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NOTES

Cloning and Partial DNA Sequencing of Two New Human Papillomavirus Types Associated with Condylomas and Low-Grade Cervical Neoplasia

ATTILA T. LÖRINCZ,* ALLISON P. QUINN, MINDY D. GOLDSBOROUGH, BRIAN J. SCHMIDT, AND GARY F. TEMPLE

Molecular Diagnostics Research, Life Technologies, Inc., Gaithersburg, Maryland 20877

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Using low-stringency Southern blot analysis and cloning in λ bacteriophage, two new human papillomavirus types (HPV-43 and HPV-44) were identified and their DNAs were cloned from vulvar tissues. The isolates were characterized by restriction endonuclease mapping and shown to be new HPV types on the basis of their minimal hybridization with all other known HPV types at high stringency. Both HPVs are most closely related to types 6, 11, and 13. HPV-43 did not exhibit any cross-reactivity with these HPV types at high stringency. HPV-44 showed minimal cross-reactivity to HPV-13, which was in the range of 20 to 25% according to liquid hybridization analysis. The deduced genomic organization of each of the two new HPVs was colinear with HPV-6b. Prevalence studies revealed that HPV-43 and HPV-44 together were found in 6 of 439 normal cervical tissues, in 8 of 195 cervical intraepithelial neoplasms, but in none of 56 cervical cancers tested thus far.

Human papillomaviruses (HPVs) are small double-stranded DNA viruses which replicate in epithelial cells (10). Approximately 15 distinct types preferentially infect the anogenital mucosa where they cause condylomas and cervical intraepithelial neoplasia (CIN) (18). Some members of this group of mucosatropic HPVs, of which the clinically most important are types 16, 18, 31, 33, and 35, are thought to play a role in the development of cervical cancer and other types of malignancies (1, 3, 9, 12–14, 19).

New HPV types in tissue samples are recognized by virtue of their cross-reactivity to other HPV probes during low-stringency Southern blot hybridization (1, 3, 9, 12–14, 19). We report here the cloning and characterization of two new HPV types detected during the analysis of approximately 1,000 anogenital tissue samples. The results of some of these studies and the characterization of two other HPV types cloned from this set of samples have been described previously (12–14, 19).

Clinical samples were digested with proteinase K and sodium dodecyl sulfate to release cellular DNA, which was then extracted with phenol-chloroform and precipitated with ethanol (16). DNA pellets were suspended in TE (10 mM Tris hydrochloride, 1 mM EDTA [pH 8.0]). DNA (5 to 10 μg) from each sample was digested with *Pst*I and electrophoresed in agarose gels. Southern blot filters were prepared on nitrocellulose and were hybridized at low stringency to identify all HPV cross-reactive sequences, as described previously (12, 14). HPV-6, HPV-11, and HPV-16 probes used in the initial hybridization were labeled with ^{32}P by nick translation. To identify specific HPV types, the filters were stripped of probe and rehybridized at high stringency with individual HPV probes.

Figure 1 shows a Southern blot analysis in which two new HPV types were initially identified, each from a separate patient. The two samples were obtained from women in the Detroit, Mich., area. One was from a vulvar biopsy (L6) that showed only hyperplasia on histopathological examination (lane 6), and the other sample (L11) was from a vulvar condyloma (lane 11). The HPVs in these samples were presumed to be new types because the signals observed with a low-stringency probing (Fig. 1A) were greatly reduced after a high-stringency rewash (Fig. 1B). These HPV-related DNAs were analyzed further to establish the best strategy for cloning with λL47 (12). The samples were digested individually with either *Eco*RI, *Bam*HI, or *Hin*dIII and were analyzed with Southern blots probed at low stringency with HPV-16 DNA. The HPV cross-reactive sequences in sample L6 were not cleaved by *Eco*RI. Two fragments of 6.3 and 1.4 kilobases (kb) were generated with *Bam*HI, and four fragments of 2.9, 2.3, 1.3, and 1.2 kb were generated with *Hin*dIII. Sample L11 produced a single 7.8-kb fragment with *Eco*RI and *Bam*HI and was not cut with *Hin*dIII.

