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Variation of Human Papillomavirus Type 6 (HPV-6) and HPV-11 Genomes Sampled throughout the World

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We examined the genomic diversity of human papillomavirus type 6 (HPV-6) and HPV-11 isolates from different parts of the world by comparing the nucleotide sequences of part of the long control region of three reference clones and 62 HPV-6 and 40 HPV-11 isolates from Africa, Europe, Asia, and North and South America. The genomic sequence of the HPV-6b reference type had to be amended by inclusion of a 94-bp segment, which is also present with minor differences in HPV-6a. Aside from two small inserts typical of all variants related to HPV-6a and three inserts found in HPV-11 variants, no major alterations to the size of the long control regions of these viruses were observed. This corrects the previous impression that these two HPV types are highly polymorphic. Altogether, 19 HPV-6 and 10 HPV-11 variant genomes could be distinguished, and most of the differences were due to point substitutions. The variants of either type were continuously connected in phylogenetic trees rather than clustered separately into subtype groups. Thirteen mutations, namely, the two HPV-6a inserts and 11 substitutions in HPV-6 or HPV-11 variants, reduced the dissimilarity between the types, but they bridged only a small fraction of the genetic distance between the two types. Genomes more obviously intermediate between HPV-6 and HPV-11 were not found and probably do not exist any more. A single HPV-11 variant was found in Africa, but otherwise, no significant correlations of HPV-6 or HPV-11 variants with geography or ethnicity of the patient cohort were found. Functional analysis of diverse enhancer variants showed activities that differed two- to threefold, and it must be considered that transcriptional differences may alter the biology or pathology of these viruses. Similar variants were found in lesions from anatomically different sites and in both benign and malignant lesions.

Papillomaviruses are causally involved in the etiology of benign and malignant neoplasia of cutaneous and mucosal epithelia (for recent reviews, see references 42 and 57). With more than 70 different types of human papillomaviruses (HPVs) known (9, 17), they are one of the most diverse groups of viruses involved in human disease. Studies of the diversity of their genomic sequences are important for establishing a database for the purpose of DNA diagnosis, genotype-phenotype studies, and phylogenetic analysis.

Taxonomically, different papillomaviruses are referred to as "types," and by definition, the DNA genome of each type differs by at least 10% of the nucleotide sequence of the three open reading frames E6, E7, and L1 from that of any other known type (9, 17). Independent isolates of the same type that have minor genomic differences have been called "variants" or "subtypes." Variants and subtypes may hold clues to understanding the evolutionary history of papillomavirus types, and toward this end it is important to find out whether intermediate genomes which link different types still exist. In epidemiological studies, sequence variation can be used as a marker for tracking the spread of the virus in contact networks through populations. An important question arising from this is how genomic diversity relates to phenotypic diversity, i.e., biological and pathological differences.

Our means of addressing some of these questions was to compare HPV type 6 (HPV-6) and HPV-11 genomes sampled throughout the world. Genomically, HPV-6 and HPV-11 are the two most closely related HPV types found commonly in mucosal epithelia. This might indicate relatively recent speciation events which could still have a record in the genomic diversity of today's viral population. As to their phenotypes, they are found in a variety of anatomical sites including the epithelia of the outer and the inner genitalia of both males and females and in the mouth and larynx. Although categorized as "low-risk" HPV types because of their preferential association with benign anogenital lesions, HPV-6 and HPV-11 are also regularly found in malignancies (5, 7, 19, 22, 38, 45, 48). The low-risk epidemiological grouping may stem from the properties of their E6 and E7 proteins, which have lower binding affinities than those of "high-risk" HPVs for the p53 and retinoblastoma tumor suppressor proteins (4, 44).

HPV-6b, the reference clone, was originally isolated from a condyloma acuminatum (18, 28), while HPV-11 was found in a laryngeal papilloma (26). Both viruses have subsequently been commonly found in both types of lesions, and they are now considered the most frequently encountered HPV types that cause benign laryngeal and genital neoplasias. In epidemiolog-

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ical studies, altered restriction patterns have been found more frequently among variant forms of HPV-6 than among any other genital HPV type. This phenomenon gave rise to the subtype classification. We were also interested to see if there was any correlation between genomic variation and disease severity or site, but most published investigations addressed individual cases or small collections of samples, and it seemed that a larger, more detailed comparison of the detected HPV-6 and HPV-11 genomes would be desirable.

MATERIALS AND METHODS

Reference clones. The original isolates of HPV-6b (18, 28) and HPV-11 (26) were obtained by one of us (H.-U.B) in the form of pBR322 clones in 1983 from Lutz Gissmann at the German Cancer Research Center, Heidelberg, Germany, and were preserved since then without further manipulations. The timing of collection and the source of the material are important because they authenticate the corrections that we propose to the original published genomic sequences (16, 51). HPV-6a (7) was supplied by M. Boshart, and HPV-6ma (39) was supplied by one of us (H.P.).

Codes of variants and origin of samples. The HPV-6- and HPV-11-containing samples were derived from independent epidemiological and diagnostic studies. Each viral variant is identified by a coding system established in previous publications of the genomic variation of HPV-16 and HPV-18 (10, 31, 46). An uppercase letter identifies the country, city, or geographic area of origin. This letter is joined with the number 6 or 11 to indicate the HPV type. The subsequent number indicates the count either in the laboratory of origin or in the Singapore laboratory. A lowercase a or b is attached for those samples in which more than one variant was found. For example, J11-2 is sample number 2 of the HPV-11-containing samples from Japan. DNAs from all samples were prepared in the country of origin with the exception of the samples from India, which were extracted in Singapore.

(i) Brazil (B). Genital warts of female patients and genital swabs from aymptomatic patients from Joao Pessoa were sampled. AM indicates cervical smears from asymptomatic patients of the Tiriyo Indians in the western Amazon. This tribe does not live in sexual isolation, and the finding of variants similar to that from the non-Indian population of Brazil is conceivable.

(ii) Germany (G). All HPV-11-containing samples were derived from laryngeal papillomas, while all HPV-6-containing samples came from genital condylomata samples at various clinics throughout Germany.

