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Presence of Catenated Human Papillomavirus Type 16 Episomes in a Cervical Carcinoma Cell Line

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Human papillomavirus (HPV) is frequently associated with cervical carcinoma and derived cell lines. In primary tissues of the carcinoma, the viral genome may be present in episomal or integrated configuration. In cell lines, however, only integrated HPV sequences have been reported. In this article, we describe the presence of episomal type 16 HPV (HPV16), demonstrated by electron microscopy and two-dimensional agarose gel electrophoresis, in a cervical carcinoma cell line, CC7T/VGH, established in 1980 in Taiwan. In CC7T/VGH, the HPV16 sequences are transcriptionally active, and at least three major HPV16 RNA species were detected in Northern blots. Results from restriction enzyme and S1 nuclease analysis suggest a composition of oligomeric HPV16 molecules in dimeric repeats. In addition, the HPV16 oligomers exist as catenated molecules of interlocking rings instead of concatemers. A monomeric copy of the HPV16 episome was cloned from a Hirt supernatant of CC7T/VGH by using a plasmid vector. Mapping and partial sequencing studies revealed an internal deletion of 163 base pairs within the L1 open reading frame. However, insertion of an A · C nucleotide pair at the deletion junction restored the otherwise frame-shifted L1 open reading frame. Two base transitions were also found within the E7 and the E1 open reading frames. Our findings suggest the need for closer examination for HPV episomal catenation in other cervical carcinoma cell lines as well as in primary carcinoma tissues of the uterine cervix and the anogenital tract. With CC7T/VGH, a way is now available for studies of many important aspects of the biology of HPV such as replication and gene expression of the extrachromosomal viral genome.

Human papillomavirus (HPV) is found ubiquitously in human bodies. An HPV genome is a circular molecule about 7,900 base pairs long, consisting of an early- and a late-gene region separated by a short stretch of noncoding sequence designated the long control region. The long control region contains most of the essential signals that regulate the propagation and the biological activities of the virus, including the viral origin of replication (42), glucocorticoid-responsive element (14), and enhancer elements (13, 16, 32). More than 45 HPV types have now been isolated, many of which are associated with both benign lesions and malignant tumors of the anogenital tract (2, 5, 10, 25, 26). The consistent presence of HPV DNA in cervical carcinomas in different regions of the world has prompted many recent studies leading to important descriptions of the physical structures of tumor-associated HPV and expression of the HPV genes in relation to the transformation activities of the virus. HPV is able to transform murine, rodent, human fibroblast, and human keratinocyte cells (19, 22, 33, 43, 45). Some of the transforming genes are transcribed from the E6/E7 open reading frames (ORFs) (3). The gene product of the E2 ORF possesses a *trans*-acting function, targeting on enhancer sequences located in the long control region (13, 16, 32). The E1 ORF is one of the longest ORFs identified on the HPV genome. Although the exact function of the E1 ORF in HPV has not been directly demonstrated, recent genetic and molecular analyses of the bovine papillomavirus (BPV) genome have assigned two separate functional domains to this ORF. The 5′ portion of E1 plays a role in the modulation of transient replication of the BPV genome, whereas the 3′ sequences seem to be essential for the establishment of stable extrachromosomal replication (27, 36). The E7 polypeptide also plays a role in BPV replication in maintaining the BPV at high copy numbers (4).

Despite the fact that many primary cervical carcinoma samples contain episomal HPV sequences (5, 9, 10, 41), cell lines derived from this cancer appear to contain only integrated HPV type 16 (HPV16) or HPV18 DNA (1, 5, 31, 38, 39, 41, 46). On transfection, the HPV genome consistently integrates into cellular DNA, often with deletions (19, 40, 45). In contrast, the BPV genome is able to replicate autonomously upon transfection into host cells (24, 27, 28, 36, 42). The absence of cell lines that support autonomous replication of the HPV genome has made studies on the replication and other biological aspects of HPV difficult. In this article, we describe the presence of extrachromosomal HPV16 DNA in a cervical carcinoma cell line established in Taiwan. This may be a major step toward the establishment of a suitable in vitro culture system for studies on the replication of HPV.

