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Two Novel Genital Human Papillomavirus (HPV) Types, HPV68 and HPV70, Related to the Potentially Oncogenic HPV39

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The genomes of two novel human papillomavirus (HPV) types, HPV68 and HPV70, were cloned from a low-grade cervical intraepithelial neoplasia and a vulvar papilloma, respectively, and partially sequenced. Both types are related to HPV39, a potentially oncogenic virus. HPV68 and HPV70 were also detected in genital intraepithelial neoplasia from three patients and one patient, respectively. Comparison with sequence data in the literature indicates that the subgenomic ME180-HPV DNA fragment, cloned from a carcinoma cell line, corresponds to an HPV68 subtype and that several HPV DNA fragments amplified by PCR from genital neoplasia represent worldwide distributed variants of HPV68 and HPV70.

More than 30 types of human papillomaviruses (HPVs) infecting the genital tract have been identified so far (8, 17). These viruses are found associated with squamous intraepithelial lesions of the uterine cervix, known as cervical intraepithelial neoplasia (CIN), which may regress, remain stable, or progress into invasive squamous cell carcinomas (23, 31). It is likely that the variability of the clinical evolution of CIN reflects the diversity of the associated HPV types (3, 23). Identification of all genital HPV types is thus an important issue to understand fully the role of HPVs in the natural history of invasive carcinoma of the uterine cervix, which is the second most frequent cause of cancer-related mortality in women worldwide (31). Obviously, such knowledge would be of significant help to clinicians for the management of patients. It is a common observation that Southern blot hybridization experiments performed in nonstringent conditions (12, 21, 26) or PCR data obtained with consensus or degenerate primers (1, 4, 11, 14, 28) reveal the presence of HPV DNA sequences different from known HPV types in genital specimens. To be recognized as a novel HPV type, an HPV isolate should share less than 90% nucleotide sequence identity with known HPV types in the E6, E7, and L1 open reading frames (ORFs), and its entire genome should be cloned (8, 17). We report here the characterization of two novel genital HPV types, HPV68 and HPV70, related to the potentially oncogenic HPV39 (2).

Cloning and restriction maps of HPV68 and -70 genomes. HPV68 was cloned from a biopsy of a low-grade CIN. HPV-related DNA sequences were detected by Southern blot hybridization of the *Pst*I-digested total DNA preparation with mixtures of ^{32}P-labeled HPV6, -11 and -42, HPV16, -18 and -33, or HPV31, -35 and -39 DNA probes. The signal was strongest with the mixture of HPV31, -35, and -39 probes, whether under nonstringent conditions of hybridization (T_m − 40°C) or after washing under more stringent conditions in which signals were reduced (T_m − 20°C) (data not shown). The full-length HPV genome was cloned after insertion into the bacteriophage lambda ZAP II DNA at the *Eco*RI site and subsequently subcloned into the Bluescript II phagemid (Stratagene, La Jolla, Calif.). Hybridization of the original DNA preparation with the cloned DNA as a probe revealed a 7.9-kb fragment after cleavage with *Bam*HI or *Eco*RI endonuclease and four to six DNA fragments after digestion with *Ava*II, *Ban*I, or *Pst*I (Fig. 1A). Cross-hybridization experiments performed under stringent conditions (T_m − 10°C) between the ^{32}P-labeled cloned HPV DNA and the DNA of known cutaneous and genital HPV types showed a strong hybridization with HPV39 and a weak hybridization with HPV18, -45, and