(iii) India (IN). All samples were from genital warts of female patients from Thiruvananthapuram (formerly Trivandrum), state of Kerala, while the variant IN6-30 was found in an oral carcinoma of a patient from the same geographical origin (3).

(iv) Italy (I). DNA was extracted from anogenital condylomata collected at the Dermatologic Clinic at the University of Siena.

(v) Japan (J). Six HPV-6-positive samples and three HPV-11-positive samples were from condylomata biopsy specimens from female patients.

(vi) New York (NY). All samples were from juvenile patients with laryngeal papillomatosis; most patients were Caucasians, but NY11-11, -12, -14 and -15 were found in African-American patients, and NY6-7 was found in a Hispanic patient.

(vii) Senegal (SN). Samples were from cervical swabs from prostitutes from Dakar.

(viii) Singapore (S). All HPV-6 and HPV-11 variants were found in cervical smears of asymptomatic patients during published studies of HPV-16 and HPV-18 infection (30, 32, 46, 54).

PCR amplification, cloning, and sequencing. Our analyses used strategies established for studies of the genomic diversities of HPV-16, HPV-18, and HPV-45 (31, 46). We amplified by PCR, subcloned into the plasmid pUC18, and determined the nucleotide sequence of a large segment of the long control regions (LCRs) of HPV-6 and HPV-11 found in the DNA samples. Toward this goal, we used the 5′ primer 5′-GGGGTACCAAAGTGGATATAGGGGA CGG-3′ and the 3′ primer 5′-GGTCTAGAAACCGGTCGCAGGTGTGTGA CC-3′. The 5′ primer was complementary to the genomic sequence from positions 7184 to 7204 within the L1 gene of HPV-6b. It was also used to amplify HPV-11 samples, although it had two mismatches to the corresponding segment of HPV-11 (genomic positions 7270 to 7290). The 5′ end of this primer contained a restriction site *Asp* 718, which was used in subcloning. The 3′ primer corresponded to the genomic positions 7869 to 7846 of HPV-6b (count following reference 51) or to positions 7963 to 7940, following the revised genomic count (see below), and to a corresponding segment of HPV-11 (positions 7901 to 7878; revised genomic count), where it gave rise to a single mismatch. It contained an *Xba*I subcloning site at its 5′ end. This primer partially overlapped with the third E2-binding site upstream of the E6 gene of genital papillomaviruses; i.e., it had a 3′ end 133 bp 5′ of the ATG codon of HPV-6b. The PCR products were cloned into pUC18 after cleavage with *Asp* 718 and *Xba*I. They were sequenced with pUC18-complementary primers or with various primers complementary to the HPV sequences (data not shown). For identification and exclusion of PCR errors, all samples were amplified and sequenced in duplicate. All point mutations that were not found in duplicate were excluded from the database. Alternative substitutions that were found twice in products of the same sample were recorded as double infections.

Computer algorithms. The nucleotide sequences of the HPV-6 subtypes, HPV-11, and the variants of both types with point mutations and insertions-deletions (indels) were phylogenetically compared by distance matrix, parsimony, and maximum likelihood algorithms in the phylogeny interference package PHYLIP 3.5 (23, 24). While there is no consensus as to how to weight indels, these were obviously important distinguishing events in our database, so we could not ignore them, as is usually done. However, it also seemed inappropriate to weight an indel as an equivalent number of individual point mutational events. Because of this, we scored each indel that was larger than 1 nucleotide as a fourfold mismatch event. This appeared to be the minimum weighting recognized by the algorithms.

CAT assays. Chloramphenicol acetyltransferase (CAT) assays were performed by following the standardizations of this technique used in the laboratory of Chong et al. (15). The LCR segments of HPV-6a, HPV-6b, HPV-11, and two variants were cloned in the form of the same PCR-generated fragments described above into the test vector ptkCATΔH/N. A total of 15 μg of each construct was electroporated into HeLa cells 48 h prior to enzymatic examination of the cellular extracts.

GenBank accession numbers. The revised genomic sequences of the HPV-6b and HPV-11 reference clones and the sequences of all variants are available by anonymous file transfer protocol (ftp) from the HPV database at the Los Alamos National Laboratory (atlas.lanl.gov).

RESULTS

Genomic sequences of the LCRs of the HPV-6a, HPV-6b, and HPV-11 reference clones. Our research concentrated on the LCRs of HPV-6 and HPV-11, because we had found in previous studies of HPV-16, HPV-18, and HPV-45 sufficient sequence variability in this region for phylogenetic and epidemiological analyses (10, 30–32, 46), and also because several DNA diagnostic studies had found LCR sequence heterogeneities (see below). Two E2-binding sites can serve as landmarks for dividing the LCR into three segments. The 5′ segment stretches from the termination codon of L1 to the first E2-binding site (genomic positions 7292 to 7641 in HPV-6b and genomic positions 7277 to 7591 in HPV-11; Fig. 1). We sequenced this segment, which contains mostly alternating G/AT repeats, only from the HPV-6a, HPV-6b, and HPV-11 reference clones. The central segment, which functionally corresponds to the epithelium-specific enhancer (12, 13, 15) (positions 7654 to 7954 in HPV-6b and positions 7604 to 7891 in HPV-11), is flanked by two single E2-binding sites. We sequenced most of this segment from all viral isolates. From the second E2-binding site to the ATG codon of the E6 gene is a segment of 131 bp which we did not analyze; it contains the replication origin and E6 promoter elements (11, 20, 53).

The bona fide sequence of the 5′ segment of the LCR of the HPV-6b reference clone has been a matter of confusion, because this region seems to give rise to frequent mutations in vivo. Furthermore, it has also been claimed that a cloning artifact occurred in the original isolate (7, 34, 37, 55). As judged by restriction fragment analysis, a segment of approximately 120 bp may have become artifactually deleted from the HPV-6b clone that was used to establish the published sequence (51). In addition, the genomic sequence of HPV-6a was apparently longer by a similar amount (7, 39). Subsequent sequencing showed that the LCR of HPV-6a was longer by 94 bp (38).

