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Characterization of Human Papillomavirus Type 66 from an Invasive Carcinoma of the Uterine Cervix

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Human papillomavirus (HPV) DNA sequences coexisting with HPV16 and HPV45 were cloned from an invasive cervical carcinoma. The cloned HPV was shown to be a novel type, named HPV66, and is related to HPV56 (an HPV detected in cervical cancer). After screening 160 anogenital biopsies, four specimens exhibited histological features of intraepithelial neoplasia and contained HPV66 sequences. Of these, three were found to be associated with another HPV type.

More than 20 types of human papillomavirus (HPV) are associated with anogenital infections, causing benign proliferations (condylomata acuminata) or premalignant lesions (intraepithelial neoplasia) (8). HPV types associated with intraepithelial neoplasia such as HPV16 and HPV18 have been detected in the majority of anogenital invasive carcinomas (30). Several studies have shown the presence of uncharacterized HPV types in 10 to 30% of HPV-positive genital lesions (1, 17, 20, 29). These HPVs need to be characterized to clarify the role of the different HPV types in genital carcinogenesis. Here, we report the molecular cloning and characterization of a novel type of HPV isolated from an invasive cervical carcinoma.

A biopsy specimen was taken from a 38-year-old patient with a stage I invasive squamous-cell carcinoma of the uterine cervix. Total tumor DNA was extracted by the guanidinium isothiocyanate-cesium chloride method (21) and tested for the presence of HPV DNA sequences by Southern blot analysis. The DNA was digested with both *Bgl*I and *Bgl*II restriction endonucleases, blotted onto nitrocellulose, and hybridized with a mixed probe consisting of ^{32}P-labeled HPV16, -18, and -33 DNAs, under nonstringent conditions (T_m − 40°C). The total molecular size of the hybridized fragments exceeded the size of the HPV genome (8 kb), suggesting the presence of more than one HPV (Fig. 1, lane a). Analysis of the tumor DNA by polymerase chain reaction with primers specific for HPV6, -11, -16, -18, and -33 (20) revealed the presence of HPV16 (9) (data not shown). The blot was dehybridized and subsequently rehybridized with HPV16 DNA probe under stringent conditions (T_m − 20°C). The hybridization signal was restricted to a band larger than 8 kb (Fig. 1, lane b). This suggested that the HPV16 genome was integrated in the tumor DNA since HPV16 DNA does

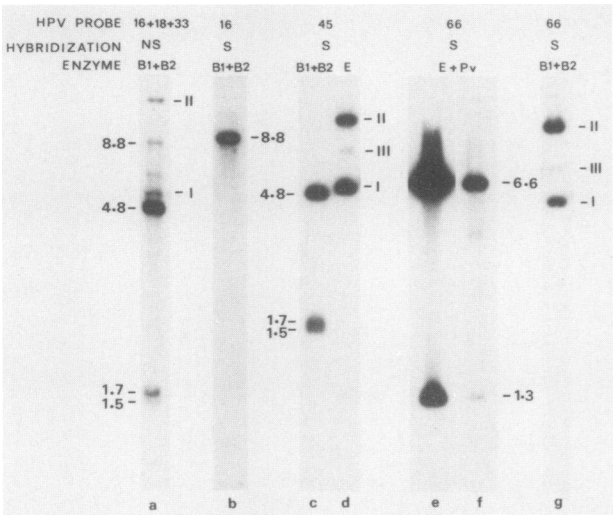

FIG. 1. Southern blot hybridization analysis of HPV DNA sequences found in an invasive carcinoma of the uterine cervix. The total cellular DNA extracted from the biopsy (6 μg; lanes a to d, f, and g) and the cloned HPV66 DNA (5 ng; lane e) were cleaved with endonucleases *Bgl*I and *Bgl*II (B1+B2), *Eco*RI (E), or with *Eco*RI and *Pvu*II (E+Pv). The fragments were separated by electrophoresis in 1% agarose gels, denatured in situ, and transferred to nitrocellulose filters. The filters were hybridized under nonstringent (NS) or stringent (S) conditions (T_m − 40°C and T_m − 20°C, respectively), by using ^{32}P-labeled DNA probes specific for HPV16, -18, -33, -45, and -66 DNAs, as indicated. The migrations of free monomeric HPV DNA molecules, forms I, II, III, are marked. The sizes of the fragments are expressed in kilobases.

