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Use of Universal and Type-specific Primers in the Polymerase Chain Reaction for the Detection and Typing of Genital Human Papillomaviruses

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By using polymerase chain reaction (PCR), we have developed a system for type-specific as well as universal detection of genital human papillomaviruses (HPVs). Primers and probes for specific detection of HPV-16, -18 and -33 were synthesized from the E7 open reading frame (ORF). They were capable of detecting corresponding HPV types with high specificity and sensitivity. Primers for detection of a broad spectrum of HPV (universal primers) were synthesized from the L1 ORF. The universal primers were shown to be capable of amplifying HPV-6b, -11, -16, -18, -33, -52b and -58. The system was applied to various cervical tissue specimens from Japanese patients. They consisted of 26 normal specimens, 18 from cervical dysplasias and 29 from cervical carcinomas. HPV was detected in none of the normal specimens. On the other hand, many of the specimens from cervical dysplasias and carcinomas were found to be positive for HPV, especially HPV-16. Except for one, all the specimens which were positive with the type-specific PCRs were also positive with the universal PCR. Furthermore, substantial numbers of specimens were found to be positive only with the universal PCR. Cloning and sequencing of DNA segments amplified by the universal primers were undertaken to characterize some of the unknown HPVs. Our PCR system may thus be useful for the specific detection of the three major types of oncogenic HPVs and also for the detection of a broad spectrum of HPVs including possibly novel HPV types.

Key words: Human papillomavirus — Cervical carcinoma — Cervical dysplasia — Polymerase chain reaction

Epidemiological studies employing techniques such as DNA hybridization and polymerase chain reaction (PCR) have now established that certain types of human papillomavirus (HPV) such as HPV-16, -18 and -33 are closely associated with the pathogenesis of cervical dysplasias and cancers.[1–7] Furthermore, the list of cancer-associated HPVs is growing rapidly.[8,9] PCR has been proved to be particularly useful for the detection of HPV types with known DNA sequences from small amounts of tissue specimens.[4–6] General primers based on highly conserved sequences have also been developed for PCR detection of a broad spectrum of HPV.[10–12] In the present study, we have developed type-specific PCR primers based on the E7 open reading frames (ORFs) of HPV-16, -18 and -33 and also PCR primers based on a conserved region in L1 ORF for the general detection of HPV. By using our PCR system, we have examined the presence of HPV DNA in a large number of cervical tissues obtained from patients with various clinical conditions. Our results have confirmed the high prevalence of HPV (especially HPV-16) in cervical dysplasias and carcinomas of Japanese patients.[3,6,7] Furthermore, a substantial number of cases were found to be HPV positive only by the universal PCR, indicating the existence of other HPV types associated with these cervical lesions. Our universal PCR would be, therefore, useful for the detection of a broad spectrum of HPV genotypes including potentially new HPV types.

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MATERIALS AND METHODS

Cells, tissues and HPV clones CaSki and HeLa are human cervical cancer cell lines with integrated HPV-16 and -18, respectively.[1] Human cervical and other tissue specimens were obtained from biopsy or surgical operations. Isolation of DNA from cultured cells and tissue specimens was carried out as described previously.[13] DNA was resuspended in distilled water and the concentration was determined by measuring absorbance at 260 nm.[13] Cloned HPV-18[14] was kindly provided by Dr. A. Hakura (Research Institute for Microbial Diseases, Osaka University) with the permission of Dr. H. zur Hausen (Krebsforschungszentrum, West Germany). Cloned HPV-33[15] was kindly provided by Dr. K. Fujinaga (Cancer Research Institute, Sapporo Medical College) with the permission of Dr. G. Orth (Institute Pasteur, France). Cloned HPV-52b[8] and HPV-58[9] were kindly provided by Dr. Y. Ito (Institute for Virus Research, Kyoto University) and Dr. T. Matsukura (National Institute of Health, Japan), respectively. Cloned HPV-1a,[16] HPV-6b,[17] HPV-11[18] and HPV-16[19] were supplied by the Japanese Cancer Research Resources

Bank. Recombinant plasmids were grown in HB101 and purified by two cycles of equilibrium centrifugation in cesium chloride after alkaline lysis as described previously.[13]

Primers Primers for PCR and oligonucleotide probes for hybridization analysis were synthesized on a DNA synthesizer (Cyclone, MilliGene/Biosearch, USA) and purified by using Nensorb Prep cartridges (E.I. du Pont de Nemours & Co., USA). Specific primers and probes for HPV-16, -18 and -33 were synthesized from the E7 ORF,[20-22] selecting sequences with low homologies to nonhomologous HPV types. Primers for general detection of a broad spectrum of HPV (universal primers) were selected from a highly conserved region in the L1 ORF detected by computer analyses of different HPV DNA sequences and synthesized from the sequence of HPV-16.[20] The nucleotide sequences of primers and probes are summarized in Table I.