To clarify the situation, we resequenced the corresponding genomic segment of the original HPV-6b isolate, which had been received by one of us (H.-U.B.) from Lutz Gissmann in 1983, i.e., directly from one of the scientists involved in the original isolation and prior to publication of the genomic sequence of HPV-6b. The sequence of this clone was identical to the published sequence of HPV-6b (51) with the exception of an additional nucleotide segment of 94 bp in the 5′ part of the

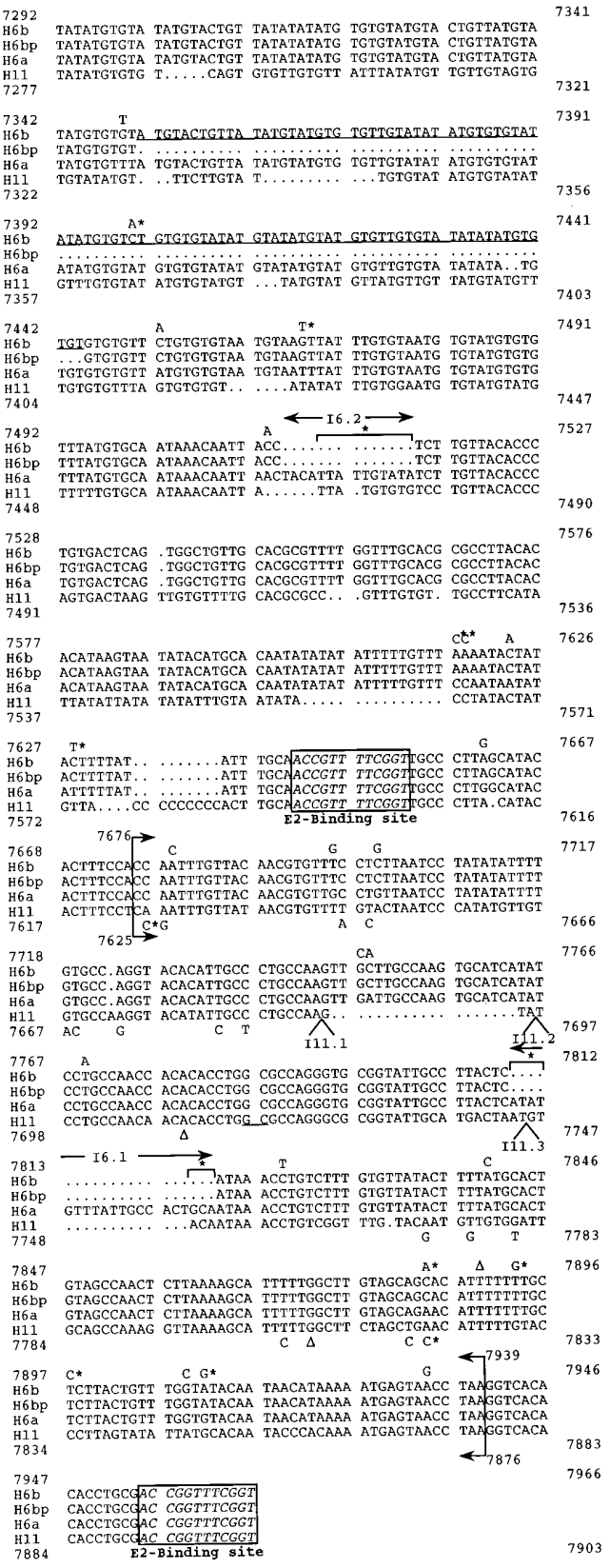


FIG. 1. Alignment of the 5′ and central part of the LCRs of HPV-6b, HPV-6a, and HPV-11. The first nucleotide of the sequences is the one following the TAA translation termination codons of the L1 genes, while the sequences end with an E2-binding site about 130 bp 5′ of the ATG of the E6 gene. The sequence

LCR, which formed part of the extensive G/AT repeat (underlined in Fig. 1). These additional 94 bp were present after position 7350 and were identical to those observed in an independent isolate and published under the code HPV-6 WV6745 (38). We conclude that this sequence was present in the HPV-6b reference type at the time of isolation and was deleted during amplification, a definite possibility according to the studies of the behavior of HPV-6 sequences in *Escherichia coli* (37). This 94-bp sequence is apparently identical to the additional 120 bp previously identified by restriction analyses, because no additional sequences were found in the LCR or in the 3′ segment of the L1 gene. We propose that this sequence be considered a bona fide part of the original HPV-6b reference genome, because a genome without this "insert" has apparently never existed in vivo. In the following, we use nucleotide numbers corrected for the addition of the 94 bp to the published sequence (Fig. 1).

We also determined the corresponding LCR sequences of the original HPV-6a clone. This clone contained a 92-bp segment that was colinear with the 94-bp addition to the HPV-6b sequence. In this segment, the HPV-6a sequence differed from HPV-6b by a C-to-A substitution at position 7400, which was previously recognized (clone HPV-6 W50 in reference 38). HPV-6a was missing one of the GT repeats, which was present in the sequence of HPV-6 W50. Because of the highly repetitive nature of this segment, this deletion is obvious only when one examines the sequences beyond the borders of the segment published previously (38). Upstream of this segment, HPV-6a differed from HPV-6b by a G-to-T transversion at position 7349. On the downstream side of the segment, HPV-6a contained an additional 14-bp insert (designated I6.2 in Fig. 1) 3′ of the genomic position 7514 of HPV-6b. The same sequence and some variation that can occur in it were recently reported, independent from our study (49).