DNA isolated from the above samples was cloned into λL47. Since it was assumed that some of the smaller fragments of the HPV DNA would not be clonable in λL47, two separate cloning procedures were performed for sample L6, using *Bam*HI or *Hin*dIII. Because sample L11 gave a single fragment of 7.8 kb only a single cloning procedure was performed with *Bam*HI. In each case, 2 μg of sample DNA and 1 μg of λL47 DNA were digested with the appropriate restriction enzyme. Ligation to produce recombinant λ molecules, packaging, and screening were performed as described previously (12). Hybridization with an HPV-16

* Corresponding author.

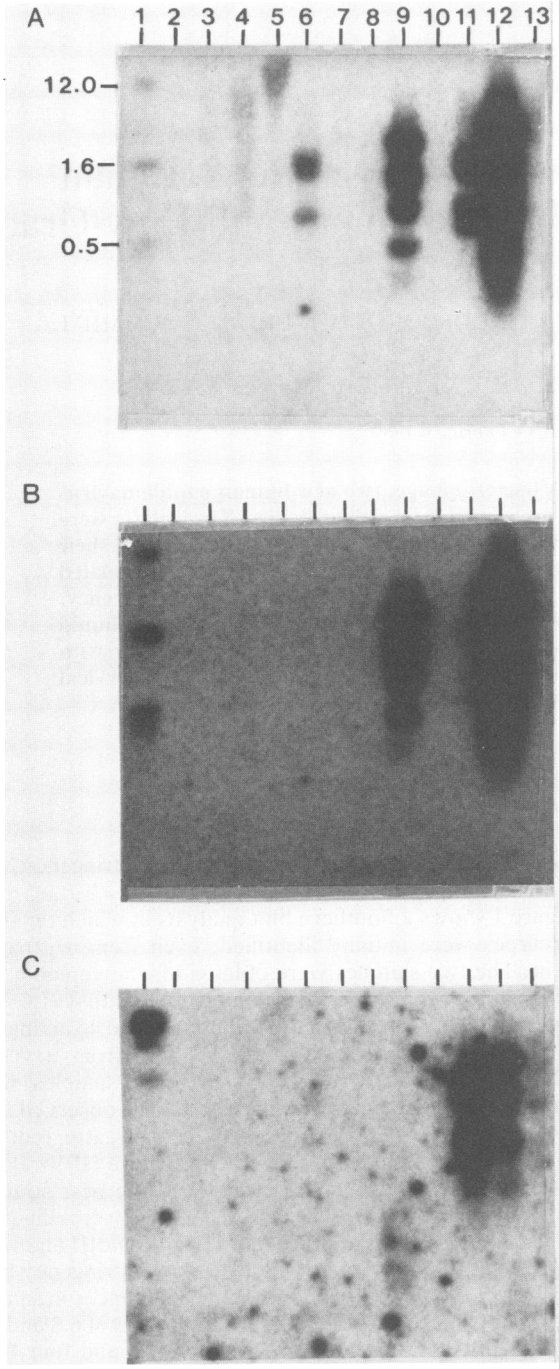

FIG. 1. Analysis of HPV types present in anogenital lesions. Total DNA was purified from 12 different clinical samples, and each was digested with the restriction endonuclease *Pst*I. Samples were electrophoresed in a 1% agarose gel. (A) A Southern blot was prepared and hybridized with a mixture of ^{32}P-labeled HPV-6b (8), HPV-11 (11), and HPV-16 (9) probes under conditions of low stringency. (B) The results of a high-stringency rewash of the blot in panel A. (C) The result of a rehybridization of the same filter with the L11 (HPV-44) clone at high stringency. Lanes: 1, molecular size markers whose sizes in kilobases are shown to the left in panel A; 2 to 13, clinical samples as follows. Lanes: 2, normal cervix; 3, normal cervix; 4, normal cervix; 5, normal cervix; 6, vulvar hyperplasia; 7, normal cervix; 8, normal cervix; 9, CIN 3; 10, normal cervix; 11, vulvar condyloma; 12, vulvar condyloma; and 13, normal cervix. Samples in lanes 6 and 11 contained unknown HPV types which were cloned and shown to be HPV-43 and HPV-44, respectively. Samples in lanes 9 and 12 contained HPV-16 and HPV-6b, respectively. All other samples had no demonstrable HPV DNA. The signal seen in lane 12 of panel C is due to some cross-reactivity between HPV-44 and HPV-6b under the hybridization conditions used in the analysis. The sample in lane 12 contained a very large amount of HPV-6b DNA (several hundred picograms), and thus the cross-reactivity is to be expected.