Polymerase chain reaction PCR was carried out essentially as described by Saiki *et al.*[23] using a Gene Amp kit and a DNA thermal cycler (both from Perkin-Elmer Cetus, USA). The primers were used at the concentration of 75 pmol. In the case of amplification with type-specific primers, each cycle consisted of denaturation at 94°C for 1 min, annealing at 60°C for 2 min and extension at 72°C for 2 min, and the cycle was repeated 35 times. In the case of amplification with universal primers, annealing in each cycle was carried out at 32°C instead of 60°C. A total of 10 μl of each reaction mixture was loaded on a 6% polyacrylamide gel. After electrophoresis, amplification products were visualized by ethidium bromide staining as described previously.[13]

Because of its extreme sensitivity, PCR is notorious for false-positive results due to contamination by DNA. We also experienced some false-positive results at the beginning of this study, but such problems were overcome by employing rigorous experimental procedures such as separation of handling of DNA samples in both time and space, keeping primers and reagents in small aliquots, frequent UV-irradiation of all tools and working spaces, etc. We also always included negative controls in each set of amplification procedures.

Restriction enzyme digestion Restriction endonuclease digestion of PCR products was performed by directly adding 10 units of each enzyme to a 10 μl PCR sample. After incubation at 37°C for 1 h, digestion products were analyzed by electrophoresis on a 6% polyacrylamide gel and staining with ethidium bromide.

Southern blot analysis DNA fragments were transferred from an electrophoresis gel onto a Hybond-N nylon membrane (Amersham, UK), and hybridization was carried out as described previously.[13] Probes were labeled with ^{32}P-ddATP using a 3′-End-Labeling kit (Amersham). The temperature for hybridization was 37°C for HPV-16 probe, 35°C for HPV-18 probe and 47°C for HPV-33 probe. The membrane was washed in 5×SSPE and 0.1% SDS at 50°C for HPV-16 probe, at 40°C for HPV-18 probe, and at 55°C for HPV-33 probe. Autoradiography was carried out overnight at −80°C with an intensifying screen.

Cloning and sequencing of universal PCR products Universal primers with 8 additional nucleotides (CCAAGCTT) at their 5′ ends to create a *Hin*dIII site (AAGCTT) were used for PCR amplification. After chloroform extraction and ethanol precipitation, amplification products were digested with *Hin*dIII, separated by gel electrophoresis, and isolated by electroelution. DNA fragments were ligated with *Hin*dIII-digested pUC118. Recombinant plasmids were grown in *E. coli* JM109, and were purified as stated above. Nucleotide sequences of inserts were determined from both directions by using a T7 Sequencing kit (Pharmacia, Sweden).

Computer analysis All matrix, homology and restriction site analyses were carried out by using DNASIS program version 6 (Hitachi Software).

RESULTS

Type-specific PCR for HPV Type-specific PCR primers and probes for HPV-16, -18 and -33 were synthesized from the published nucleotide sequences of the E7 ORF (Table I).[20-22] As shown in Fig. 1a, the primers specific for HPV-16 amplified products of about 200 bp only from cloned HPV-16 DNA or CaSki DNA. The amplified bands were close to the expected size (203 bp), hybridized with the HPV-16 specific probe (Fig. 1d), and had diagnostic *Pvu*II and *Eco*RI sites (data not shown). Similarly, primers specific for HPV-18 amplified bands from cloned HPV-18 DNA and HeLa DNA with the size of about 250 bp, close to the expected size of 244 bp (Fig. 1b). The amplified products hybridized with the HPV-18 specific probe (Fig. 1e), and had the expected diagnostic *Hin*fI and *Pvu*II sites (data not shown). Primers for HPV-33 amplified a band of about 250 bp only from cloned HPV-33 DNA (Fig. 1c). The band was of a size close to the expected 240 bp, hybridized with the HPV-33 specific probe (Fig. 1f), and had the expected diagnostic *Eco*RI and *Hin*cII sites (data not shown). These results clearly demonstrated that our type-specific primers were capable of amplifying sequences from corresponding HPV types with high specificity.