The central part of the LCR of HPV-6a contained a 20-bp insert (designated I6.1 in Fig. 1) and four substitutions (38) in a region corresponding to the epithelium-specific transcriptional enhancer (12, 13, 15). This 20-bp insert was subsequently observed in all samples from Germany (see below) that had been diagnosed by *Pst*I restriction digestion as containing HPV-6a. We conclude that this insert (and probably the 14-bp insert as well) are linked to the sequences generating the *Pst*I restriction pattern of HPV-6a. We take the presence of this insert as a marker for the HPV-6a subtype, independent of other variations in substitutions linked to it, and throughout this report we refer to genomes with this insert as variants of HPV-6a. HPV-6ma, a subtype isolated from a condyloma of the mamilla, has a partial duplication of the LCR (39) but was otherwise indistinguishable from HPV-6a in all sequence elements discussed above.

coded HPV-6bp refers to the published (51) sequence of HPV-6b, which we amended by a 94-bp segment, which is underlined in the sequence designated HPV-6b. The published sequence of HPV-11 (16) was amended by a 2-bp insertion (positions 7717 and 7718; underlined). The genomic segment determined and evaluated for all variants is indicated by two brackets with arrowheads. All substitutions and single-base-pair deletions in the HPV-6b or the HPV-11 genome that were observed in any variant are indicated above or below the sequences, respectively. HPV-6a and HPV-6b differ by the indels I6.1 and I6.2, which are indicated by lines with two arrowheads. The positions of three inserts found in variants of HPV-11 are shown by open triangles designated I11.1 to I11.3; the sequences of these inserts are given in Fig. 4. As discussed in detail in the text, asterisks at substitutions or indels indicate those mutations that can be interpreted as bridging between the prototype sequences of HPV-6b and HPV-11. Nine of these mutations were found in the HPV-6a reference clone, and a 10th one (at position 7893) was found in the HPV-6a variants AM6-1 and B6-1.

The subtype HPV-6c has not been isolated in the form of a plasmid, but its characteristic *Pst*I restriction pattern has been described previously (27). The German sample G6-8 showed this characteristic *Pst*I restriction pattern diagnostic of HPV-6c. Sequence analysis showed that G6-8 (and, by inference, HPV-6c) contained the 20-bp insert and a point mutational pattern similar to those in several variants of HPV-6a (see below). We conclude that while HPV-6a and HPV-6b show significant differences from one another, HPV-6c is more closely related to HPV-6a. Partial genomic sequences have been published from a clone termed HPV-6vc (47, 48), which was not available during our study. This clone differed by 74 bp rather than by 94 bp from the HPV-6b clone, and it was reported to have had 15- and 19-bp inserts (instead of 14- and 20-bp inserts) at positions and with sequences similar to those of the inserts I6.2 and I6.1. Several additional point mutations indicate its relationship to, but distinction from, HPV-6a.

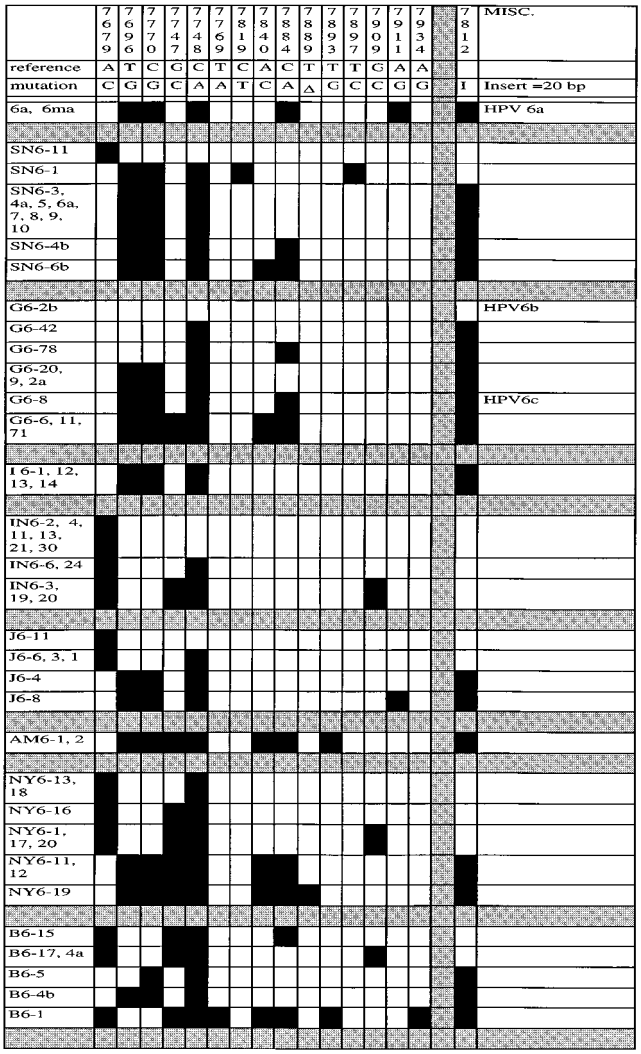
We also reexamined the nucleotide sequence in the LCR of the HPV-11 reference clone. This sequence differed from the published sequence by a GC insertion following a G at position 7716. This sequence is difficult to read because of extreme compression due to the presence of 16 G/C base pairs in an 18-bp segment. This addition altered the count of the genomic sequences of HPV-11 variants 3′ of position 7716, which all contained this sequence, by 2 bp. This 2-bp addition to HPV-11 also eliminated a potential 2-bp indel that would have been a difference between HPV-6 and HPV-11.

Point mutations and indels in the LCR of 62 HPV-6 isolates. To study genomic variation between HPV-6 isolates, we determined all mutations between the genomic positions 7676 and 7939 (revised count as shown in Fig. 1), i.e., starting 22 bp downstream of the 5′ E2-binding site and ending 15 bp upstream of the 3′ E2-binding site. This segment has a size of 264 bp in HPV-6b and a size of 284 bp in HPV-6a (Fig. 1).