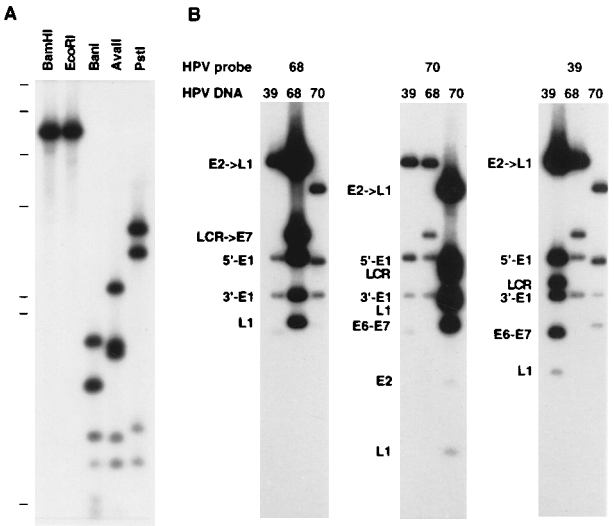

FIG. 1. (A) Blot hybridization analysis of HPV68 DNA sequences found in a CIN. The total cellular DNA extracted from the biopsy (1.5 μg) was cleaved with different endonucleases, as indicated. The fragments were separated by electrophoresis in a 1% agarose gel, denatured in situ, and transferred to a nylon membrane (Amersham, Les Ullis, France). The membrane was hybridized under stringent conditions (T_m − 10°C), using a ^{32}P-labeled HPV68 DNA probe. The migration of λ DNA *Hin*dIII fragments is indicated on the left. (B) DNA sequence homology among HPV39, -68, and -70 as analyzed by blot hybridization. Cloned HPV DNAs were excised from plasmid sequences by digestion with *Bam*HI (HPV70) or *Eco*RI (HPV39 and -68) endonucleases, purified, and cleaved with *Pst*I endonuclease. Blot hybridization experiments were performed in stringent conditions (T_m − 10°C), using an HPV68, HPV70, or HPV39 DNA probe, as indicated. The ORFs contained in the HPV DNA fragments corresponding to each probe (see Fig. 2) are indicated on the left.

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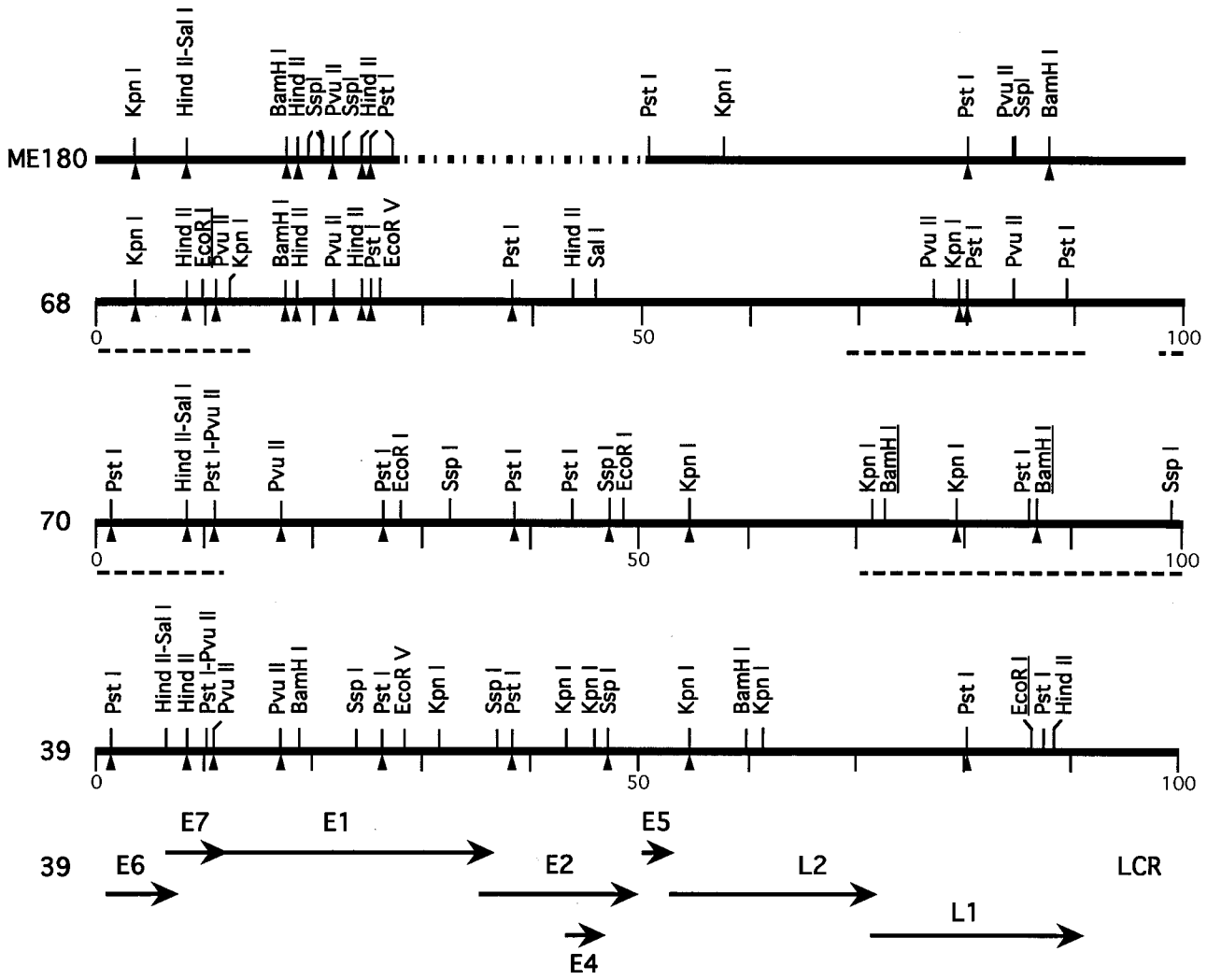