Fig. 2 shows the experiments carried out to determine the detection sensitivity of each type-specific PCR by using cloned HPV-DNA serially diluted without or with cellular DNA. The detection sensitivities were mostly similar under both conditions, even though the intensities of specifically amplified bands were reduced and the levels of nonspecific amplifications were increased in the

Table I. The Nucleotide Sequences of PCR Primers and Probes

		Base position
HPV-16E7		
sense primer	+5′-AGCTCAGAGGAGGAGGATGA-3′	652–671
anti-sense primer	−5′-GGTTTCTGAGAACAGATGGG-3′	835–854
probe	+5′-TGTGCGTACAAAGCACACAC-3′	761–780
diagnostic fragment	*Pvu*II-*Eco*RI	683–825
HPV-18E7		
sense primer	+5′-GAGCCCCAAAATGAAATTCC-3′	635–654
anti-sense primer	−5′-CAAAGGACAGGGTGTTCAGA-3′	859–878
probe	+5′-TAATCATCAACATTTACCAG-3′	721–740
diagnostic fragment	*Hin*fI-*Pvu*II	687–851
HPV-33E7		
sense primer	+5′-AGGACACAAGCCAACGTTAA-3′	578–597
anti-sense primer	−5′-AGTTGCTGTATGGTTCGTAG-3′	798–817
probe	+5′-CCGGCCAGATGGACAAGCAC-3′	689–708
diagnostic fragment	*Eco*RI-*Hinc*II	652–779
Universal		
sense primer	+5′-GAATATGATTTACAGTTTATTTTTCA-3′	6741–6766
anti-sense primer	−5′-GAAAACTTTTCCTTTAAAT-3′	6985–7003

Base positions are those from the original reports.[20–22]

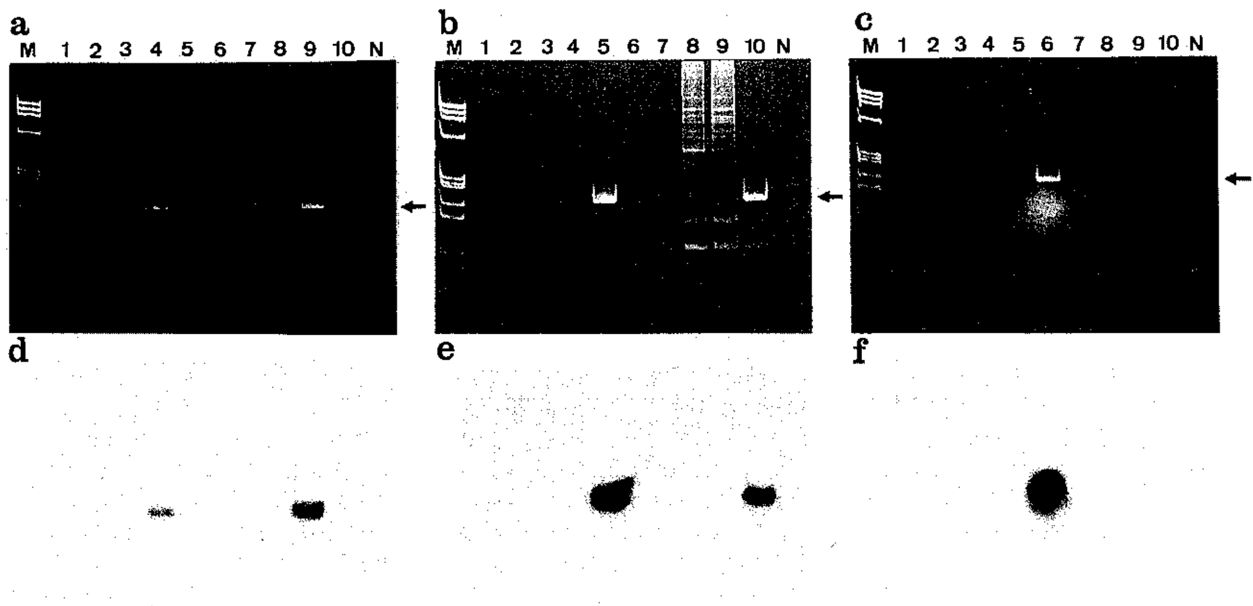

Fig. 1. Specificity of type-specific PCR. PCR was carried out on DNA samples from various recombinant HPV plasmids (1 pg) and cells (300 ng) by using type-specific primers. PCR products were detected by ethidium bromide staining after electrophoresis (a, b, c). DNA bands were then transferred to filter papers by blotting and hybridized with ^{32}P-labeled type-specific probes (d, e, f). For details, see "Materials and Methods." a and d: PCR with HPV-16 primers and hybridization with HPV-16 probe. b and e: PCR with HPV-18 primers and hybridization with HPV-18 probe. c and f: PCR with HPV-33 primers and hybridization with HPV-33 probe. Lanes are as follows. M: PhiX174 DNA digested with *Hae*III, 0.25 μg; 1: HPV-1a; 2: HPV-6b; 3: HPV-11; 4: HPV-16; 5: HPV-18; 6: HPV-33; 7: HPV-52b; 8: DNA from normal human lymphocytes; 9: DNA from CaSki; 10: DNA from HeLa; N: no DNA. The arrows indicate specifically amplified bands.