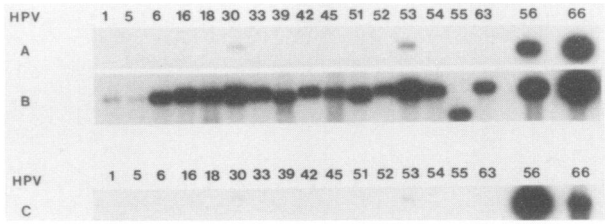

FIG. 2. Analysis of the extent of similarity among HPV DNAs by Southern blot hybridization. The purified cloned DNAs (5 ng) of HPVs associated with cutaneous (HPV1 and HPV5) or genital (HPV6, -16, -18, -30, -33, -39, -42, -45, -51, -52, -53, -54, -55, -63, -56, and -66) lesions were electrophoresed in 1% agarose gels in the presence of human placenta DNA (1 μg). Samples were blotted onto nitrocellulose membranes and hybridized under either stringent (T_m − 10°C) (A and C) or nonstringent (T_m − 40°C) (B) conditions, with ^{32}P-labeled DNA probes specific for HPV66 (A and B) and HPV56 (C).

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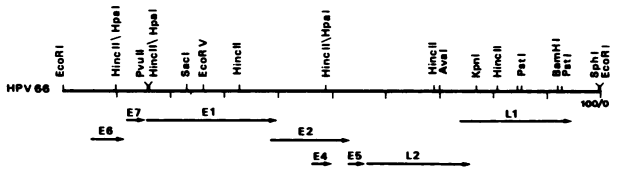

FIG. 3. Physical map of HPV66 DNA. The single *Eco*RI site was taken as the origin. The HPV66 map was aligned with the genetic map of HPV16 by DNA sequencing. The open reading frames corresponding to the early (E) and late (L) genes are indicated at the bottom of the figure. The enzymes *Bgl*I, *Bgl*II, *Hin*dIII, *Sma*I, and *Xba*I have no cleavage sites in HPV66 DNA.

not contain *Bgl*I and *Bgl*II restriction sites (25). The fragments of 1.5, 1.7, and 4.8 kb (Fig. 1, lane a) might correspond to the *Bgl*I-plus-*Bgl*II pattern of HPV45 DNA (19). This was confirmed by hybridization with ^{32}P-labeled HPV45 DNA under stringent conditions (Fig. 1, lane c). After hybridization of *Eco*RI-digested tumor DNA (no *Eco*RI site in HPV45), only bands corresponding to supercoiled circular (form I), open circular (form II), and linear (form III) molecules were observed (Fig. 1, lane d). It appeared, therefore, that HPV45 was present in an episomal form in this tumor.

Two bands at the positions of forms I and II were detected in the *Bgl*I-plus-*Bgl*II-digested tumor DNA, indicating the presence of additional episomal HPV molecules (Fig. 1, lane a). This HPV was found to have a single *Eco*RI site (data not shown). We proceeded to isolate this HPV by constructing a genomic library of the tumor DNA in bacteriophage lambda zap II (Stratagene, La Jolla, Calif.) (26) by using *Eco*RI endonuclease. The recombinant phages carrying the cloned HPV DNA were identified by plaque hybridization by using ^{32}P-labeled DNA of HPV16, -18, and -33 as a mixed probe, under nonstringent conditions (T_m − 40°C). The HPV DNA was subsequently subcloned into plasmid pBR322. After cleavage with a mixture of *Eco*RI and *Pvu*II endonucleases, the cloned HPV DNA and the tumor cellular DNA yielded fragments of identical sizes (with a sum of 8 kb), by using the cloned HPV DNA as a probe (Fig. 1, lanes e and f). This indicated that the complete genome of the HPV had been cloned. Hybridization of the *Bgl*I-plus-*Bgl*II-digested tumor DNA with the ^{32}P-labeled cloned DNA confirmed the presence of this HPV as free episomal DNA molecules (Fig. 1, lane g).