Analysis of 62 HPV-6 LCR isolates from patients from Africa, Asia, Europe, and North and South America identified 19 HPV-6 variants which differed from one another by 14 substitutions, one single-nucleotide deletion, and the 20-bp indel that differentiates the HPV-6b and HPV-6a reference clones (Fig. 2). The most diverse variants differed from one another by nine mutations. Interestingly, this number is similar to the maximal diversity of about 5% found between the most diverse variants of HPV-16 and HPV-18 (30, 46). No mutations were found within the 20-bp insert, which commonly occurred together with the three substitutions at positions 7696, 7770, and 7748. These three substitutions also occurred without the 20-bp insert in the African HPV-6b variant SN6-1. SN6-1 can be viewed as intermediate between HPV-6a and HPV-6b variants, but it is set apart by the unique substitutions at positions 7819 and 7897. Two mutations at positions 7747 and 7748 were found in both HPV-6a and HPV-6b variants. A frequent substitution at position 7679 was normally found in HPV-6b variants with the exception of the Brazilian variant B6-1. Surprisingly, we found only a single isolate that had the reference sequence of HPV-6b.

The 94-bp segment in the 5′ part of the LCR which was outside the region studied here was apparently present in all samples because we did not observe any size differences in the amplified products. This was unexpected, because a segment of 74 bp rather than 94 bp is present in this genomic region of subtype HPV-6vc (48), and occasionally, similar or identical deletions (compared with the sequence of HPV-6a) have been observed in other patient cohorts (45, 50).

These observations and their phylogenetic analysis (Fig. 3) suggest that the genomic variation of HPV-6 isolates represents a continuum of events rather than the strict dichotomy



I6.1 = ATATGTTTATTGCCACTGCA

FIG. 2. Genomic variation in the LCRs of HPV-6 isolates sampled in four continents. The figure represents all substitutions, a single-base-pair deletion, and the 20-bp insertion I6.1 found in a segment of the LCR that is indicated in Fig. 1. This segment correlates functionally to the epithelium-specific enhancer of HPV-6. The sequence of I6.1 appears at the bottom of the figure. Black squares indicate the presence of mutations, and white squares indicate the absence of mutations. Geographic origins are abbreviated as follows: SN, Senegal; G, Germany; I, Italy; IN, India; J, Japan; AM, Amazon of Brazil; NY, New York; B, Brazil (urban). In Singapore, we found only HPV-11 variants, which are indicated by the letter S in Fig. 4.

suggested by restrictions patterns, unless one attributes a much higher weight to the event that gave rise to the 20-bp indel than to substitutional changes. No geographic clustering of mutations was observed, because several variants in Africa, Asia, Europe, and the Americas were found to be identical or related forms. This is in contrast to the findings with HPV-16 and HPV-18, which showed specificities of certain variants for geographic regions or ethnic groups.

Point mutations and indels in the LCRs of 40 HPV-11 isolates. To study HPV-11 variation, we sequenced the LCR segment homologous to that studied for HPV-6, which spanned 252 bp between genomic positions 7625 to 7876. Altogether, we observed 10 different LCR variants which differed from one another by 17 substitutions and three indels (Fig. 4).

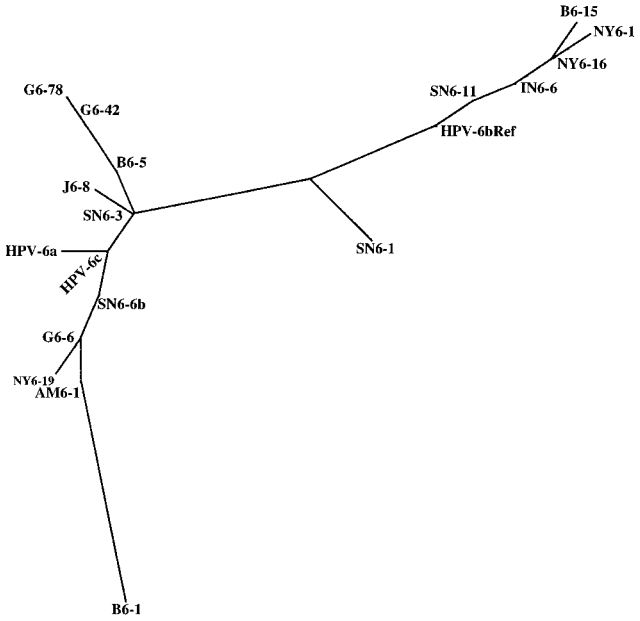
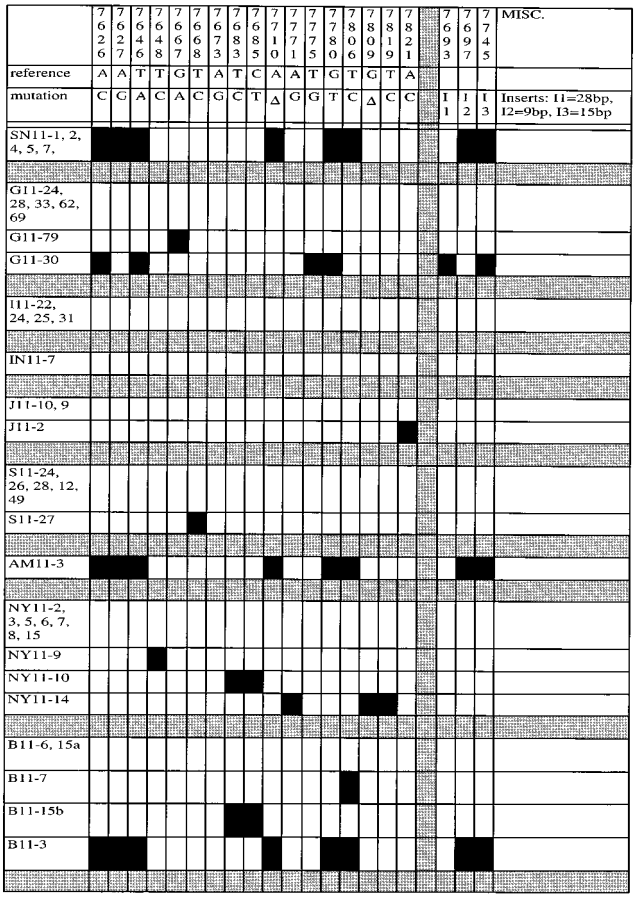


FIG. 3. Relationship among HPV-6 variants. Phylogenetic evaluation of the sequences shown in Fig. 2 was done by the method of maximum likelihood. All isolates positioned on the branch to the right can be interpreted as variants of the HPV-6b subtype, while all of those to the left (including SN-6.1 and HPV-6c) would be variants of HPV-6a. The distances in the center of the figure are inflated, because they were generated by a 4-bp weighting of the 20-bp indel I6.1. This weighting was the minimum necessary to reflect the plausible hypothesis that this indel was generated in a single event during the evolution of HPV-6.