TABLE 1. Prevalence of HPV-43 and HPV-44 in cervical samples from the United States[a]

Diagnosis	No. of biopsy specimens containing DNA sequences of:		Total no. of samples
	HPV-43	HPV-44	
Normal squamous epithelium[b]	1	5	439
Metaplastic squamous epithelium	0	1	55
CIN 1	4	3	112
CIN 2	0	1	55
CIN 3	0	0	28
CIN (total)	4	4	195
Carcinoma[c]	0	0	56

[a] All specimens were from different women. None of the specimens which contained either HPV-43 or HPV-44 DNA had any other detectable HPV types.

[b] Seventy-five of these normal samples were biopsies, the other 354 were exfoliated cervical cells.

[c] Of the 56 cancer specimens, 48 were squamous carcinoma, and 8 were adenocarcinomas.

probe under low-stringency conditions revealed from 1 to 10 plaques per cloning procedure from approximately 2×10^5 plaques screened for each sample. All plaques containing DNA that was cross-reactive with the HPV-16 probe were characterized by restriction endonuclease mapping and Southern blot analysis. Each cloning procedure revealed only one type of λ recombinant; thus a representative member from each group was cloned into pBR322 for further analysis. Sample L6 gave two different clones, one with a 6.3-kb *Bam*HI insert and one with a 2.9-kb *Hin*dIII insert. Sample L11 gave a single type of clone with a 7.8-kb *Bam*HI insert.

To ensure that these clones were new HPV types, they were used as probes to hybridize with Southern blot filters containing DNAs of all the known HPV types at that time (HPV-1 to HPV-42 [2, 18]). Southern blot analysis of the HPV types that were available in our laboratory, using the HPV DNA clone from sample L11 as probe, is shown in Fig. 2. Filters with the remaining HPV types were obtained from E.-M. de Villiers and G. Orth (data not shown). At high stringency, no hybridization was seen between the L6 clones and any of the other known HPV types. A weak hybridization signal was seen between the L11 clones and HPV-13 (Fig. 2). To quantitate the degree of homology between L11 and HPV-13, standard liquid hybridization analysis using S1 nuclease was performed (11, 17). Experiments in which either HPV-13 or L11 DNA were used as the driving probes revealed hybridization kinetics indicating 20 to 25% homology (data not shown). Because the standard for a new HPV type is less than 50% homology with the most closely related known HPV type (4), the HPV clones from samples L6 and L11 were considered new HPV types and were designated HPV-43 and HPV-44, respectively.