The degree of similarity between the newly cloned HPV DNA and the DNA of HPV types 1 to 56 (8) was examined by Southern blot hybridization under different conditions of stringency. At high stringency (60% formamide; T_m − 10°C), the ^{32}P-labeled, newly cloned HPV DNA showed a strong cross-hybridization with the DNA of HPV56, an HPV type associated with cervical cancer (16), and a weak cross-hybridization with the DNAs of HPV30 (14) and HPV53 (12) (Fig. 2A). Similar results were observed when HPV56 DNA

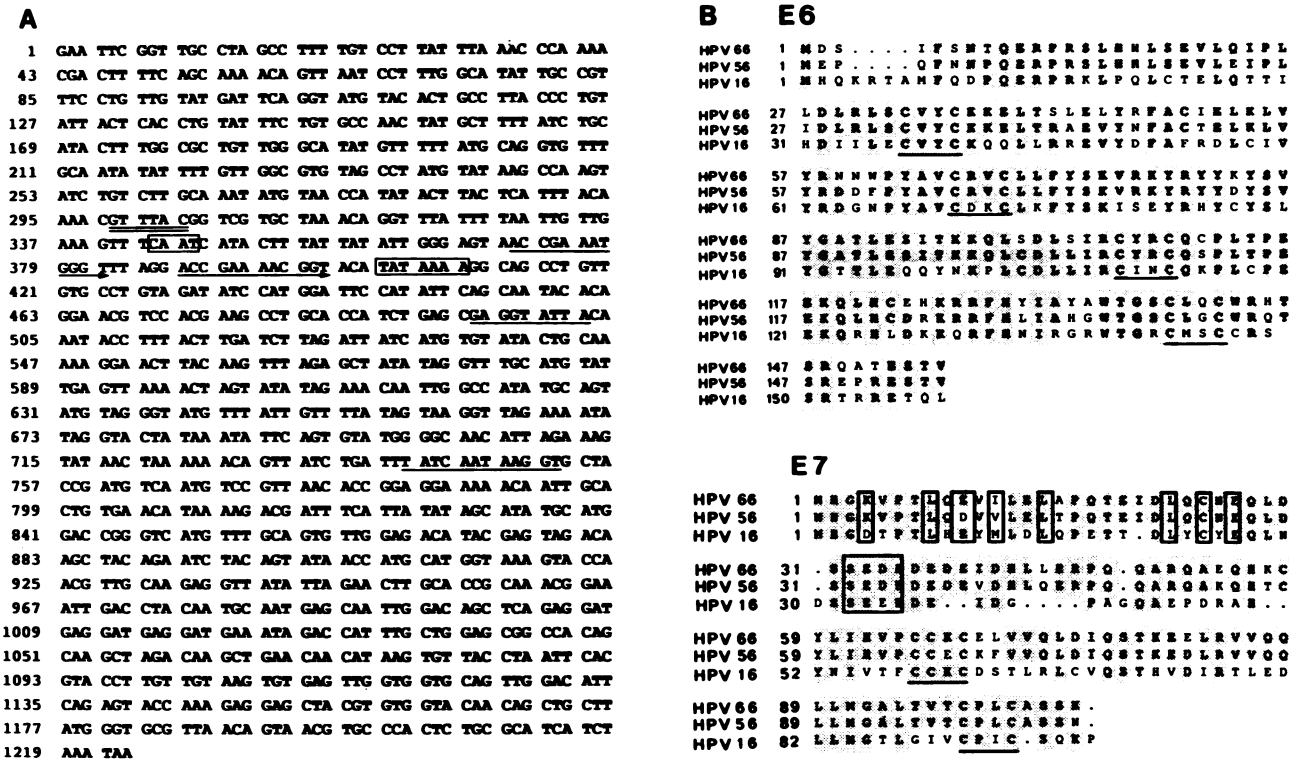

FIG. 4. (A) Nucleotide sequences of the 3′ end of long control region and the E6 and E7 open reading frames of HPV66. The predicted E6 and E7 genes begin at nucleotides 437 and 907 and terminate at nucleotides 901 and 1221, respectively. The putative *Hpa*I site used to align the papillomavirus sequences is double underlined (7). The TATA and CAAT sequences are boxed. The $ACCN_6GGT$ binding motifs of the E2 gene products are indicated by arrows. The putative consensus sequences of splice donor and acceptor sites are underlined. (B) Alignment of the deduced amino acid sequences of E6 and E7 proteins from HPV16, -56, and -66. Residues that are identical between the aligned regions are shaded. The putative zinc finger motifs (Cys-X-X-Cys) (6) are underlined. The minimal regions needed for binding HPV16 E7 to p105-RB (18) and the corresponding regions in HPV56 and HPV66 are boxed.