FIG. 2. Physical maps of HPV68 and -70 DNAs and their alignment with the maps of HPV39 and ME180 sequences. The cloning restriction sites are underlined. Cleavage sites conserved between at least two genomes are indicated by arrowheads. Dashes underline sequenced regions of HPV68 and -70 DNAs. The missing sequences of ME180 DNA (19) are noted by a dashed line. The physical (2) and genetic (30) maps of HPV39 represented at the bottom. The enzymes *Ava*I, *Bgl*II, *Cla*I, *Hin*dIII, *Sac*I, *Sma*I, *Ssp*I, *Xba*I, and *Xho*I have no cleavage sites in HPV68 DNA. The enzymes *Ava*I, *Bgl*I, *Bgl*II, *Eco*RV, *Hpa*I, *Pvu*I, *Sac*II, *Sma*I, *Xba*I, and *Xho*I have no cleavage sites in HPV70 DNA.

-59 (data not shown) (8a). A physical map was constructed from the study of DNA cleavage patterns obtained with 17 restriction endonucleases (Fig. 2). Partial nucleotide sequence data (see below) confirmed the localization of the involved restriction sites and allowed the map to be aligned with that of the HPV39 genome (2, 30).

HPV70 was cloned from a biopsy of a vulvar papilloma from an immunosuppressed renal allograft recipient. In a first attempt, we had cloned a 6.8-kb *Bam*HI fragment that showed a 16% cross-hybridization with HPV39 DNA as evaluated by liquid-phase hybridization experiments, suggesting that this isolate represented a novel HPV type (2). Heteroduplex analysis had allowed the alignment of the restriction maps of the HPV39 genome and the 6.8-kb fragment and had shown that the missing sequences corresponded to ORF L1 (2). Due to the very small amount of DNA available, direct cloning of the lacking *Bam*HI fragment was not possible. We thus amplified the missing sequences by a nested PCR method. To design the primers, we sequenced the region flanking the *Bam*HI sites within the 6.8-kb fragment by the dideoxy chain terminator method (22). The primers used for the first step of PCR were located 280 nucleotides upstream of the 5′ *Bam*HI site (5′-GGCGAAGGTTGTCAATACAG-3′) and 360 nucleotides downstream of the 3′ *Bam*HI site (5′-ACCAGGACAAAC ATATACAG-3′). Amplification was performed with an automated thermal cycler (Hybaid, Teddington, United Kingdom) in 100-μl reaction mixtures containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 250 μM (each) deoxynucleoside triphosphate, 30 pmol of each primer, 1.25 U of *Taq* polymerase (AmpliTaq DNA polymerase; Perkin-Elmer Cetus, Norwalk, Conn.), and 1 μg of the biopsy DNA. After an initial denaturation at 94°C for 5 min, each of the 20 cycles consisted of a 1-min denaturation step at 95°C, 2 min of primer annealing at 55°C, and 2 min of chain extension at 72°C. The extension of all amplified products was completed by a final extension step of 3 min at 72°C. A second amplification was performed on the PCR products (2 μl) under the same conditions, using primers located 71 nucleotides upstream of the 5′ *Bam*HI site (5′-CCTAAGGTGTCTGCATATCA-3′) and 66 nucleotides downstream of the 3′ *Bam*HI site (5′-AACT