I11.1 = CAACACACCTGCCAAGTACATATTGCCC
I11.2 = TATATAATG
I11.3 = GGTTGTGTTATCTCC

FIG. 4. Genomic variation in the LCRs of HPV-11 isolates sampled in four continents. The sequences of the three inserts I11.1 to I11.3 are indicated at the bottom of the figure. For other details, see the legend to Fig. 2.

The most diverse variants differed by 10 mutations, similar to the 5% maximal diversity seen in the HPV-6, HPV-16, and HPV-18 variants. No substitutions were found within the three inserts, which occurred in two different combinations in 8 of the 40 isolates. One of these variants, SN11-1, was the only one found in the African population; it was found only twice more in Brazilian populations with African ethnic elements or sexual contacts with them. In contrast to the rarity of the HPV-6b reference clone, the HPV-11 reference clone was the most abundant genomic form and was present in 26 of the 40 samples. The phylogenetic analysis of these 10 HPV-11 variants is represented in Fig. 5.

Relationships among 19 HPV-6 and 10 HPV-11 variants. The 264-bp HPV-6 and the 252-bp HPV-11 LCR segments, whose intratype diversities had been studied, can be easily aligned and are clearly homologous. The HPV-6b and HPV-11 alignments differ from one another by 46 substitutions, two large indels (19 and 7 bp), and two small 1-bp indels. Seven of 14 intratype point mutation sites in HPV-6b and 5 of 15 intratype point mutation sites in HPV-11 coincided with intertype point mutation sites. The coincidence of the intertype and the intratype variable sites is almost certainly due to selection pressure that confines mutations to neutral sites because many of these mutations do not alter the known transcription factor-binding sites for nuclear factor I (NFI), AP-1, and TEF-1 in this region. Only four of the seven coincident variable sites in the HPV-6 variants and two of the five coincident variable sites in the HPV-11 variants had mutations which bridged the distance between HPV-6 and HPV-11 (see legend to Fig. 1). Thus, from the absence of genomes more obviously intermediate between HPV-6 and HPV-11, one concludes that the HPV-6 and HPV-11 variants that can be found today do not contain a substantive record of the evolutionary pathway from their common ancestor. The consequence of this is that today HPV-6 and HPV-11 are genomically easily distinguishable, natural taxonomic units.

Functional differences between the enhancers of HPV-6 and HPV-11 variants. Papillomavirus variants may show phenotypic differences due to genomic changes in any part of the genome, and it is difficult to pinpoint the relevant mutations unless one undertakes the laborious task of sequencing the

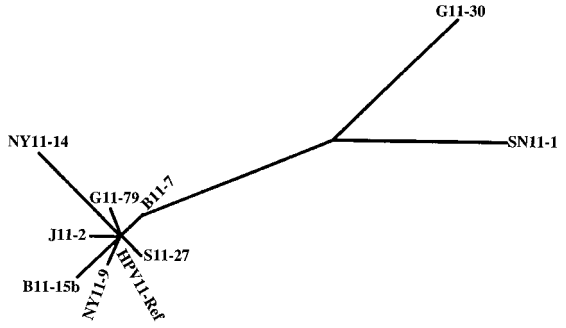


FIG. 5. The relationship among HPV-11 variants. Phylogenetic evaluation of the sequences shown in Fig. 4 was done by the maximum likelihood method.

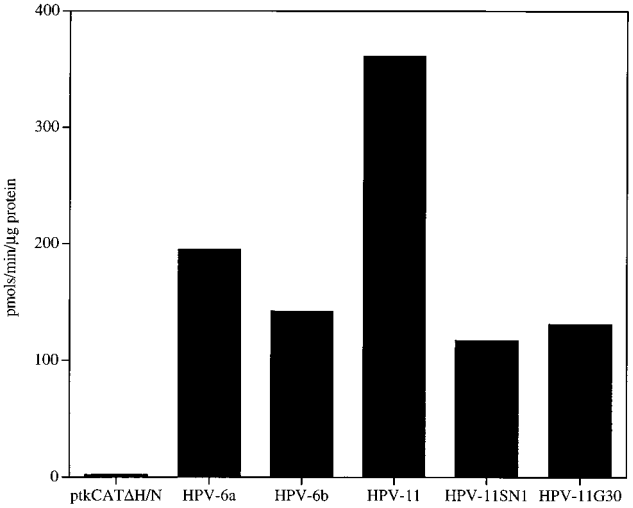


FIG. 6. Enhancer activities of five HPV-6 and HPV-11 variants cloned into the vector ptkCATΔH/N, which served as a negative control.

whole genome of a large number of variants. But since HPV-6 and HPV-11 variants showed significant differences due to indels in the LCR, we decided to compare the most diverse variants for their enhancer functions. We did not analyze all variants, because many of them differed only by one or a few substitutions of sequences that are not believed to constitute transcription factor-binding sites. In particular, we examined the HPV-6a and HPV-6b subtypes, because the indel difference between the two genomes involves a half palindromic NFI-binding site. We also analyzed the HPV-11 reference genome and the two HPV-11 variants SN11-1 and G11-30. The sequences of both of these variants differed from the reference HPV-11 sequence by two insertions, which contain sequences similar to those of published binding sites for the transcription factors NFI, TEF-1, and TEF-2, which are relevant for HPV gene expression (15, 36). Additional copies of these three sites are present elsewhere in the enhancers of HPV-6 and HPV-11.

