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# Isolation and Characterization of a Novel Human Papillomavirus Type 6 DNA from an Invasive Vulvar Carcinoma

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**Human papillomavirus type 6 (HPV-6) DNA was detected in a rapidly growing vulvar verrucous carcinoma and two recurrent tumor samples. The viral DNA (HPV-6vc) was molecularly cloned and found to have a high degree of DNA sequence homology to HPV-6b DNA. Comparison of restriction endonuclease cleavage patterns between HPV-6b and HPV-6vc genomes and DNA sequencing analysis demonstrated an additional 106 bases in the HPV-6vc genome. These additional nucleotides were located in the noncoding region of the viral genome which contains the putative viral DNA replication and early gene transcriptional control elements. Seventy-four of the additional 106 nucleotides were found as one insert in the purine–thymidine-rich region 3′ to the end of the L1 open reading frame. This 74-base-pair addition had homology with viral sequences immediately upstream to it and to poly(dG-dT) sequences found in the human genome including the conserved repeated sequences in human DNA (EC1) and in the human cardiac muscle actin gene. Two smaller inserts, 19 and 15 nucleotides, were found upstream from the transcriptional control elements and demonstrate homology with regions of human alpha and gamma interferon genes.**

Human papillomaviruses (HPVs) have been proposed as causative agents in a number of malignant tumors (28). Evidence implicating HPVs as the etiologic agent in these malignancies includes the observation of condyloma acuminata (anogenital warts) progressing into squamous cell carcinomas (5) and the detection of HPV DNA in tumor samples (7, 21).

DNA sequences homologous to HPV type 6 (HPV-6) have been detected in verrucous carcinomas (19), a nonmetastasizing invasive tumor, and other invasive squamous cell carcinomas of the anogenital tract (5). In the present study, a subtype of HPV-6 DNA was isolated from a rapidly growing vulvar verrucous carcinoma. The patient, an otherwise healthy 36-year-old female, had a 16-year history of condyloma acuminata. Comparison of the newly isolated viral DNA (designated HPV-6vc) with the well-characterized HPV-6b genome, which was cloned from a benign anogenital wart (6, 24), revealed extensive homology in DNA sequence and genome organization. However, significant sequence divergence as well as three inserts were detected in the noncoding region of the HPV-6vc genome which contains the putative viral origin of replication and early gene transcriptional regulatory functions (24).

Three samples of a recurrent tumor were removed from a patient with a rapidly growing vulvar verrucous carcinoma during a 6-month period. Unintegrated HPV-6 DNA sequences were detected by Southern blot analysis (25, 27) in the high- and low-molecular-weight DNA extracted (13) from all three tumor biopsies. No additional HPV DNA fragments were observed, at either stringent or relaxed hybridization conditions (14), when HPV-11 or HPV-16 DNAs were used as hybridization probes. Low-molecular-weight DNA was extracted from the primary tumor sample (13), cleaved with *Bam*HI, and ligated into the *Bam*HI arms of Charon 28. Recombinant phage containing HPV DNA were detected in the resulting library by using $^{32}$P-labeled HPV-6b DNA as a molecular probe (4). HPV-6vc DNA was subcloned into the *Bam*HI site of pBR322 (pHPV-6vc). Recombinant plasmids and bacteriophage DNAs were grown and purified by standard procedures (16).

The intact HPV-6b genome was constructed by recircularization of the two cloned subgenomic fragments (6). Analysis of the digestion patterns of HPV-6b and HPV-6vc by using multiple restriction endonucleases revealed that HPV-6vc was larger than HPV-6b (8.0 versus 7.9-kilobases). The difference in size was located between the HPV-6b *Hpa*I site (position 6650) and the HPV-6b *Hpa*II site (position 7862; Fig. 1). This region on the HPV-6b genome includes the 3′ end of the major capsid protein (L1) open reading frame (ORF), the putative viral origin of replication, and the early gene transcriptional control region (24).

Southern blot analysis indicated no difference in the thermal stability of the DNA duplex between HPV-6b and HPV-6vc DNAs under experimental conditions (melting temperature −2°C) that allowed a 1 to 2% base mismatch (14). The HPV DNAs used for hybridization probes were first purified from flanking pBR322 DNA sequences.