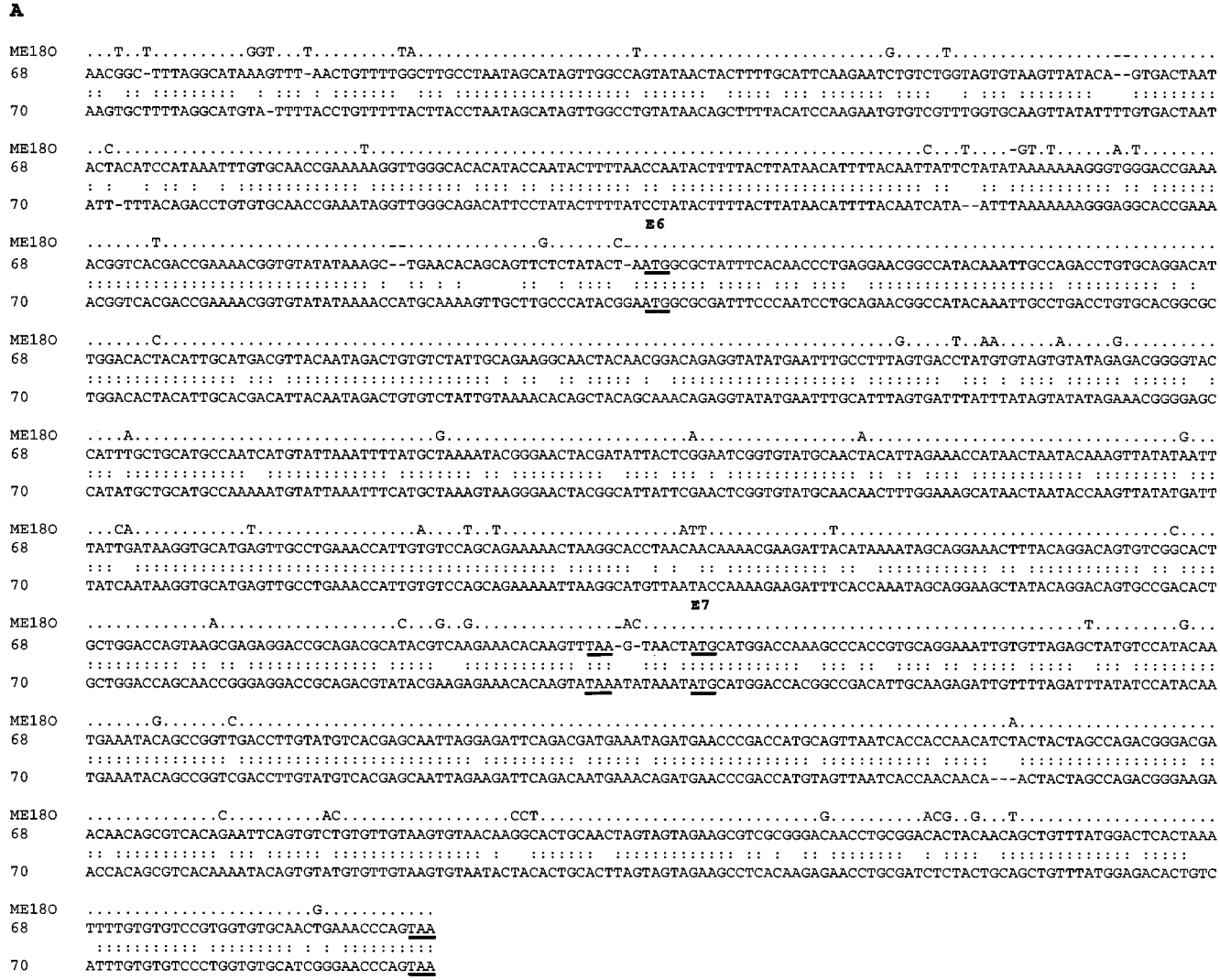

FIG. 3. Nucleotide sequence alignments of the 3′ end of the long control region, the E6 and E7 ORFs (A), and the L1 ORF (B) of HPV68 and -70 DNAs. The initiation and stop codons of the E6, E7, and L1 ORFs are underlined. The nucleotide sequence of ME180-HPV DNA (19) is given above the HPV68 sequence. Dots indicate identical nucleotides. Dashes represent spaces included for alignment purposes. The L1 sequence of the HPV70 NO87 isolate is represented beneath the HPV70 sequence, between the two *Bam*HI sites underlined by a thick line. Substituted nucleotides in variants reported in the literature (Table 2) are indicated by lowercase letters for HPV68 (above the ME180 sequence) and HPV70 (beneath the NO87 sequence) at positions 15, 16, 81, 84, 99, 146, and 172 for the X02 isolate (11), 1245 for L1AE1, LVX160, and CP141 (16, 18, 25), and 1283 and 1398 for CP141 (18). Variants were identified by sequencing a 211- or a 450-nucleotide fragment amplified by PCR, using L1C1 and L1C2 (11) or MY11 and MY09 (14) primers, respectively. The positions of primers are indicated. L1C1 ends four nucleotides upstream of the L1 ATG.

GATCTAGTTCTGTACT-3′). The 1.1-kb *Bam*HI fragment obtained after enzymatic digestion of the PCR products was cloned into Bluescript II phagemid. Sequencing of three independent recombinant plasmids yielded the same nucleotide sequence, rendering unlikely the introduction of nucleotide substitutions by the *Taq* DNA polymerase. These sequence data and those on E6 and E7 ORFs (see below) were used, together with the restriction map of the 6.8-kb fragment (2), to establish a physical map of HPV70 and to align it with the maps of HPV39 and -68 DNAs (Fig. 2).