MATERIALS AND METHODS

Cell line. Cell line CC7T/VGH (abbreviated herein as CC7T) was established from a biopsy specimen of a cervical carcinoma, stage IIIb, of a Chinese woman at the Veterans General Hospital, Taipei, in 1980. Establishment of the cell line, growth characteristics, and tumorigenicity have been briefly described (6, 21). The cells of CC7T are epithelial in shape and are tumorigenic in nude mice. However, CC7T cells do not form colonies in soft agar (21). The cells have been in culture since 1980 and are maintained in Dulbecco modified Eagle medium with 10% fetal calf serum. No detectable cytological and molecular changes were observed

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in the cell line during 12 months of intensive studies in our laboratory.

DNA and RNA preparations. To obtain Hirt supernatants (17), CC7T cells were grown to confluency and lysed in 0.6% sodium dodecyl sulfate–10 mM EDTA (pH 7.5). NaCl (5 M stock solution) was added to the lysate to a final concentration of 1 M and the mixture was kept at 4°C overnight. The supernatant was collected from the lysate after centrifugation at 12,000 rpm for 30 min and was extracted with buffered phenol. DNA was harvested by ethanol precipitation. If further purification of the DNA was desired, the volume of the supernatant obtained after the 12,000-rpm, 30-min centrifugation was made up to 8 ml with 10 mM Tris hydrochloride–1 mM EDTA (pH 7.4). CsCl (8.0 g) was dissolved in the solution, 0.8 ml of ethidium bromide stock solution (10 mg/ml) was added, and the DNA was banded in a TFT65.13 rotor (Kontron) at 52,000 rpm for 20 h at 20°C. Fractions (200 μl) were collected after banding, and samples were spotted onto GF/A filters for visualization under UV light. Ethidium bromide-stained fractions were pooled, and ethidium bromide was removed by several extractions with isopropanol. In most cases, however, CsCl banding of CC7T Hirt extract was carried out without ethidium bromide. DNA-containing fractions were revealed by staining of GF/A filter disk in ethidium bromide solution after the gradient fractions were spotted onto the filters.

Recombinant plasmid DNA was prepared and purified in CsCl gradients as described previously (29). For use as a hybridization probe, an HPV16 sequence (7.9 kilobase [kb]) was cleaved from an HPV16 clone (provided by H. zur Hausen), with *Bam*HI and purified from low-temperature agarose gel.

Total RNA was prepared from CC7T cells by essentially the guanidinium-cesium chloride procedure of Chirgwin et al. (7).

Gel electrophoresis and Southern and Northern (RNA) blot analysis. Southern blot analysis was performed as previously described (8, 9). For the two-dimensional gel electrophoresis experiments (18, 44), CC7T DNA was first digested with *Hin*dIII and electrophoresed in two separate lanes in a 0.4% agarose gel at 6.5 V/cm for 4.5 h. One of the lanes was cut out, mounted horizontally onto a 1% agarose gel, and electrophoresed at 3.5 V/cm for 8.5 h in 89 mM Tris (pH 8.0)–89 mM boric acid–0.2 mM EDTA. The other lane from the 0.4% gel was then aligned with the 1% gel slab and blotted onto a membrane filter for hybridization.

For Northern blot analysis, 20 μg of glyoxylated total RNA was electrophoresed in a 1.2% agarose gel in 10 mM $NaHPO_4/Na_2HPO_4$ (pH 6.8). Blotting of RNA onto nitrocellulose membranes, hybridization, and the wash conditions that followed were similar to the conditions used in Southern hybridization.

Enzymatic reactions. Restriction endonuclease digestion of DNA samples was carried out as recommended by the suppliers. S1 nuclease digestion was carried out in 33 mM sodium acetate–50 mM NaCl–0.03 mM $ZnSO_4$ (pH 4.5) at 37°C for 10 min. The samples were then applied to a 0.8% agarose gel for immediate electrophoresis.