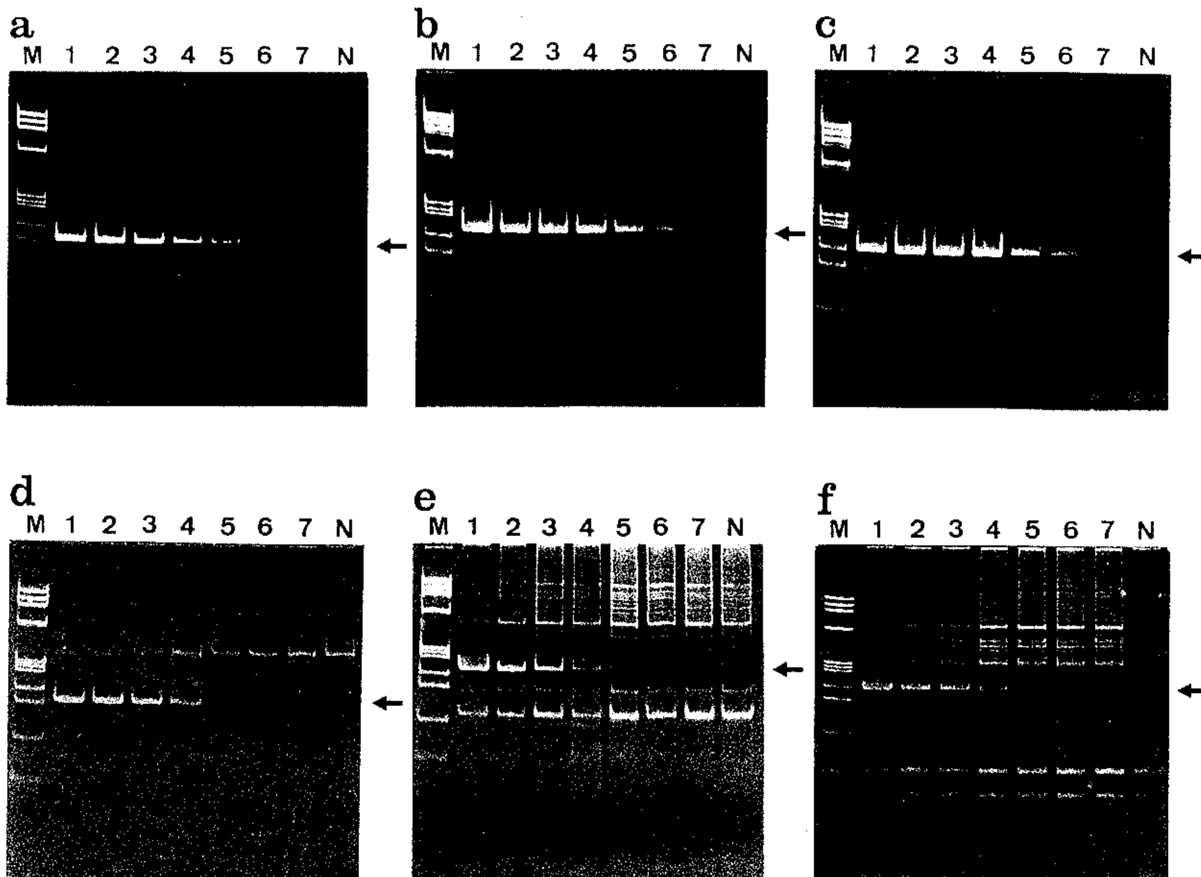

Fig. 2. Detection sensitivity of type-specific PCRs. Cloned HPV DNA of the corresponding type was serially diluted (10-fold dilutions from 10 pg to 0.01 fg per assay, 10 pg corresponds to about 10^6 copies of HPV DNA) without (a, b, c) or with (d, e, f) cellular DNA (300 ng per assay) prepared from normal human lymphocytes. PCR products were detected by ethidium bromide staining after electrophoresis. For details, see "Materials and Methods." (a, d): PCR with HPV-16 primers; (b, e): PCR with HPV-18 primers; (c, f): PCR with HPV-33 primers. M: PhiX174 DNA digested with *Hae*III, 0.25 μg. N: no HPV DNA. The arrows indicate specifically amplified bands.

presence of cellular DNA, especially at low HPV-DNA concentrations. PCR for HPV-16 produced a band detectable by ethidium bromide staining if the reaction mixture contained about ten copies of HPV-16 DNA. PCRs for HPV-18 and -33 were more efficient and capable of producing detectable bands if as few as ten to one copies of HPV DNA were present in the reaction mixture. Subsequent Southern blotting hybridizations with specific probes confirmed the specificity of PCR amplifications but did not significantly increase the detection sensitivity (data not shown).