Of the 19 cleavage sites mapped on the HPV68 genome, 4 sites were found conserved in HPV39 and -70 DNAs, 1 site was found conserved in HPV39, and 1 site was found conserved in HPV70. Among the 20 restriction sites mapped on the HPV70 genome, 4 additional sites were found to be shared with HPV39 DNA (Fig. 2). The relationship between HPV39, -68, and -70 DNAs was further shown by cross-hybridization experiments performed under stringent conditions (T_m − 10°C), using the excised cloned HPV39, -68, and -70 DNAs digested with *Pst*I endonuclease (Fig. 1B). Most of the DNA fragments were detected with heterologous probes, even when they displayed much weaker signals than with homologous probes. As anticipated from the presence of conserved *Pst*I sites, fragments with the same sizes were common to two or three of the HPV types, namely, the larger HPV39 and -68 *Pst*I fragment encompassing E2, L2, and L1 ORFs and the HPV39, -68, and -70 *Pst*I fragment containing the 3′ end of E1 (Fig. 1B).

Comparison of nucleotide sequences of E6, E7, and L1 ORFs of HPV39, -68, and -70. To determine the nucleotide sequences of the E6, E7, and L1 ORFs (Fig. 3A and B), we sequenced appropriate restriction fragments of HPV68 and HPV70 DNAs subcloned in pBluescript II. Sequence determi-