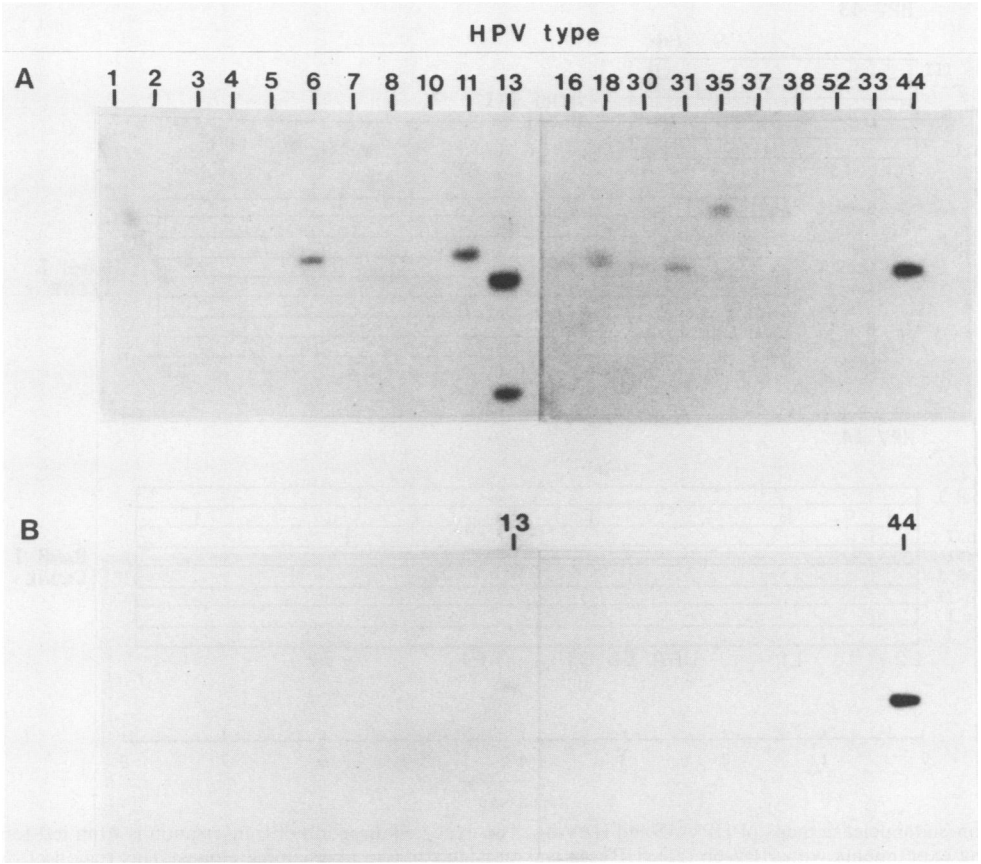

FIG. 2. Analysis of homology between HPV-44 and other cloned HPVs. HPV DNAs were liberated from their plasmid vectors, and 10 ng per lane was electrophoresed in a 1% agarose gel. The DNAs were transferred to nitrocellulose and hybridized at low stringency (panel A) with a ^{32}P-labeled HPV-44 DNA fragment isolated by gel electrophoresis from the λ clone. This probe did not react with any vector sequences that may have been present with the HPV DNAs being tested. The HPV types in each lane are shown at the top of panel A. At low stringency, HPV-44 showed strong cross-reactivity with HPV-6b, HPV-11, and HPV-13. In contrast, at high stringency (panel B), HPV-44 showed no cross-reactivity to HPV-6b or HPV-11, only minimal cross-reactivity with the 5.5-kb *Bam*HI fragment of HPV-13, and no cross-reactivity with the 1.76-kb *Bam*HI fragment. Some of the HPV DNAs for this experiment were released from their vector DNAs as single fragments of approximately 8 kb with the following enzymes: HPV-1, -3, -4, -6, -11, -16, -30, and -44 with *Bam*HI; HPV-2, -18, -31, -37, -38, and -52 with *Eco*RI. Other types were liberated from their vectors as subgenomic fragments with the following enzymes: HPV-10 and -13 with *Bam*HI; HPV-5 with *Eco*RI; HPV-7 and HPV-33 (type 33b, cloned by A. Lorincz et al., unpublished data) with *Hin*dIII; and HPV-8 with *Bam*HI plus *Eco*RI. HPV-35 was not liberated from vector sequences and hence migrated larger than the expected size.

HPV-43 was obtained as a 6.3-kb *Bam*HI fragment and a 2.9-kb *Hin*dIII fragment. Restriction endonuclease analysis and hybridization studies demonstrated that these fragments shared 1.6 kb of DNA and revealed the relative orientation of the fragments (Fig. 3). Furthermore, we deduced that the total amount of unique HPV sequences represented by the two overlapping HPV-43 clones is 7.6 kb. The HPV DNA cloned from sample L11 (HPV-44) is a single *Bam*HI fragment of 7.8 kb that was mapped with a number of different restriction endonucleases (Fig. 3).

The genomic organizations of HPV-43 and HPV-44 were deduced by hybridization analysis as previously described (12), using subgenomic fragments of HPV-6b as probes. HPV-43 was subjected to restriction endonuclease digestion with *Hin*dIII plus *Pst*I, and HPV-44 was digested with *Nco*I. The resulting DNA fragments were separated by gel electrophoresis and blotted onto nitrocellulose filters. Five identical filters containing the various restriction endonuclease digests of HPV-43 and HPV-44 were hybridized at low stringency with the five electrophoretically purified DNA fragments of HPV-6b generated by using *Bam*HI plus *Eco*RI plus *Pst*I (21). The five fragments representing the entire genome of HPV-6b hybridized to the two HPV types, and each contiguous HPV-6b fragment hybridized with a specific contiguous HPV-43 or HPV-44 fragment. The deduced positions of the recognized open reading frames (ORFs), as shown in Fig. 3, are consistent with the positions of the E6 ORF established by nucleotide sequencing (Fig. 4). Thus, the sequence arrangements of HPV-43 and HPV-44 are consistent with the typical HPV genome.