Universal PCR for genital HPV A single set of universal primers suitable for general detection of various genital HPV types was synthesized from a highly conserved region in the L1 ORF using the sequence of HPV-16[20] (Table I). As shown in Fig. 3, our universal primers were capable of amplifying bands of about 250 bp from a broad spectrum of genital HPV types such as 6b, 11, 16, 18, 33, 52b and 58, even though the efficiency of amplification was not equal among different HPV types (it was especially low with HPV-11).

The detection sensitivity of the universal PCR was determined by using serially diluted HPV-16 DNA. Without cellular DNA, amplification products detectable by ethidium bromide staining were produced when the reaction mixture contained about 1,000 copies of HPV-16 DNA (Fig. 4a). In the presence of cellular DNA, the sensitivity seemed to be reduced by about 10-fold (Fig. 4b).

Detection of HPV in cervical tissue specimens DNA samples obtained from cervical tissue specimens taken from patients with various clinical conditions were sub-

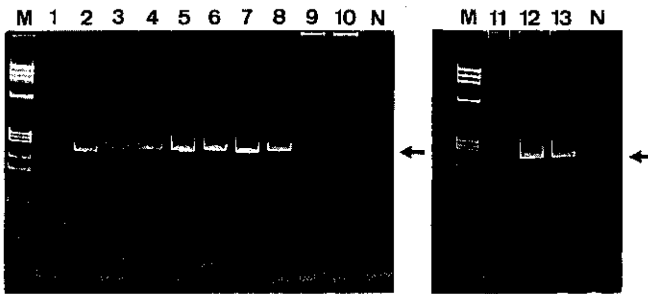

Fig. 3. PCR with universal primers. PCR was carried out with HPV DNA of various types and cellular DNA by using universal primers. PCR products were detected by ethidium bromide staining after electrophoresis. For details, see "Materials and Methods." Lanes are as follows. M: PhiX174 DNA digested with *Hae*III, 0.25 μg; 1: HPV-1a, 1 pg; 2: HPV-6b, 1 pg; 3: HPV-11, 100 pg; 4: HPV-16, 1 pg; 5: HPV-18, 1 pg; 6: HPV-33, 1 pg; 7: HPV-52b, 1 pg; 8: HPV-58, 1 pg; 9: pUC119, 1 pg; 10: pBR322, 1 pg; 11: DNA from normal human lymphocytes, 300 ng; 12: DNA from CaSki, 300 ng; 13: DNA from HeLa, 300 ng; N: no DNA. The arrows indicate specifically amplified bands.

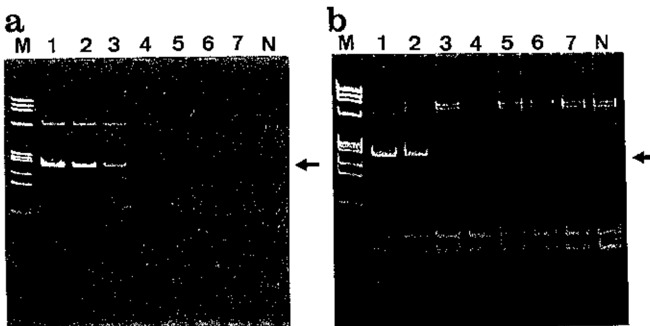

Fig. 4. Detection sensitivity of universal PCR. PCR was carried out with serially diluted HPV-16 DNA (10-fold dilutions from 10 pg to 0.01 fg per assay, 10 pg corresponds to about 10^6 copies of HPV DNA) without (a) or with (b) cellular DNA (300 ng) prepared from normal human lymphocytes. PCR products were detected by ethidium bromide staining after electrophoresis. For details, see "Materials and Methods." M: PhiX174 DNA digested with *Hae*III, 0.25 μg; N: no DNA. The arrows indicate the specifically amplified bands.

jected to PCR amplifications using the type-specific and universal primers. Representative results are shown in Fig. 5, and the results for all 73 cases are summarized in Table II.