Sequencing reactions. For determination of the nucleotide sequence, a reaction protocol modified from that of Hattori and Sakaki (15) with double-stranded DNA templates was used as previously described (8). Synthetic oligonucleotides (20-mers) with sequences located close to the target regions were used as polymerization primers.

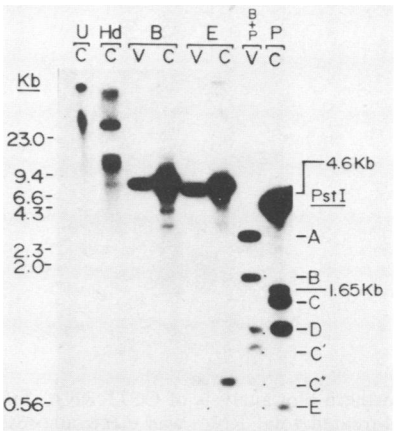

FIG. 1. Restriction analysis of cellular DNA from CC7T. CC7T cellular DNA (C) and DNA from an HPV16 clone (V) were electrophoresed in a 0.8% agarose gel either undigested (U) or after digestion with *Hin*dIII (Hd), *Bam*HI (B), *Eco*RI (E), or *Pst*I (P). The DNA fragments were blotted onto a nitrocellulose membrane and probed with ^{32}P-labelled HPV16 DNA. The size markers (*Hin*dIII-digested λ DNA) and the prototype *Pst*I fragments (*Pst*I-A to -E) are shown. The respective sizes of the *Pst*I-A through -E fragments are 2.82, 1.78, 1.56, 1.06, and 0.48 kb. The *Pst*I-F band (0.22 kb) had run off the gel and is not shown. The *Pst*I-C fragment of the HPV16 genome was cleaved by *Bam*HI, which was used in the cloning of HPV16, into C′ and C″ subfragments (0.91 and 0.64 kb, respectively).

RESULTS

Presence of episomal and integrated HPV16 sequences in CC7T. Total cellular DNA was prepared from CC7T and examined in a Southern blot for the presence of HPV16 sequences (Fig. 1). When undigested, only high-molecular-weight DNA hybridized with HPV16 probe, and no discrete bands were discernible. However, on digestion with the noncutting *Hin*dIII restriction enzyme, distinct HPV16 bands were observed, again in the high-molecular-weight region. The single-cutting *Bam*HI produced a very intense 7.9-kb band, similar in size to the band produced by the *Bam*HI-digested HPV16 prototype genome. Two smaller bands of much lower hybridization intensities were also present. Digestion with *Eco*RI, which normally cleaves the viral genome twice, produced the two prototype fragments and other new but minor bands. Cleavage of the HPV16 DNA by *Pst*I produced six fragments (designated *Pst*I-A through -F) with sizes ranging from 2.82 to 0.22 kb (Fig. 1). *Pst*I-digested CC7T DNA produced only four prototype bands (*Pst*I-C through -F). The *Pst*I-A and -B fragments were absent, replaced by two novel bands (4.6 and 1.65 kb). Since the 4.6-kb fragment has the added size of the *Pst*I-A (2.82 kb) and the *Pst*I-B (1.78 kb) fragments, it could have arisen by a deletion of the *Pst*I site (nucleotide position 879), which separates these two fragments. The other new *Pst*I fragment (1.65 kb) was probably a form of junctional sequence. This band disappeared on second digestion with *Bam*HI (data not shown), indicating the presence of an internal *Bam*HI site. Northern blot analysis of CC7T RNA detected at least three major HPV16 transcripts (Fig. 2), indicating that the HPV16 sequences in CC7T are transcriptionally active.