Several hundred cervical specimens obtained from previous studies (14, 19) (A. Lörincz et al., unpublished data) were analyzed for the presence of these HPV DNAs. All specimens were obtained from the Washington, D.C., and Detroit, Mich., metropolitan areas. DNA was extracted from these specimens and was digested with *Pst*I for Southern blot analysis as previously described (12, 14). The results of these studies are shown in Table 1. HPV-43 and HPV-44 together were found in 8 of 195 (4%) CINs, but they were not detected in 56 cancers. As described for many other HPV

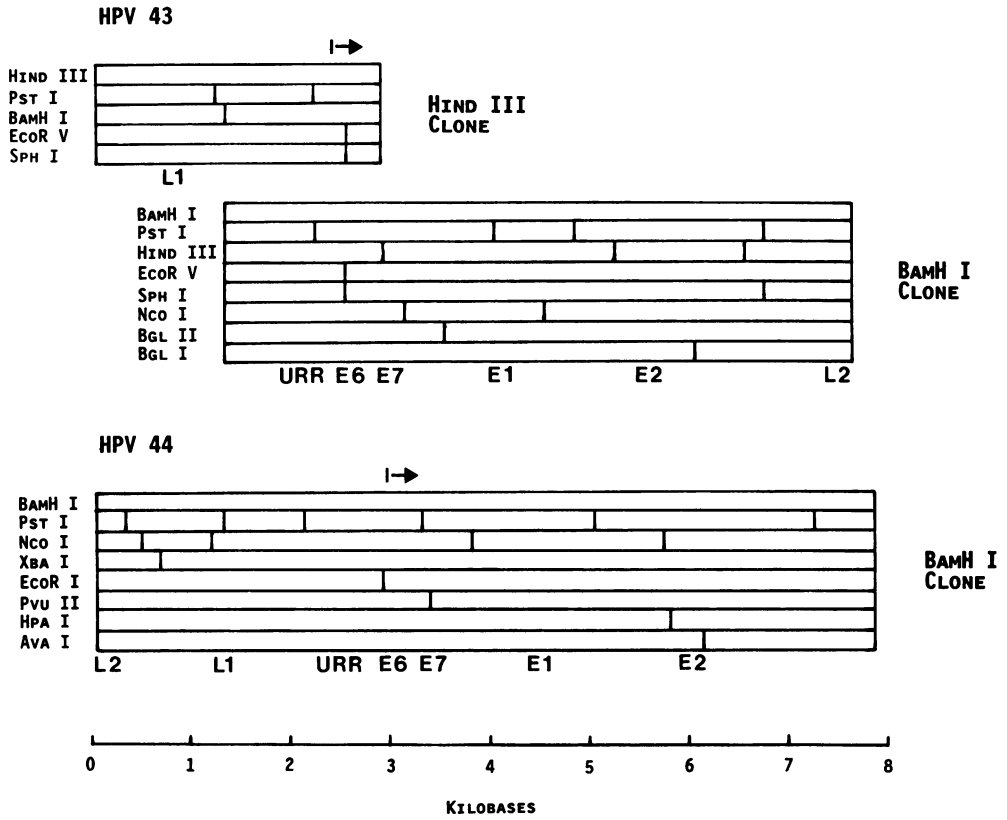

FIG. 3. Restriction endonuclease maps of HPV-43 and HPV-44. The expected direction of transcription is from left to right on the basis of homology mapping experiments with HPV-6b (21). HPV-43 was obtained as two overlapping clones. This region of overlap is the area bounded by the left end of the *Bam*HI clone and the right end of the *Hin*dIII clone, as revealed by the restriction endonuclease patterns and by Southern blot analysis (data not shown). HPV-43 was not cut by *Eco*RI, *Sal*I, or *Sst*I; HPV-44 was not cut by *Bgl*I, *Bgl*II, *Cla*I, *Eco*RV, *Hin*dIII, *Nru*I, *Sal*I, *Sph*I, *Sst*I, or *Xho*I. The midpoints of the recognized HPV ORFs are indicated by the letters and numbers immediately below each restriction endonuclease map. The beginning and terminating positions of the ORFs can be approximated by referring to the ORF map of HPV-6b (21). The putative control regions of HPV-43 and HPV-44 are shown by the letters URR (upstream regulatory region). The vertical lines above each map indicate the approximate positions of the initiating methionines of the E6 protein, predicted from the nucleotide sequence data; the arrows point toward the stop codons.

types (14, 15, 19), HPV-43 and HPV-44 were also detected in normal cervical epithelium. These two HPVs combined were found in 6 of the 439 normal cervical tissues. Although HPV-43 and HPV-44 were cloned from two vulvar biopsy specimens, there was an insufficient number of these samples to conduct a prevalence survey. They were, nevertheless, detected in several other benign vulvar lesions.