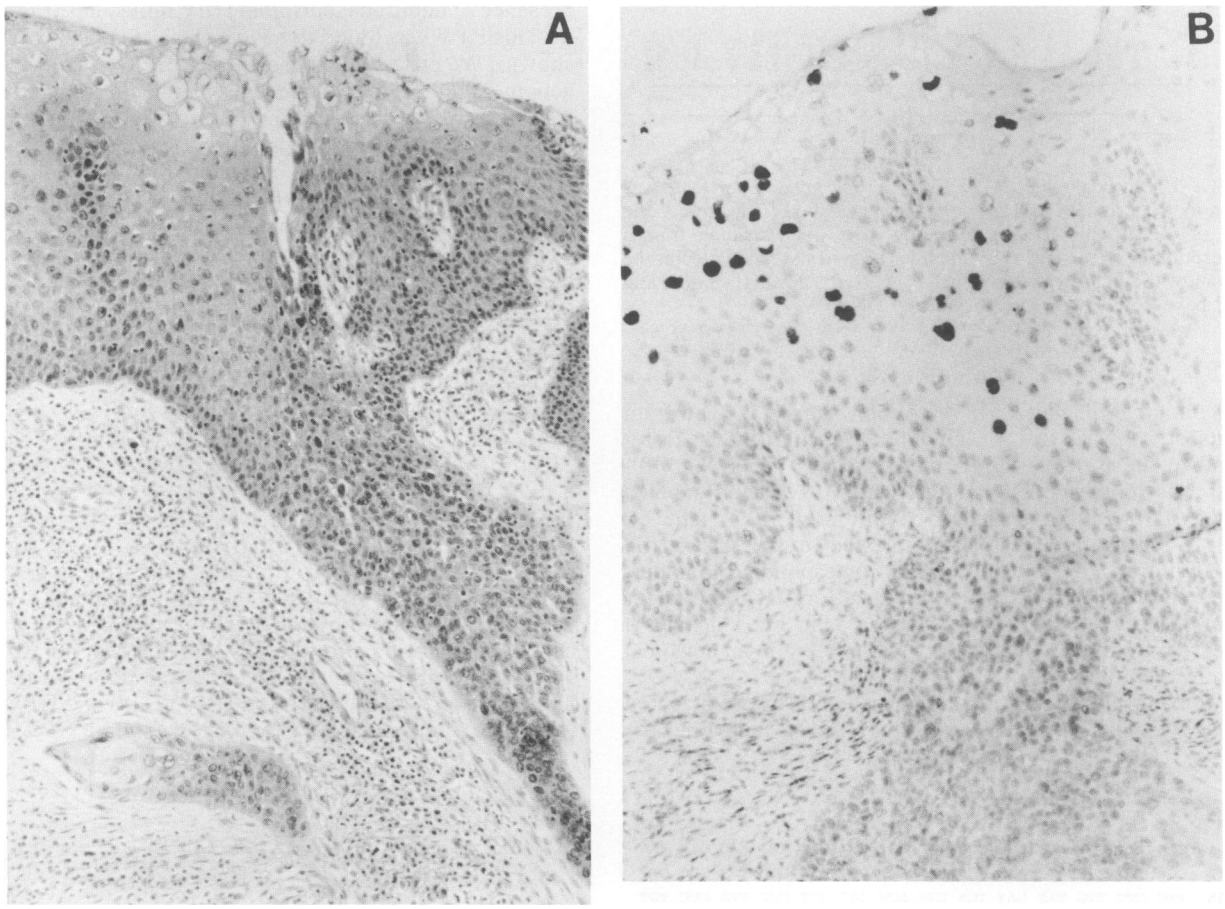

FIG. 5. Detection of HPV66 DNA in tumor specimen by in situ hybridization. A small biopsy was fixed with Carnoy's fluid and embedded in paraffin. Sections (5 μm thick) were used for histology and in situ hybridization. (A) Histological features. An invasive squamous-cell carcinomae merges from a low-grade intraepithelial neoplasia. Hematoxylin-and-eosin staining was used. Magnification, ×124. (B) In situ hybridization. HPV66 DNA molecules are detected in the nuclei of differentiating cells of the intraepithelial neoplasia after hybridization with a tritiated HPV66 DNA probe by using conditions previously described (11). Magnification, ×140.