No HPV DNA was detected by the type-specific PCRs or the universal PCR in any of the histologically-classified normal cervical tissue specimens obtained from 24 patients with myoma uteri and two patients with

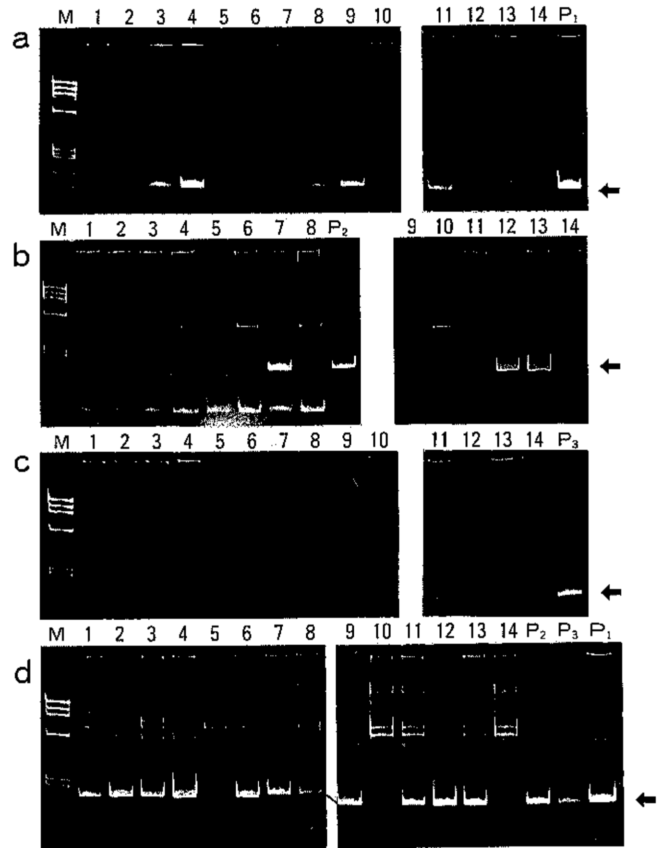

Fig. 5. Detection of HPV in DNA samples obtained from various cervical tissue specimens by type-specific and universal PCRs. PCR was carried out with DNA from clinical samples (500 ng) and cell lines (300 ng) by using the type-specific and universal primers. PCR products were detected by ethidium bromide staining after electrophoresis. For details, see "Materials and Methods." (a) PCR with HPV-16 primers. (b) PCR with HPV-18 primers. (c) PCR with HPV-33 primers. (d) PCR with universal primers. Lanes 1 to 14 correspond to individual clinical samples. P1: DNA from CaSki; P2: DNA from HeLa; P3: DNA from the recombinant HPV-33 plasmid, 1 pg; M: PhiX174 DNA digested with *Hae*III, 0.25 μg. The arrows indicate the specifically amplified bands.

Table II. Detection of HPV DNA in Various Cervical Tissue Specimens by Using Type-specific and Universal PCRs

Diagnosis	Total	PCR positive (%) for			
		16	18	33	universal
Normal	26	0	0	0	0
Dysplasia	18	5 (28)	2 (11)	0	11 (61)
mild	5	0	1 (20)	0	1 (20)
moderate	3	0	0	0	2 (67)
severe	10	5 (50)	1 (10)	0	8 (80)
Carcinoma	29	13 (45)	5 (17)	2 (7)	24 (83)

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          6897                                                        6956
HPV-16:   GTAA---CCCAGGCAATTGCTTGTCAAAAACATACACCTCCAGCACCTAAAGAAGATGATCCC
          ValThr---GlnAlaIleAlaCysGlnLysHisThrProProAlaProLysGluAspAspPro

samples:  GTAACATCCCAGGCAATTGCTTGTCAAAAACATACACCTCCAGCACCTAAAGAAGAT---CCC
          ValThrSerGlnAlaIleAlaCysGlnLysHisThrProProAlaProLysGluAsp---Pro
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Fig. 6. Comparison of the L1 nucleotide sequence and the deduced amino acid sequence of the two Japanese HPV-16 samples with those of the reported HPV-16.[20] Gaps are indicated by broken lines and insertions are underlined.