Sequence analyses were performed of approximately 600 base pairs in the region of the E6 ORFs of HPV-43 and HPV-44 (Fig. 4). A comparison of the partial nucleotide sequences to the published sequences of HPV-6b (21) (Fig. 4A) and other HPV types (data not shown [5–7, 22]) revealed that in the E6 region, HPV-43 is most related to HPV-6b, being identical to 63% of the nucleotide bases. HPV-44 also is most related to HPV-6b and is identical to 74% of the nucleotide bases. HPV-43 and HPV-44 share 61% identical nucleotides in this region.

Analysis of the nucleotide sequences in Fig. 4A show the predicted proteins encoded by the E6 ORFs of HPV-6b, -43, and -44 to be 150, 155, and 150 amino acids long, respectively. Comparison of the E6 ORFs (Fig. 4B) reveals that the HPV-43 E6 protein has 55% of its amino acids identical to those of the HPV-6b E6 protein, similarly HPV-44 has 73% identical amino acids to HPV-6b. HPV-43 and HPV-44 have 52% identical residues.

The potential to produce a different E6 protein, E6* (20), by alternative mRNA splicing has been regarded as a possible feature of HPV types associated with malignant lesions. The proper splice sites to produce an E6* message are found in HPV-16 (22), HPV-18 (5), HPV-31 (M. D. Goldsborough, D. DiSilvestre, G. F. Temple, and A. T. Lörincz, unpublished data), and HPV-33 (6), whereas they are absent in HPV-6b (21) and HPV-11 (7). Analysis of the sequence of HPV-43 and HPV-44 for the E6* splice sites reveals that both HPV types contain the required consensus GT splice donor in the proper location (nucleotide 233 in HPV-43 and 229 in HPV-44). The consensus splice acceptor, AG, is present in HPV-43 at nucleotide 413, but is absent (CG) in HPV-44 (nucleotide 409). Since neither of these HPV types was detected in the 56 cancers or the 28 CIN 3, the E6* splice donor and acceptor sites may not be fully predictive of high-risk HPV types. In all sequenced HPV types associated with cervical cancer, the splice donor site has an AGGT motif and the splice acceptor site has an AGGT motif (underlined residues are invariant and critical for splicing). In HPV-43, the splice donor motif is AAGT and the acceptor