was used as a probe (Fig. 2C). However, when the hybridization was carried out under conditions of lower stringency (20% formamide; T_m − 40°C), the newly cloned HPV DNA showed a strong cross-hybridization with the DNAs of the genital HPV types and a weak cross-hybridization with the DNAs of cutaneous HPV types, as illustrated for HPV1 and HPV5 (Fig. 2B). No cross-hybridization was shown under stringent conditions between the cloned HPV and HPV types 57 to 65 (8a). The extent of similarity between the newly cloned HPV and HPV56 was estimated as 40% (mean value of four independent experiments) by using liquid-phase hybridization at saturation followed by S1 endonuclease digestion (15). By using different restriction enzymes, the physical map of HPV66 was constructed (Fig. 3), and it was clearly different from that of other known types. There were no conserved restriction sites found between HPV66 and the published HPV56 physical maps. According to the convention for the classification of HPV isolates (5), an HPV is considered as a new type if it exhibits less than 50% cross-hybridization with other HPV types. Therefore, the cloned HPV was designated as a new type, named HPV66.

The E6 and E7 genes of HPV16 were found to have transforming and immortalizing activities (13), and their products are able to form complexes with the cellular p53 protein (28) and retinoblastoma tumor suppressor gene product (p105-RB) (10, 18), respectively. Alignment of the physical map of HPV66 with the genetic map of HPV16 was deduced from electron microscopy analysis of the heteroduplex molecules (2) (data not shown). HPV66 E6 and E7 genes were located in an *Eco*RI-*Sac*I fragment (Fig. 3). This fragment was subcloned in M13 phage, and the complete DNA sequences of these two open reading frames, as well as the 3′ end (436 nucleotides) of the long control region, were determined by using the dideoxy chain termination method (Fig. 4A) (22). In the long control region, several conserved sequence elements, known to be involved in the regulation of HPV expression, were identified, including the TATA and CAAT boxes and two consensus-binding motifs of the viral E2 transregulating proteins (6). In the E6 region, potential splice donor and acceptor sites were found, signifying the existence of an intron, the splicing of which generates a truncated E6 protein. This has been recognized as a common feature of HPV types associated with genital carcinoma (24, 27).

Comparison of the nucleotide sequences of E6 and E7 of HPV66 and HPV16 (25) revealed 56 and 54% similarity, respectively. Alignment of their deduced proteins showed the presence of 50 and 54% identical amino acids in E6 and

E7, respectively (Fig. 4B). A region was found in the HPV66 E7 protein sharing homology with the p105-RB binding motif of HPV16 (18). Nine of the 12 amino acids known to be essential for binding to p105-RB were identical (Fig. 4B) (18). A similar comparison between HPV66 and HPV56 (16) in the E6 and E7 regions showed that the two HPVs shared 84.5 and 93% nucleotides and 81.8 and 88.6% identical amino acids (Fig. 4B), respectively. Thus, HPV66 and HPV56 are much more closely related than anticipated from liquid-phase hybridization studies. Similar results were previously found for other HPV types, such as HPV6 and HPV11 (4) or HPV32 and HPV42 (3). It is hoped, therefore, that the accumulation of nucleotide sequences of HPVs will provide new bases for their classification.

In this article we have shown that HPV66 was detected along with HPV16 and HPV45 in a biopsy taken from a cervical cancer. To address the role of HPV66 in the development of the tumor, in situ hybridization experiments were performed on tumor sections by using tritiated HPV16, HPV45, or HPV66 DNA probes. The specimen showed a low-grade intraepithelial neoplasia which continues as a poorly differentiated invasive carcinoma (Fig. 5A). High levels of HPV66 DNA vegetative replication were detected in nuclei of terminally differentiating cells of the intraepithelial neoplasia (Fig. 5B). No hybridization signals were found in deeper layers or in the carcinoma. This was expected since these cells are most likely to contain viral episomes in low number (less than 50) which are undetectable by in situ hybridization (23). The inability to detect HPV16 and HPV45 DNA might be due to their presence in another region of the tumor specimen. The in situ detection of HPV66 DNA in a lesion with histological evidence for malignant transformation suggests an oncogenic potential for this virus. This was further supported by identifying HPV66 sequences in DNA isolated from precancerous lesions, by using Southern blot hybridization. Upon screening 160 anogenital biopsies with probes specific for 20 HPV types, HPV66 was found in three cervical lesions diagnosed as high-grade cervical intraepithelial neoplasia and one penile intraepithelial neoplasia. In three of these four specimens, HPV66 was found associated with another HPV type (HPV16, HPV51, or HPV58).

In conclusion, HPV56 and HPV66 can be classified as members of an HPV group associated mainly with genital neoplasia.

Nucleotide sequence accession number. The nucleotide sequence accession number M75123 was assigned to HPV66.

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