Table III. PCR Cloning and Sequencing of Unidentified HPV Detected by Universal Primers

Sample	Diagnosis	Type from sequence	Level of homology (%)
1	carcinoma	18	217/218 (99.5)
2	carcinoma	58	218/218 (100)
6	severe dysplasia	52b	218/218 (100)
33	carcinoma	52b	218/218 (100)
44	moderate dysplasia	58	217/218 (99.5)

ovarian cancer. On the other hand, among 18 samples from patients with cervical dysplasia, five (28%) were positive for HPV-16 and two (11%) were positive for HPV-18. The combined positive rate in the three type-specific PCRs was 39% (7/18). All the samples positive in the type-specific PCRs were also positive in the universal PCR. Furthermore, four extra samples were found to be HPV-positive in the universal PCR. The positive rate obtained with the universal PCR was, therefore, 61% (11/18). Similarly, among 29 samples from patients with cervical carcinoma, 13 (45%) were positive for HPV-16, five (17%) were positive for HPV-18, and two (7%) were positive for HPV-33. One sample was doubly positive for HPV-16 and -33. The combined positive rate in the three type-specific PCRs was, therefore, 66% (19/29). All these positive samples except for one were also positive in the universal PCR. The one exception was a sample that was positive for HPV-16 in the type-specific PCR. Furthermore, five extra samples were detected to be HPV-positive only by the universal PCR. The positive rate in the universal PCR was, therefore, 83% (24/29).

Nucleotide sequences of universal PCR amplification products PCR cloning and sequencing of DNA segments amplified by the universal primers would provide useful information for indentification of HPV types detectable only by the universal PCR. To examine this possibility, we first carried out PCR cloning of two DNA samples from cervical carcinoma specimens that were positive for HPV-16. The sequences of the two clones were found to be identical to each other, and also very similar to the corresponding region of the original HPV-16 sequence,[20] indicating that the amplified products were indeed derived from HPV-16. There are, however, interesting differences in the determined sequence from that of the original report (Fig. 6). Three nucleotides (CAT) are inserted at 6900, and three nucleotides (GAT) are deleted at 6948 (or 6951). The former changes bring about insertion of a serine, while the latter bring about deletion of an aspartic acid (Fig. 6).

We next applied PCR cloning to samples containing unknown HPV types. As shown in Table III, five samples have so far been determined. Sample 1 unexpectedly showed 99.5% homology to HPV-18 with one synonymous base change. Samples 6 and 33 were 100% homologous to HPV-52b (Y. Ito, personal communication). Samples 2 and 44 were 100% and 99.5% homologous to HPV-58, respectively (T. Matsukura, personal communication). The latter had a single mutation resulting in a conservative amino acid change (serine to threonine).

DISCUSSION

PCR primers designed for the detection of specific types of HPV such as HPV-16, -18 and -33 have been reported.[4-6] They were mostly based on sequences from the E6 ORF. In the present study, we have synthesized new type-specific PCR primers based on the sequences from the E7 ORF (Table I). E7 is most actively transcribed in human cervical cancer cells containing HPV genomes and is considered to take the major role in HPV-induced cell transformation and carcinogenesis.[24] PCRs for detection of E7 DNA sequences, therefore, would not only complement those designed for detection of E6 sequences but also may provide better information concerning the possible involvement of HPV in human cancers. Furthermore, our E7 primers for each HPV type are capable of highly specific amplification with a high efficiency (Figs. 1 and 2). Therefore, HPV typing with our PCRs can be done at the level of ethidium bromide staining without any further hybridization analysis, whereas some of the previous PCRs apparently required hybridization analyses with type-specific probes, presum-

ably because of low amplification efficiencies or some cross-amplifications among different HPV types.[4, 6]

PCR primers useful for detection of a broad spectrum of HPV genotypes (universal primers) have also been reported.[10-12] Gregoire *et al.*[10] described consensus primers derived from the E1 ORF. In order to facilitate annealing with various HPV types, their primers had degenerate bases in several positions and inosine residues in places of four-base permutations. Each primer is in fact a mixture of many oligonucleotides. PCR by using these primers was capable of detecting HPV-1a, -5, -6b, -8, -11, -16, -18, and -33. Consensus primers derived from the L1 ORF were also reported. Manos *et al.*[11] described consensus L1 primers that were also degenerate in several positions to accommodate base differences among various HPV types. Their primers were shown to be capable of amplifying HPV types such as 6, 11, 16, 18 and 33. Snijders *et al.*[12] prepared two sets of consensus primers from the L1 ORF. One set was designed for the detection of genital types of HPV and amplified sequences from HPV-6b, -11, -13, -16, -18, -30, -31, -32 and -33, while the other set was designed for the detection of skin-associated types and amplified sequences from HPV-1a and -8.