Overlapping deletion mutants were constructed in the noncoding region of the HPV-6vc genome. Plasmid DNA (pHPV-6vc) was linearized at the *Hpa*I site, digested with BAL 31 nuclease for various periods of time, and digested with *Eco*RI to provide a defined site on pBR322 for sequencing, and the overhanging ends were repaired by using the Klenow fragment of DNA polymerase I (16). The blunt-ended molecules were recircularized by using T4 DNA ligase and used to transform *Escherichia coli* HB101 to ampicillin resistance. Deletion mutants were isolated, and plasmid DNAs were sequenced by the dideoxy method of Sanger et al. (23) and with a synthetic pBR322 *Eco*RI site polynucleotide primer.

The HPV-6vc genome corresponding to HPV-6b positions 6650 to 510 was sequenced. There were two single-base changes on the HPV-6vc genome corresponding to HPV-6b positions 6650 through 7288 (the end of the L1 ORF). The

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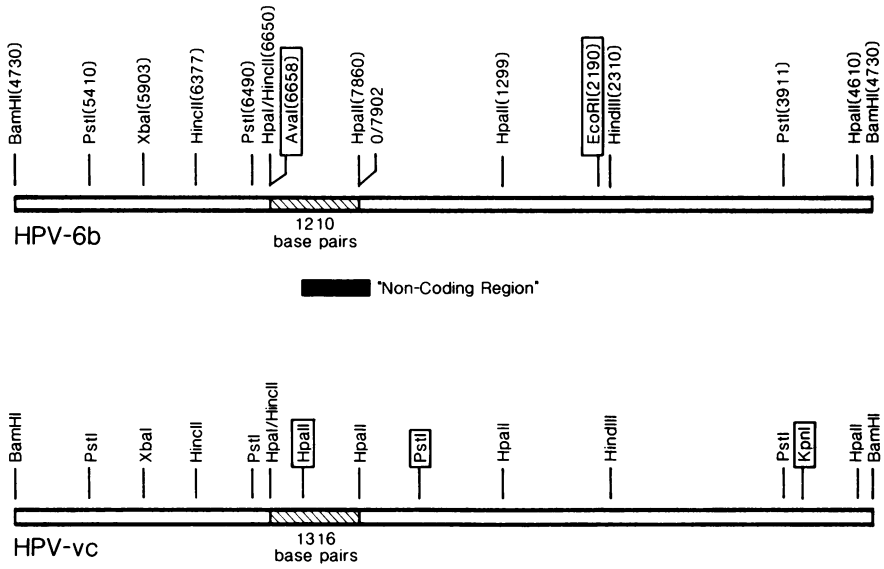


FIG. 1. Comparison of the restriction endonuclease maps of HPV-6b and HPV-6vc. The endonuclease sites which are unique to one genome are in boxes. The region of noted size difference between the two genomes is indicated by hatch marks. The HPV-6b noncoding region is shown in relation to the viral genomes.

first change, at position 6661, removed the HPV-6b *Ava*I site, and the second change, at position 7219, created an extra *Hpa*II site on HPV-6vc (Fig. 1 and 2). Neither base change altered the amino acid sequence in the L1 ORF.

Three inserts, consisting of 74, 19, and 15 base pairs (bp), were found between HPV-6b positions 7348 and 7721 (Fig. 2). The largest insert (HPV-6b position 7348) contained 74 bp, the first 60 of which repeated HPV-6b positions 7302 to 7363 (45/60 match), a region rich in alternating purine-thymidine nucleotides. The 24-nucleotide tandem repeat reported by Schwarz et al. (24) at HPV-6b positions 7292 and 7316 was disrupted slightly, and the resultant configuration yielded a 23-nucleotide triple repeat unit (5′-TATGTACTG TTATPuTPuTPuTGTPuT-3′) at positions 7302 and 7326 and position 1 of the insert (Fig. 3). A high degree of DNA sequence homology was detected between this 74-bp insert and poly(dG-dT)-rich regions of the human genome (Fig. 3). DNA sequence comparisons were made by using the DNA sequence data bank of the National Biomedical Research Foundation (2).