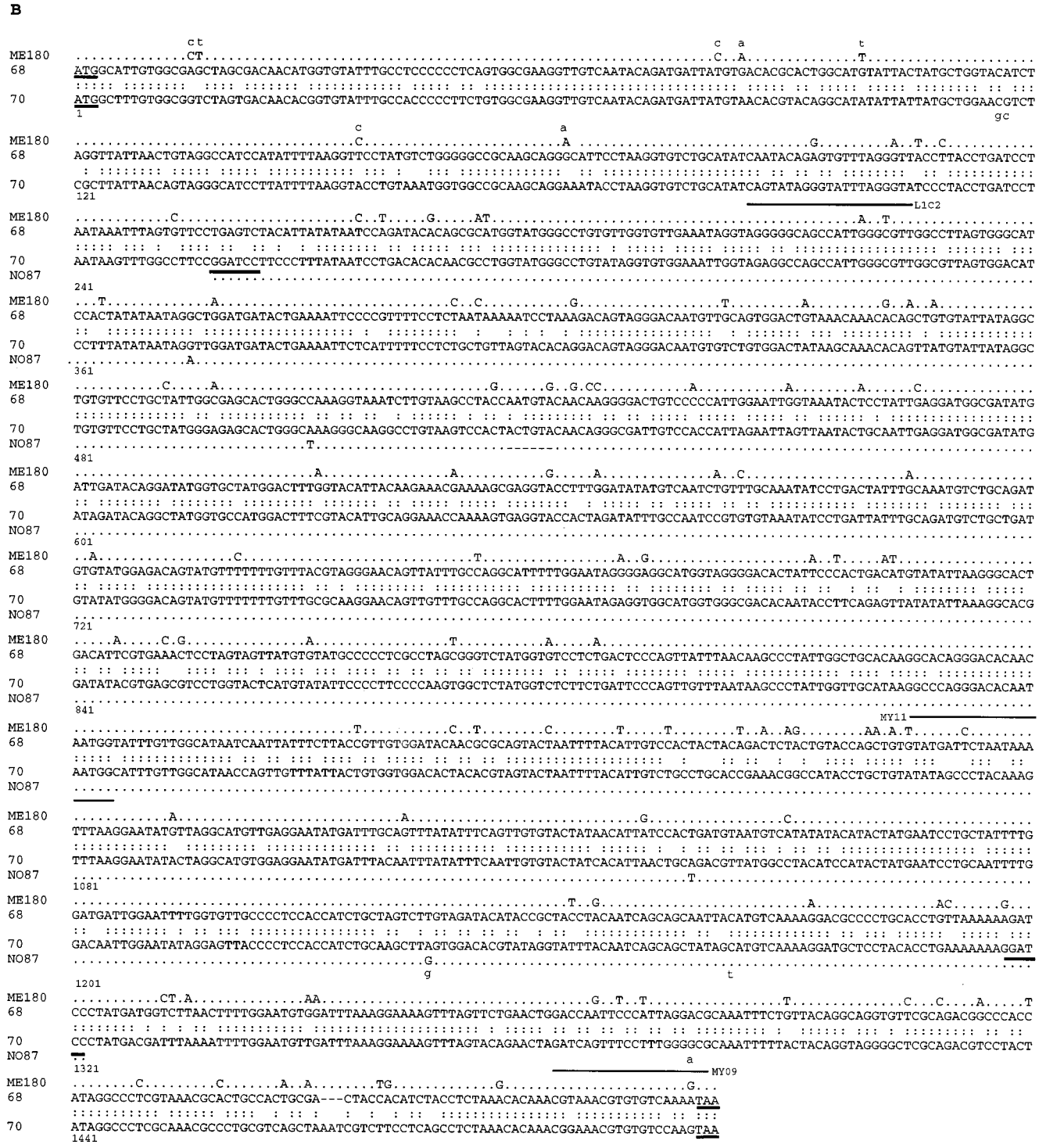

FIG. 3—*Continued.*

nation was performed in both orientations by the dideoxy method (22), first using universal primers (Stratagene) and then synthetic oligonucleotides (Genset, Paris, France) chosen from newly established sequences. On the whole, 3,168 nucleotides were sequenced for HPV68, encompassing the 3′ end of L2 ORF, the L1 ORF, the 5′ and 3′ ends of the long control region, the E6 and E7 ORFs, and the 5′ end of the E1 ORF (Fig. 2). A 3,283-nucleotide segment, from the 3′ end of the L2 ORF to the 5′ end of the E1 ORF, was sequenced for the HPV70 genome (Fig. 2). Sequence comparison was done with the Sequence Analysis Software Package (Genetics Computer Group Inc., Madison, Wis.). Pairwise alignment of HPV39 (30), -68, and -70 sequences, performed with the FASTA program, disclosed 82% nucleotide identity between HPV68 and

TABLE 1. Percentage of identity of nucleotide and deduced amino acid sequences

Compared HPV sequences	% Identity						
	Nucleotide sequence				Amino acid sequence		
	3′-LCR[a]	E6	E7	L1	E6	E7	L1
HPV68 vs HPV39	72	87	89	81	85	83	87
HPV68 vs ME180	86	94	95	93	93	89	95
HPV70 vs HPV39	71	87	86	81	82	76	86
HPV68 vs HPV70	68	85	85	81	82	76	86
HPV68 vs HPV18	54	70	72	75	63	61	75
HPV70 vs HPV18	55	72	75	75	66	64	76

[a] LCR, long control region.