In the present study, we have also synthesized universal primers from the L1 ORF. We used only a single set of primers based on the HPV-16 sequence. Our primers are derived from regions entirely different from those of Manos *et al.*[11] Our 3′ primer, however, partly overlaps the 5′ primer used by Snijders *et al.*[12] Our strategy has been to carry out annealing at a relatively low temperature (32°C) to facilitate hybridization of primers even to HPV sequences with considerably low homologies. We were able to demonstrate that, by using this strategy, the single set of primers could amplify the corresponding target sequences from a broad spectrum of HPV types such as 6b, 11, 16, 18, 33, 52b and 58 (Fig. 3). HPV-52b and -58 are both recent isolates from Japanese patients with cervical carcinoma[8, 9] and it is an important confirmation that our universal PCR is capable of detecting these new HPV types. Our universal PCR is thus simple and economical (using only a single pair of primers).

By using our type-specific and universal PCRs, we have examined the presence of HPV in DNA from a total of 73 cervical tissue specimens obtained from biopsy or surgical operations. HPV was detected in none of the 26 histologically classified normal cervical tissue specimens by any of the three type-specific PCRs (Table II). The absence of HPV DNA in these normal samples was further confirmed by the universal PCR. These results, therefore, strongly suggest that HPV infection is quite rare in normal cervical tissues in Japan. Recently, by using filter *in situ* hybridization, Yokota *et al.* also found quite low incidences of HPV infections in normal cervices of Japanese women (1.8% and 0.6% for HPV-16 and -18, respectively).[7] On the other hand, many of the tissue specimens from cervical dysplasias and carcinomas were detected to be HPV-positive by the type-specific PCRs (Table II). HPV-16 was the most prevalent HPV type. In the case of dysplasia, HPV-16 also seemed to be associated with more severe cases. Detection of HPV-18 was less frequent. Infection by HPV-33 seemed to be very rare. It may be suggested that infection by HPV-16 is more likely to lead to more severe dysplasias and eventually to carcinomas than those by other types. These results are quite consistent with previous reports.[3, 6, 7] All these HPV-positive samples except for one were also scored positive by the universal PCR. Furthermore, about 20% of cervical dysplasias and carcinomas were positive only with the universal PCR. These results indicate that substantial fractions of Japanese cases with cervical dysplasias and carcinomas are infected by HPV types different from the three major types.[3]

Our finding that HPV infection was quite rare in normal cervical tissues contrasts with some previous studies in other countries which, also employing the PCR technique, showed quite high prevalence rates of HPV infection in normal cervical tissues.[5, 25] Such differences may be partly due to some geographical and/or racial factors. One must also be cautious about results obtained by PCR, since the technique is notorious for false-positivity due to laboratory contamination. Indeed, some of the papers were later retracted.[25] Furthermore, a recent study employing primers flanking HPV cloning sites (anti-contamination primers) showed much lower HPV prevalence rates in cervical scrapes and biopsies cytologically classified as normal.[26] However, it should be remembered that the normal cervical tissues used in the present study were obtained from patients who had undergone hysterectomy for myoma uteri or ovarian cancer, and such patients might not represent the general normal population. Furthermore, PCR techniques with higher detection sensitivities, such as two-step PCR might increase the rate of HPV detection.

We cloned and sequenced universal PCR amplification products from two carcinoma specimens that were found positive for HPV-16 by the type-specific PCR. The nucleotide sequences of the two clones were identical and also quite similar to that of the corresponding region of the original HPV-16.[20] There were, however, interesting differences between our sequences and the original HPV-16 sequence (Fig. 6). Insertion of three nucleotides at one site and deletion of three nucleotides at another were found in both of our samples. Surprisingly, the same mutations were recently reported in the L1 ORF of HPV-16 derived from a human cervical keratinocyte cell line W12 established from a patient with cervical dysplasia in the UK as the only differences from the

original sequence.[27] Therefore, the L1 sequence of HPV-16 derived from two Japanese patients and W12 cell line might be in fact the major form.

The PCR cloning and sequence analysis were also applied to the universal PCR products of five samples detected to contain unknown HPV types (Table III). One sample was found to contain HPV-18. The reason why this sample was negative in the type-specific PCR could not be determined, since no DNA was available for further analysis. One severe dysplasia and one carcinoma were found to contain HPV-52b, whereas one mild dysplasia and one carcinoma contained HPV-58. HPV-52b and HPV-58 were both recently isolated from Japanese patients with cervical carcinoma.[8, 9] Even though their sequence data have not been published, we could carry out homology analysis using data provided in personal communications (Y. Ito and T. Matsukura, respectively). These results further confirm the close association of these two HPV types with cervical dysplasia and carcinoma of Japanese women.

In conclusion, the combined use of type-specific and universal PCRs as described in the present study allows both specific and general detection of genital HPV types. HPV types detected only by the universal PCR can be further analyzed by sequencing of the amplified region. Our present strategy would, therefore, be useful for large-scale clinico-epidemiological studies of HPV infection and also for identification of novel HPV genotypes.

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