The 15-bp insert at HPV-6b position 7418 resembled nucleotide sequences found in the human gamma interferon (26) gene (13/15 match). Two nucleotides were deleted from the HPV-6b genome at positions 7419 and 7420. The 19-bp

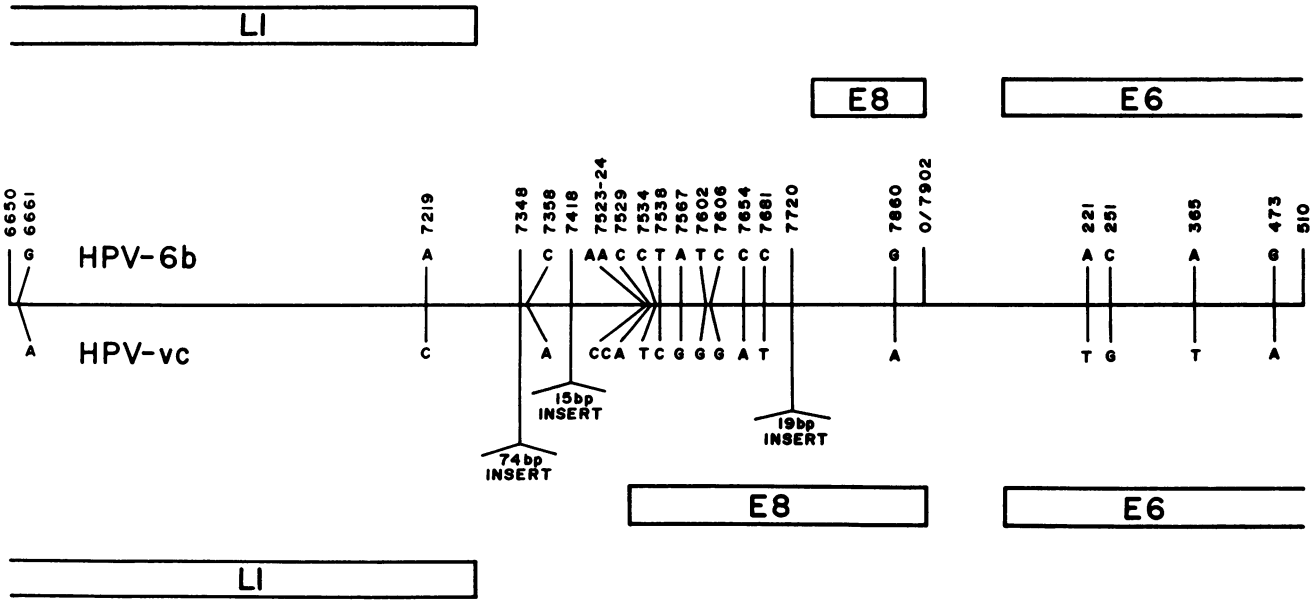


FIG. 2. Schematic representation of the inserts and single nucleotide changes which are located in the region of the HPV-6vc genome corresponding to HPV-6b nucleotide positions 6650 through 510. The positions and nature of each nucleotide change and the three inserts are indicated in relation to the L1, E6, and E8 ORFs. The extension of the E8 ORF on HPV-6vc is also diagramed.

