. 1976. Deoxyribonucleic acid polymerase from the extreme thermophile *Thermus aquaticus*. *J. Bacteriol.* 127:1550-1557.
6. Cole, S., and R. Streeck. 1986. Genome organization and nucleotide sequence of human papillomavirus type 33, which is associated with cervical cancer. *J. Virol.* 58:991-995.
7. Cole, S. T., and O. Danos. 1987. Nucleotide sequence and comparative analysis of the human papillomavirus type 18 sequence. *J. Mol. Biol.* 193:599-608.
8. Dartmann, K., E. Schwarz, L. Gissmann, and H. zur Hausen. 1986. The nucleotide sequence and genome organization of human papillomavirus type 11. *Virology* 151:124-130.
9. de Villiers, E.-M., L. Gissmann, and H. zur Hausen. 1981. Molecular cloning of viral DNA from human genital warts. *J. Virol.* 40:932-935.
10. Durst, M., L. Gissmann, and H. zur Hausen. 1983. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proc. Natl. Acad. Sci. USA* 80:3812-3815.
11. Erlich, H. A., D. H. Gelfand, and R. H. Saiki. 1988. Specific DNA amplification. *Nature (London)* 331:461-462.
12. Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction fragments to high specific activity. *Anal. Biochem.* 132:6-13.
13. Fuchs, P. G., F. Girardi, and H. Pfister. 1988. Human papillomavirus DNA in normal, metaplastic, preneoplastic and neoplastic epithelia of the cervix uteri. *Int. J. Cancer* 41:41-45.
14. Fuchs, P. G., F. Girardi, and H. Pfister. 1989. Human papillomavirus 16 DNA in cervical cancers and in lymph nodes of cervical cancer patients: a diagnostic marker for early metastases? *Int. J. Cancer* 43:41-44.
15. Gissmann, L., V. Diehl, H. J. Schultz-Coulon, and H. zur Hausen. 1982. Molecular cloning and characterization of human papillomavirus DNA derived from a laryngeal papilloma. *J. Virol.* 44:393-400.
16. Hames, B. D., and S. J. Higgins. 1985. Nucleic acid hybridisation: a practical approach. IRL Press, Oxford.
17. Heilman, C. A., M.-F. Law, M. A. Israel, and P. M. Howley. 1980. Cloning of human papillomavirus genomic DNAs and analysis of homologous polynucleotide sequences. *J. Virol.* 36:395-407.
18. Kurman, R. J., M. H. Schiffman, W. D. Lancaster, R. Reid, A. B. Jenson, G. F. Temple, and A. T. Lorincz. 1988. Analysis of individual human papillomavirus types in cervical neoplasia: a possible role for type 18 in rapid progression. *Am. J. Obstet. Gynecol.* 159:293-296.
19. Lancaster, W. D., R. J. Kurman, and A. B. Jenson. 1986. Papillomaviruses in anogenital neoplasms, p. 153-183. In A. A. Luderer and H. H. Weetall (ed.), *The human oncogenic viruses*. Humana Press, Clifton, N.J.
20. Law, M.-F., W. D. Lancaster, and P. M. Howley. 1979. Conserved sequences among the genomes of papillomaviruses. *J. Virol.* 32:199-207.
21. Lorincz, A. T., W. D. Lancaster, and G. F. Temple. 1986. Cloning and characterization of the DNA of a new human papillomavirus from a woman with dysplasia of the uterine

- cervix. *J. Virol.* **58**:225–229.
22. Lorincz, A. T., A. P. Quinn, M. D. Goldsborough, B. J. Schmidt, and G. F. Temple. 1989. Cloning and partial DNA sequencing of two new human papillomavirus types associated with condylomas and low-grade cervical neoplasias. *J. Virol.* **63**:2829–2834.
 23. Lorincz, A. T., A. P. Quinn, W. D. Lancaster, and G. F. Temple. 1987. A new type of papillomavirus associated with cancer of the uterine cervix. *Virology* **159**:187–190.
 24. Lorincz, A. T., G. F. Temple, R. J. Kurman, A. B. Jenson, and W. D. Lancaster. 1987. Oncogenic association of specific human papillomavirus types with cervical neoplasia. *J. Natl. Cancer Inst.* **79**:671–677.
 25. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 26. Manos, M. M., Y. Ting, D. K. Wright, A. J. Lewis, T. R. Broker, and S. M. Wolinsky. 1989. The use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. *Cancer Cells* **7**:209–214.
 27. Ohtsuka, E., S. Matsuki, M. Ikehara, Y. Takahashi, and K. Matsubara. 1985. An alternative approach to deoxyoligonucleotides as hybridization probes by insertion of deoxyinosine at ambiguous codon positions. *J. Biol. Chem.* **260**:2605–2608.
 28. Pfister, H. 1987. Relationship of papillomaviruses to anogenital cancer. *Obstet. Gynecol. Clin. North Am.* **14**:349–361.
 29. Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**:487–491.
 30. Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim. 1985. Enzymatic amplification of β -globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230**:1350–1354.
 31. Schwarz, E., M. Durst, C. Demankowski, O. Lattermann, R. Zech, E. Wolfspurger, S. Suhai, and H. zur Hausen. 1983. DNA sequence and genome organization of genital human papillomavirus type 6b. *EMBO J.* **2**:2341–2348.
 32. Seedorf, K., G. Krammer, M. Durst, S. Suhai, and W. Rowe-kamp. 1985. Human papillomavirus type 16 DNA sequence. *Virology* **145**:181–185.
 33. Shah, K. V., and L. Gissmann. 1989. Experimental evidence on oncogenicity of papillomaviruses, p. 105–111. In N. Munoz, F. X. Bosch, and O. M. Jensen (ed.), Human papillomaviruses and cervical cancer. International Agency for Research on Cancer, Lyon, France.
 34. Shibata, D., N. Arnheim, and W. J. Martin. 1988. Detection of human papillomavirus in paraffin-embedded tissue using the polymerase chain reaction. *J. Exp. Med.* **167**:225–230.
 35. Shibata, D., W. J. Martin, and N. Arnheim. 1988. Analysis of DNA sequences in forty-year old paraffin-embedded thin-tissue sections: a bridge between molecular biology and classical histology. *Cancer Res.* **48**:4564–4566.
 36. Shimoda, K., A. T. Lorincz, G. F. Temple, and W. D. Lancaster. 1988. Human papillomavirus type 52: a new virus associated with cervical neoplasia. *J. Gen. Virol.* **69**:2925–2928.
 37. Tidy, J., K. H. Vousden, P. Mason, and P. J. Farreh. 1989. A novel deletion within the upstream regulatory region of episomal human papillomavirus type 16. *J. Gen. Virol.* **70**:999–1004.
 38. Young, L. S., I. S. Bevan, M. A. Johnson, P. I. Blomfiend, T. Bromidge, N. J. Maitland, and C. B. J. Woodman. 1989. The polymerase chain reaction: a new epidemiological tool for investigating cervical human papillomavirus infection. *Br. Med. J.* **298**:14–18.