HPV39, 79% identity between HPV39 and HPV70, and 81% identity between HPV68 and HPV70. The percentage of identical nucleotides in E6, E7, and L1 ORFs varies between 81 and 89% (Fig. 3A and B; Table 1). This warrants the recognition of three distinct HPV types. The percentage of identity of the deduced amino acid sequences varies between 76 and 87%. The three E6 proteins have the same size (158 amino acids), whereas the sizes of the E7 proteins (109 amino acids for HPV39 and -70 and 110 amino acids for HPV68) and L1 proteins (505 amino acids for HPV39 and -68 and 506 amino acids for HPV70) differ by one amino acid. The 3′ end of the long control region (about 300 nucleotides) shows a higher nucleotide sequence variation (Fig. 3A; Table 1). Furthermore, the long control region of HPV70 (see accession number below) is 114 nucleotides longer than that of HPV39 (30), due to insertions or deletions in the 5′ region. When compared with HPV18 (6), a highly oncogenic related type, HPV39, -68, and -70 showed a percentage of identical nucleotides in E6, E7, and L1 ORFs varying from 70 to 75%, with identical amino acids ranging from 61 to 76% (Table 1).

The ME180-HPV DNA sequence, a 5,993-bp fragment cloned from the cervical carcinoma-derived ME180 cell line, has been reported to be related to HPV39 (19). The unavailability of the complete genome precluded its recognition as a new HPV type (19). When compared, HPV68 and ME180 nucleotide sequences show 93% identity for the 3,168 nucleotides analyzed and 93 to 94% identity in the E6, E7, and L1 ORFs (Fig. 3A and B; Table 1). Amino acid sequence identity varies from 89 to 95% for the encoded proteins (Table 1). HPV subtypes are defined by a nucleotide sequence variability of 2 to 10% in the E6, E7, and L1 ORFs, whereas variants show a sequence variability of less than 2% (4, 5, 7, 8). The prototypical HPV68 and ME180-HPV DNA sequences may thus be considered two subtypes, HPV68a and HPV68b, respectively. It is worth stressing that, when restriction maps for nine endonucleases are compared (Fig. 2), only 8 of the 16 HPV68 sites and the 18 HPV ME180 sites were found conserved.

To evaluate the evolutionary relationships between HPV68, -70, -39 and ME180 and the related HPV types 18, 45, and 59 (6, 15, 20), the deduced amino acid sequences of the E6 and L1 ORFs were aligned, using the Clustal W program (9, 27), and phylogenetic trees were generated with the Phylogenetic Inference Package (PHYLIP 3.5.) (24). The trees were rooted taking HPV51 (13) as an outgroup. The same trees were obtained by both maximum sequence parsimony analysis and distance matrix analysis. Bootstrap resampling (100 replicates) indicates a 91.5 to 94.1% confidence level for the grouping of HPV39, -68, and -70. As illustrated for E6 amino acid sequences (Fig. 4), HPV68 appears more related to HPV39 than

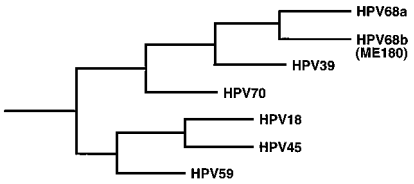

FIG. 4. Phylogenetic relationships among HPV18-related types. A tree was constructed from the comparison of aligned E6 proteins, using maximum parsimony algorithms in the PHYLIP 3.5 package (24). The tree was rooted using HPV51.

to HPV70. All three viruses, together with HPV types 18, 45, and 59, constitute one of the branches of the subgroup containing HPV types associated with high-grade CIN and invasive cancer (29).