A

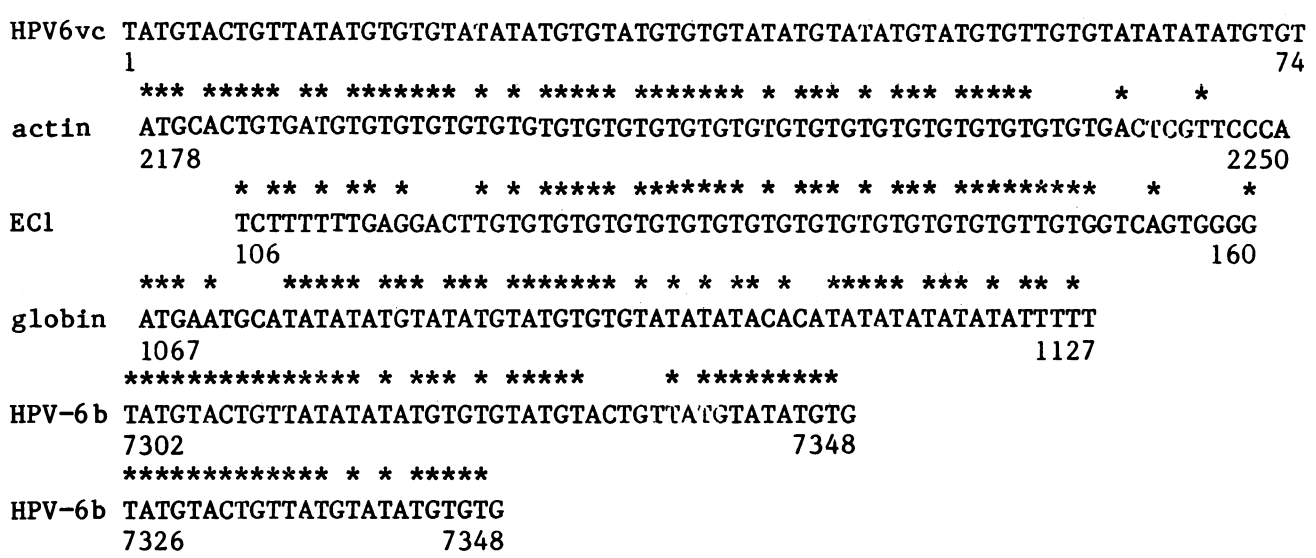


FIG. 3. DNA sequence homology between the HPV-6vc 74-bp insert (HPV-6b position 7348) and the human cardiac muscle actin gene (10), EC1 repeated sequences (17), and the beta globin gene (18) are compared. The 23-bp repeat sequence found at position 1 of the HPV-6vc 74-bp insert and at HPV-6b positions 7302 and 7326 is also shown.

insert at HPV-6b position 7720 was homologous with sequences found in the human alpha interferon (8) gene (15/19 match). The 19-bp insert also contained a direct repeat of the HPV-6b heptanucleotide sequence 5′-TATTGCC-3′ found 15 nucleotides upstream from the start of this insert and repeated five bases into the insert yielding a 13-bp intervening sequence.

Twelve single-base changes were found in HPV-6vc DNA in the region corresponding to HPV-6b positions 7349 to 7862. The most notable difference was the change TAA to TCC at position 7522. Removing the TAA stop codon created an ORF from position 7498 which spanned the HPV-6vc 19-nucleotide insert and included the putative HPV-6b E8 ORF (Fig. 2). To date, no mRNA transcripts have been observed in this region of the viral genome (15).

An additional 550 bp were sequenced 3′ to the *Hpa*II site at HPV-6b position 7862. This region of the viral genome included the early gene transcriptional control signals and most of the E6 ORF. Four single-base changes were detected at HPV-6b positions 221, 251, 365, and 473. The mutation at position 221 (C to G) created an extra *Pst*I site on HPV-6vc. These four-point mutations do not change the putative E6 polypeptide amino acid sequence.

The three inserts detected in the noncoding region of the HPV-6vc genome could have arisen from recombination with host DNA or by sequence duplication. Sequence duplication in the region of the 74-bp insert is consistent with findings of sequence duplications in simian virus 40 (3) and polyomavirus (22) enhancer regions. In addition, there have been reports of papillomaviruses undergoing genetic alterations in vivo (1, 9, 20).

Poly(dG-dT) sequences are found frequently in the human genome (10, 17, 18) and have been shown capable of forming Z-DNA structures and enhancing gene expression (11, 12). The poly(dG-dT) region found in and around the 74-bp insert may allow the molecule to assemble in the Z-DNA conformation and act as an enhancer element for the viral genome. In addition, the poly(dG-dT) sequences of the viral genome could stimulate genetic recombination as postulated for a class (EC1) of conserved human repeat sequences (17).

Experiments are under way to investigate both the transcription of the HPV-6vc E8 ORF and to determine whether the inserts found in the HPV-6vc genome enhance transcriptional activity.

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