Preliminary Southern blot analysis with total DNA showed the presence of multiple copies of HPV16 sequences in CC7T. Since most or all of the viral sequences were

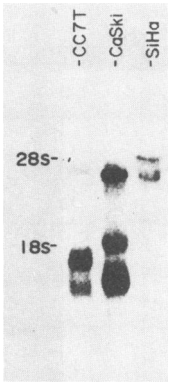

FIG. 2. Northern blot analysis of CC7T RNA. In each lane, 20 μg of glyoxal-treated total RNA was electrophoresed in a 1.2% agarose gel. The RNA was blotted onto nitrocellulose membrane and probed with radiolabeled HPV16 DNA. RNA preparations from the well-characterized cervical carcinoma cell lines CaSki and SiHa were also included as references.

probably retained, as shown in the *Pst*I and *Bam*HI digests, the genome multiplicity could be in extrachromosomal or in integrated form, or both. To distinguish between these possibilities, *Hin*dIII-digested CC7T DNA was analyzed by two-dimensional agarose gel electrophoresis (Fig. 3), which allows separation of circular DNA molecules from linear DNA species (18, 44). The CC7T DNA was first digested with *Hin*dIII to obtain better electrophoretic resolution. Since the HPV16 genome does not contain *Hin*dIII cleavage sites, episomal HPV16 molecules would not be affected if they were present. On electrophoresis in a 1% agarose gel in the second dimension followed by Southern blot analysis, the continuous trail of linear DNA hybridized with the HPV16 probe, indicating integrated HPV16 sequences (Fig. 3). In addition, distinct spots were also found corresponding to the major HPV16 bands present in the first dimension. These spots represented nicked and/or supercoiled circular HPV16 molecules in the CC7T DNA. Thus, CC7T contains HPV16 DNA in both integrated and extrachromosomal forms.

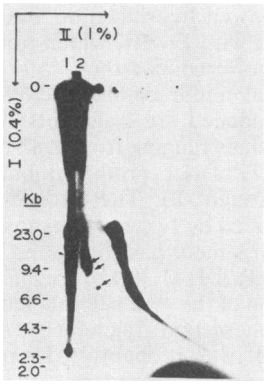

FIG. 3. Analysis of CC7T DNA in two-dimensional gel electrophoresis. CC7T cellular DNA was first digested with *Hin*dIII and then electrophoresed in a 0.4% agarose gel in the first dimension followed by electrophoresis in a 1% agarose gel in the second dimension. A gel track from the first dimension (lane 1) and the gel slab from the second dimension (lane 2) were aligned and blotted for hybridization with an HPV16 probe. The size markers indicate mobilities for the first-dimension electrophoresis only. The major spots representing circular DNA molecules are indicated by arrows.

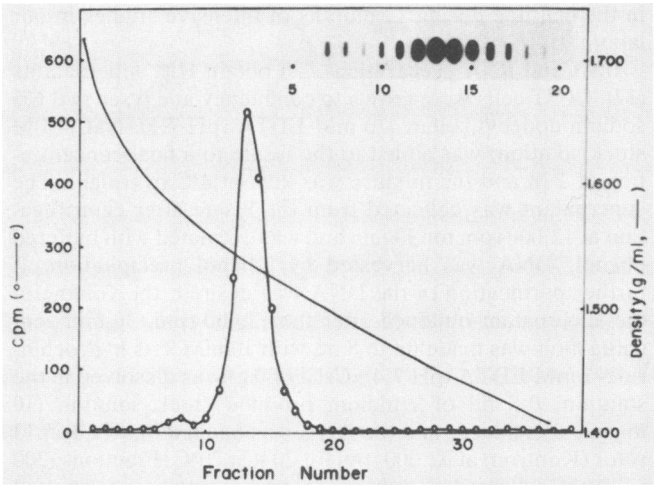

FIG. 4. Analysis of a Hirt extract of CC7T in a CsCl density gradient. The Hirt extract of CC7T cells was centrifuged in a CsCl gradient in the presence of ethidium bromide as described in Materials and Methods. Fractions (0.2 ml) were collected from the bottom of the tube, and fixed samples were loaded onto a nitrocellulose membrane in slots. The HPV16-containing fractions were determined by hybridization and autoradiography. The insert at the top shows the HPV16-positive region of the autoradiogram. The slots were then cut out, and the radioactivity in each slot was determined by scintillation counting. The results are shown on the left-hand scale. The density profile of the gradient was determined from the refractive index of each fraction and is shown on the right-hand scale.