Prevalence, variability, and pathogenicity of HPV68 and HPV70. In the course of screening about 3,000 genital samples for the presence of HPV DNA sequences, HPV39-related sequences were detected in specimens of six patients by Southern blot hybridization. HPV68 was found in four specimens: the low-grade CIN from which the prototype was cloned, a low-grade CIN from a human immunodeficiency virus-seropositive patient, a low-grade intraepithelial neoplasia of the vagina from a renal allograft recipient, and a penile Bowenoid papule. All four HPV68 isolates showed the *Pst*I cleavage pattern illustrated in Fig. 1A. HPV70 was detected in the two other specimens, the vulvar papilloma from which the prototype was cloned and a low-grade CIN. The HPV70 isolate from this lesion, referred to as NO87, showed a distinct *Pst*I cleavage pattern. To characterize the L1 region of the NO87 isolate, the 1.1-kb *Bam*HI fragment was amplified by the PCR method and cloned, as described above for the prototypical HPV70. Compared with the prototypical nucleotide sequence, this fragment (1,059 bp) showed four silent nucleotide substitutions and a six-nucleotide deletion affecting codons 179 and 180 (Fig. 3; Table 2). One of the four nucleotide substitutions involved a *Pst*I site. Since the two isolates differ by less than 1% in the L1 region, it is likely that they represent variants.

Discussion. In this study, we have characterized two HPV types related to HPV39, a potentially oncogenic genital virus (2, 30). Compared with HPV39, found in about 3.5% of HPV-positive cervical specimens (1, 1a, 3), HPV68 and HPV70 have been seldom detected in our screening series, and three of the six positive specimens originated from immunosuppressed patients. In spite of this low prevalence, sequence data on DNA fragments of the L1 region obtained by PCR amplification, using consensus or degenerate primers, indicate the occurrence of HPV68 and HPV70 worldwide (10, 11, 16, 18, 19, 25) (Table 2). Two HPV68 isolates, X02 (11) and 1111 (10), are identical or closely related to ME180-HPV (19) (Fig. 3B; Table 2). The substantial differences observed between ME180 and the prototypical HPV68 (7% nucleotide divergence) are thus unlikely to result from the long-term maintenance of the ME180 cell line in tissue culture but rather support the existence of two subtypes, HPV68a and HPV68b (ME180-HPV). Similarly, five isolates identified by others (11, 16, 18, 25) are closely related to HPV70 (Fig. 3B; Table 2). All isolates show a nucleotide sequence variability of less than 1% compared with the prototype described in this paper. Three isolates identified in distinct parts of the world, LVX160 found twice (16) and L1AE1 (25), show the same nucleotide sequence and differ from the prototype by 1 out of the 454 nucleotides sequenced. Furthermore, a Swedish isolate was found to be identical to the French NO87 variant for the 1,059 nucleotides

TABLE 2. Evidence for additional HPV68 and -70 isolates from published L1 ORF nucleotide sequence data

HPV isolates (reference)	Origin	Diagnosis[a]	Variable vs total nucleotides[b]	Amino acid changes[b]
HPV68				
ME180 (19)	United States	SCC	110/1,518	27 amino acids
X02 (11)	Japan	CIN III	7/204	Ala-6→Ser
			0/204[c]	None
1111 (10)	Zaire	CIN	3/335[c]	None
HPV70				
NO87 (this study)	France	CIN I	10/1,064	del Thr-179, Val-180
X11 (11)	Japan	SCC	2/204	Thr-39→Ser
L1AE1 (25)	United States	Normal	1/454	None
LVX160 (16)	Brazil	Unknown	1/454	None
	Singapore	Unknown		
CP141 (18)	United States	Normal	3/454	Ala-428→Val

[a] SCC, invasive cervical squamous cell carcinoma; CIN I or III, cervical intraepithelial neoplasia grade I or III.
[b] Compared with the prototypes described in this study. Variable nucleotides are detailed in Fig. 3.
[c] Compared with ME180 isolate.

compared, both isolates displaying the same deletion of two adjacent codons (Thr-179 and Val-180) in the L1 protein (8b). These data point to the stability of HPV70 variants and indicate that the genetic variability in the coding regions of HPVs involves not only point mutations but also insertion or deletion events.

Although only a few HPV68 and -70 isolates have been identified so far, two of them, ME180 and X11, have been isolated from invasive cervical carcinomas (11, 19) and three have been isolated from CIN lesions (10, 11) (Table 2). Moreover, these isolates have been identified in Europe, Africa, North and South America, and Asia (Table 2). Thus, HPV68 and HPV70 should be considered to have worldwide distribution and to be potentially oncogenic, HPV39-related genital HPV types.

Nucleotide accession numbers. The nucleotide sequence accession numbers X67160, X67161, and U22461 have been assigned to HPV68 and HPV70.

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