Figure 6 summarizes the data from the CAT assays. The enhancers of these HPV-6 and HPV-11 variants led to a 59-fold (SN11-1) to 190-fold (HPV-11) stimulation of CAT expression. Four of these constructs showed only minor variation, while the HPV-11 reference sequence exceeded the activity of the LCRs of the other clones by a factor of two to three. The reduction of the activity of the sequences of both HPV-11 variants in comparison with that of the HPV-11 reference sequence was unexpected because the inserts I11.1 and I11.3 seemed to constitute a gain rather than a loss of transcription factor-binding sites.

DISCUSSION

Variation in the LCRs of HPV-6 and HPV-11 isolates and the terms "subtype" and "variant." The term "subtype" appeared in the papillomavirus literature at a time when restriction pattern analysis was the principal tool for characterizing papillomavirus genomes. It was based on the observation of significant variations in the restriction pattern observed after DNA cross-hybridization between two HPV genomes under stringent conditions. Although the term subtype is not strictly defined, it has the connotations that (i) the different restriction patterns would correlate with significant genomic differences, which (ii) might correlate with significantly different phenotypes. It also seems to imply that (iii) the genome of a subtype would not have originated during the course of the infection of a single patient, but that the genomes of closely related subtypes coexist elsewhere in the viral population. Lastly, (iv) the term implies a major genotypic gap between subtypes; otherwise, each isolate with even a single point mutation would qualify as a subtype, and one would then have hundreds of subtypes of each HPV type. In contrast to this, we applied the term "variant" to reflect the findings made with HPV-16 and HPV-18 (10, 46), that many isolates differ only by a few mutations and, when these are taken together, seem to reflect a continuum of divergent genomes clustered around some progenitor genome that may now be extinct.

We believe that the phenomena observed with HPV-6 and HPV-11 isolates are consistent with the connotations of the term "variant." All HPV-6 and HPV-11 isolates can be seen as points along a continuum of genomic changes. Major phenotypic differences may exist between variants, but this is not supported by experimental evidence or clinical observations.

Two indels and 11 substitutions indicate variants that are possible intermediates between HPV-6 and HPV-11. The 51 "genital" HPV types that are presently known are clustered together at the deepest level of the phylogenetic tree (6, 8, 9). Among them are several pairs of closely related types, which presumably had a common ancestor in the more recent past. Among the common types that infect mucosal epithelia, the two most closely related pairs are HPV-18 and HPV-45 (genomic dissimilarity of 18.6%) and HPV-6 and HPV-11 (genomic dissimilarity of 17.2%). Variants of HPV-18 and HPV-45 from Africa contain mutations that place them in between the two types, such that the dissimilarity is reduced by about one-third. In contrast, in the central segment of the LCR, we could identify only a few substitutions that link HPV-6 and HPV-11. Thus, extant variants contain only a faint trace of the common ancestry of HPV-6 and HPV-11.

Interestingly, however, the single 20-bp insertion I6.1 found in HPV-6 variants, which we took as a defining criterion for the HPV-6a subtype, brackets the 7-bp insertion in HPV-11 in the HPV-6b–HPV-11 alignment, and 5 of these 7 nucleotides are identical. This insertion is linked to the three substitutions at genomic positions 7884, 7893, and 7911, which are present in many HPV-6a variants and which further reduce the distance between HPV-6 and HPV-11. Beyond this, HPV-11 differs from HPV-6b by an insertion in the 5′ half of the LCR outside the phylogenetically evaluated fragment, which is nested within the insert I6.2 of the HPV-6a–HPV-6b alignment. In addition to these five mutations, the 5′ half of the LCR of HPV-6a contains five additional substitutions resulting in nucleotides found in HPV-11. These 10 mutations (and 3 others that link HPV-6 and HPV-11) are indicated in Fig. 1 by asterisks. We take these 10 mutational differences as evidence that HPV-6a variants are closer than HPV-6b variants to the common root with HPV-11. It is obvious, however, that these mutations still bridge less than 10% of the genetic distance between HPV-6 and HPV-11. It is not understood why variants more similar to the common precursor of these two types no longer exist or are extremely rare in human hosts.

HPV-6 and HPV-11 variants show only minor correlations with geographic origin and ethnicity of the patient population. Tracking of a pathogen by its genomic diversity is a powerful tool for addressing epidemiological questions. Sequence diversity in HPV-16 and HPV-18 has pointed to an origin and speciation of these viruses in Africa and subsequent spread with further diversification by ancient human migrations (30, 46). Disappointingly, the study of HPV-6 sequences revealed no such scenario, because phylogenetically diverse genomes like SN6-3 and SN6-11 were found in African, Caucasian, and

east Asian patients. It might be significant that SN11-1 was the only HPV-11 variant found in the African cohort. This variant was found only twice elsewhere, and this was in two Brazilian patients from Joao Pessoa and the Amazon who may have become infected with African variants, because a large fraction of the population of Brazil has an African origin. The limited amount of diversity found in HPV-6 and HPV-11 variants strengthens the concept of a very slow evolutionary change of papillomavirus genomes. Nevertheless, because of the extreme intermixing of variants in ethnic populations, the evidence for the geographical origin of HPV-6 and HPV-11 and coevolution with human races has been obliterated. It cannot be decided whether this intermixing of viral variants occurred recently, i.e., subsequent to the spread of humans through all continents, or alternatively, whether HPV-6 and HPV-11 diversification was already advanced before that. Our findings establish, however, a database of the genomic variation of HPV-6 and HPV-11 as a powerful tool for epidemiological research, because it allows one to reconstruct contact networks of infected individuals (32, 33). In contrast, traditional epidemiological analyses can only allow one to infer the overall behavior of a pathogen in a population.