When a Hirt extract of CC7T was analyzed in a CsCl gradient, HPV16 sequences were separated into one major band and one minor band (Fig. 4). However, the minor band was barely detectable in most cases, making it difficult to analyze its nature. The fractions representing the major band were pooled and further analyzed by gel electrophoresis (Fig. 5). Undigested DNA showed an electrophoretic pattern similar to that of *Hin*dIII-digested total DNA. Digestion with *Bam*HI, *Eco*RI, and *Pst*I of the DNA reproduced the intense prototype bands found in the total DNA digests, whereas the minor bands were now absent. In the *Pst*I digest, the novel 4.6-kb band was still present, but the 1.65-kb band observed in the total DNA experiments was now missing, suggesting a chromosomal origin.

Direct evidence for the presence of episomal HPV16 molecules in CC7T was obtained by electron microscopic

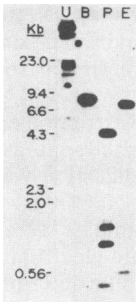

FIG. 5. Restriction and Southern blot analysis of CsCl gradient-purified Hirt extract of HPV16 DNA in CC7T. The DNA was digested with *Bam*HI (B), *Pst*I (P), *Eco*RI (E), or undigested (U) before electrophoresis in a 0.8% agarose gel and analysis by Southern blotting.

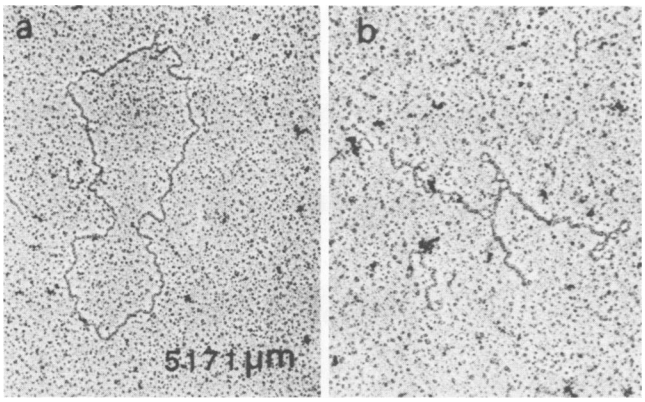

FIG. 6. Electron micrographs of HPV16 episome in CC7T. CC7T Hirt supernatant was first subjected to CsCl gradient purification without ethidium bromide, and the HPV16 fractions were pooled and dialyzed extensively. The electron microscopic work was carried out as described by Register et al. (34). (a) A relaxed molecule measuring 5.171 μm. (b) A supercoiled molecule.

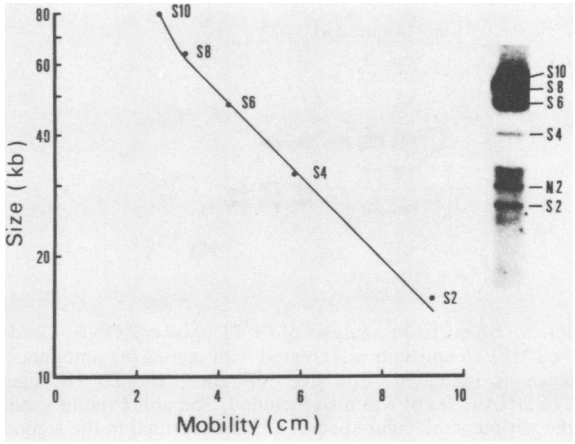

FIG. 7. Semilogarithmic plot of electrophoretic mobilities against the oligomeric sizes of the HPV16 oligomers as resolved in a 0.8% agarose gel (insert). Prefixes S and N indicate supercoiled and nicked molecules, respectively, whereas the numericals indicate the predicted number of HPV16 genomic units per molecular species.

analysis of gradient-purified CC7T DNA (Fig. 6). Both supercoiled and relaxed circles were observed. Unexpectedly, measurable relaxed circles were of a size greater than the monomeric length but were consistently 0.1 μm (approximately 0.3 kb) short of a dimeric molecule. This result suggests that the HPV16 episomal molecules present in CC7T are composed of dimeric units (see below).