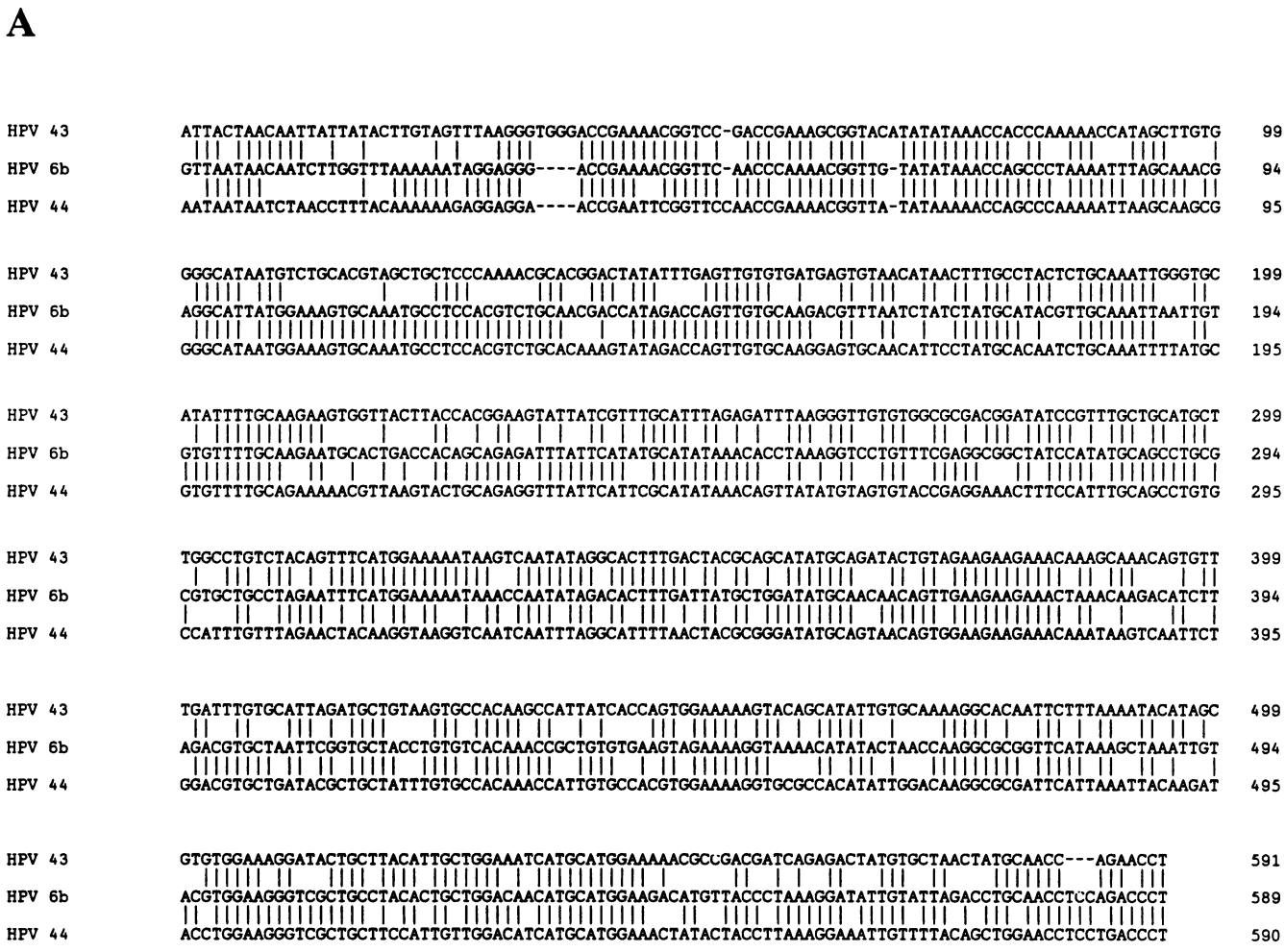

FIG. 4. DNA sequence in the E6 region of HPV-43 and HPV-44. To prepare DNA for sequencing, subgenomic fragments containing the E6 ORFs were made blunt by S1 nuclease and subcloned in both orientations into M13mp19. Nested deletions were prepared by exonuclease III digestion of each subgenomic fragment. The resulting deleted DNAs were cloned and sequenced by using the chain-terminating dideoxy method. The sequences shown are based on sequencing data obtained from both DNA strands. (A) Nucleotide sequence comparisons of HPV-43 with HPV-6b and HPV-6b with HPV-44. Sequence comparisons begin at nucleotide 1 as defined for HPV-6b (21). The E6 ORFs of HPV-6, -43, and -44 begin at nucleotides 30, 32, and 70 and terminate at nucleotides 554, 571, and 555, respectively. The E7 ORFs of HPV-6b, -43, and -44 begin at nucleotides 440, 499, and 486, respectively; the exact ends of the E7 ORFs for HPV-43 and HPV-44 are unknown because the complete nucleotide sequences were not obtained. The numbers to the right of the sequences are the numbers of nucleotides in each line. (B) Predicted amino acid sequence of the E6 protein of HPV-6b, -43, and -44. Putative DNA-binding motifs (Cys X X Cys) in the E6 proteins are underlined. The additional motif present only in HPV-43 and HPV-44 is underlined and identified with four asterisks.

motif is AGAT. Thus, HPV-43 may not be able to synthesize an E6* protein-coding mRNA due to changes in the conserved nucleotides adjacent to the invariant splice donor-acceptor determinants. Experiments to test the ability of the potential splice sites in HPV-43 to produce a functional E6* mRNA have not been performed.

Thus, HPV-43 and HPV-44 appear to be relatively uncommon HPV types associated principally with low-grade lesions. These HPV types may resemble HPV-6b, -11, and -42 in that their DNAs are rarely, if ever, detected in cervical cancers (2, 11, 14, 19). Consistent with this association is the finding that the DNA sequences of HPV-43 and HPV-44 appear most closely related to those of HPV-6b and HPV-11.

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