Lesions from different anatomic sites and pathologically differing lesions contain similar HPV-6 and HPV-11 variants. Several examples of genotypic variation in HPV types that may lead to phenotypic changes have been described. Deletions or substitutions in the YY1 sites in HPV-16 lead to elevated levels of expression of the virus-transforming genes (43), but it is believed that these mutations occurred in situ rather than representing a particular subpopulation of viral variants. Substitutions in the L2 gene lead to antigenic diversity of the HPV-6 and HPV-16 variants (56). No functional differences have been found in transformation and transcription assays between an HPV-6 genome from a benign lesion and one from a malignant lesion (22). Although most mutations are likely to be silent, because they either do not alter amino acid sequences, lead only to conservative changes (10, 21, 25, 35), or do not affect *cis*-responsive elements (32), they may nevertheless be linked to functionally significant mutations, and epidemiological observations concerning a variant may point to such a connection.

It will be necessary to examine a larger number of clinical samples than was examined here or to sequence the whole genome of variants to identify such functional alterations. On a preliminary level, however, we have not found an association between particular variants and disease severity or site. A total of 16 of 19 laryngeal samples (all except samples NY11-9, NY11-14, and NY6-19) contained variants that were also found in genital swabs or condylomata, as was the case with five of the seven German HPV-11-containing laryngeal samples; HPV-11 prototype sequences were found elsewhere in genital samples from the patients from whom the laryngeal samples were obtained. Also, a malignant oral lesion from a patient in India contained the same variants as benign genital lesions of the same patient cohort.

Transcriptional elements in the LCRs of HPV-6 and HPV-11 and genomic variants. The central segments of HPV-6 and HPV-11 LCRs contain enhancers, which are epithelium specific and, apparently, even tissue site specific (12, 13, 15, 52). They are activated similarly to the enhancers of HPV-16 and HPV-18 by the binding of AP-1, NFI, TEF-1, TEF-2, oct-1, and possibly, other factors (1, 14, 15, 36). The 5′ segments of the LCRs of HPV-6 and HPV-11 contain transcription termination signals. Considering the conserved size and several conserved sequence elements of this part of the LCRs in all genital HPV types, there are probably additional unknown functions. For HPV-6, but not yet for other genital HPV types, transcriptional up- and down-modulatory functions have been detected (2, 55). Some of these functions may be explained by the affinity of the G/AT repeats for transcription factors like TEF-2 (14). We have compared the LCRs of five very divergent HPV-6 and HPV-11 variants by examining the transcription modulatory functions of their 5′ and central LCR segments.

Functional testing of a segment with the 5′ LCR sequences and the epithelium-specific enhancer gave different values of transcriptional activation, i.e., a stimulation of 59- to 97-fold for HPV-6a, HPV-6b, and two HPV-11 variants and a 2- to 3-fold higher value for the HPV-11 reference type. This latter value was unexpected, because HPV-11 has fewer transcription factor-binding sites than variants G11-30 and HPV-6b, but it might be explained by steric restrictions at the transcription factor-binding sites rather than by their mere additive accumulation. At this stage, it would be speculative to base pathological or epidemiological interpretations on these differences, but it may point toward the potential diversity of transformation efficiency or spread of these viruses because of these small differences in transcription functions.

Slippage, a mutational mechanism important for the generation of diversity among papillomaviruses. HPV-6b and HPV-11 differ from one another by about 17.2% of their total genomic nucleotide sequence. Most of this difference is due to substitutional exchanges, while about 2.3% of the dissimilarity is due to gaps (31 gaps totaling 187 bp, with the exact number varying with details of the alignment), where insertions or deletions had occurred in either of the two viruses. Because of the need for functional conservation, insertions and deletions are more frequent in noncoding regions (19 gaps in about 1 kb of the two noncoding regions) than in genes (12 gaps in about 7 kb). An inspection of these gaps shows that 5 of those in the genes and about 14 of those in the LCR have sequences that are direct repeats of or contain sequence elements similar to those of flanking genomic sequences. Research in other systems has revealed that misalignment-mediated DNA synthesis errors (slippage) is a likely mechanism for generating this type of mutation (for reviews and references, see references 40 and 41).

Inspection of the sequences of HPV-6 and HPV-11 and their variants suggests that slippage may also be a frequent source for generating variation in these two papillomavirus types. Examples are the inserts I6.1 present in HPV-6a and I11.1 present in G11-30 and the sequence between the genomic positions 7745 and 7763 of HPV-6, which is missing in HPV-11, because these segments contain nucleotide stretches of 5 to 8 bp in length that are repeated adjacently. Slippage can give rise to indels as small as a single nucleotide in situations in which the deleted or inserted nucleotide is identical to flanking nucleotides (40, 41), which is the case for the HPV-6 mutation at position 7789 and the HPV-11 mutation at position 7809. It can also lead to large duplications of up to several hundred base pairs and was most likely responsible for generating certain large "rearrangements" in the LCR (7, 39, 50). It seems that the extensive G/AT repeats in the 5′ part of the LCR as well as some short repeated motifs like NFI-binding sites (TT GGC) can enhance the probability of initiating these events. The G/AT repeats may grow or shrink by a mechanism similar to that which generates the GT repeats in human chromosomal DNA. The Z-DNA-forming potential of such sequences may support this mechanism (29).

It would be a mistake, however, to consider these sequence elements as targets undergoing an extremely high rate of mutation, and thus to picture HPV-6 and HPV-11 as viruses with

a highly unstable genome. While the reiteration of G/AT in the 5′ part of the LCR would seemingly make it an ideal substrate for undergoing slippage-mediated indel events, it appears only to have led to the alteration of a single 2-bp repeat during the diversification of HPV-6a and HPV-6b. We can also discount major alterations in the size of this sequence in different variants, although this region was not routinely sequenced in all variants, because we did not observe size differences in the amplimers, which included this part of the LCR. It was mentioned above, however, that a partial deletion of this segment may have given rise to the subtype HPV-6vc (47, 48) and that two other laboratories may have occasionally observed similar genomes (45, 50). If one adopts estimates for the time over which HPV variant diversification occurred from our studies of HPV-16 and HPV-18 evolution, one sees that little alteration of the majority of HPV-6 and HPV-11 genomes has occurred because of slippage over many thousands of years. On the other hand, while the viral population is probably not changing at a very rapid rate, it is likely that variations among individual isolates regularly originate from partial duplications stimulated by repeated sequences (7, 19, 39, 50).

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