Reconstruction experiments showed that the amount of HPV16 DNA present in CC7T per cell was similar to that found in another cervical carcinoma cell line, CaSki (data not shown), i.e., about 500 to 600 copies per cell (39). Although exact determination was not possible, estimation from analysis of extensively washed high-molecular-weight DNA spooled from ethanol precipitation of a total DNA preparation suggested integration of 50 copies or fewer of HPV16 genome in the cellular DNA (data not shown). It was not determined whether the HPV16 integration was at a single site or at multiple regions of the chromosomes. The minor bands in the *Bam*HI and *Eco*RI digests observed in Fig. 1 could represent the virus-cell junctional fragments of the integrants, whereas the relatively intense 1.65-kb *Pst*I fragment could be the junctional fragment between the repeating integrated HPV16 units which contained rearrangements and was thus amplified.

Physical characteristics of the episomal HPV16 molecules in CC7T. CsCl gradient-purified episomal HPV16 DNA from CC7T always showed several discrete bands on gel electrophoresis (Fig. 5), which is indicative of oligomeric HPV16 molecules. When the mobilities of these bands were plotted on a semilogarithmic scale (30), a smooth curve was obtained only if the band with the highest mobility was assumed to represent a dimeric size (S2) of 15.8 kb and other bands were taken as repeats of this dimeric unit (Fig. 7). A straight line was obtained for up to octameric (S8) length with deviation from linearity beyond. Thus, the oligomeric HPV16 sequences in CC7T are circular molecules with dimeric repeats of 8 to 10 copies per molecule. This conclusion is consistent with the observation of only dimeric circles in electron micrographs (Fig. 6), although supertwisted molecular structures were also observed (data not shown) which could represent oligomeric species larger than the dimeric length. We also observed in gel electrophoresis that the oligomers were not present in equal quantities (Fig. 7; see gel patterns). The S2, S6, and S8 bands were the predominant species, whereas the S4 and S10 species were the minor fractions. In some DNA preparations, the S4 species was present only in a very low amount or was not detectable (lane 1 of Fig. 8).

The oligomeric viral genome may exist as concatemeric molecules or as a catenated form of interlocking rings. To distinguish between these two structures, a CC7T episomal preparation was analyzed in a *Bam*HI partial digestion (Fig. 8). The rationale of this experiment was that if the oligomers were concatemers, partial digestion with increasing concentrations of *Bam*HI, which cleaves HPV16 only once, would produce a series of linear oligomeric fragments with a 7.9-kb stepwise decrease in length ranging from about 80 kb (S10) to

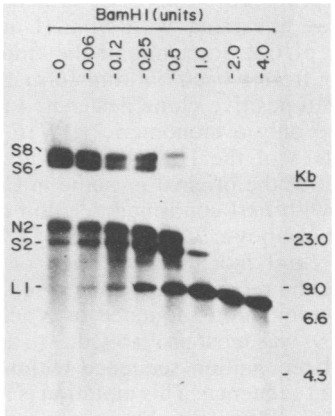

FIG. 8. *Bam*HI partial digestion of CC7T episomal DNA. Episomal HPV16 DNA prepared from Hirt supernatant and purified in a CsCl gradient was subject to *Bam*HI digestion with increasing enzyme concentrations. In each track, CC7T DNA was treated with 0 to 4.0 U of *Bam*HI for 30 min at 37°C. The digestion products were separated in a 0.8% agarose gel for Southern blot analysis. The prefix L represents linear molecules. Other prefixes are as explained in the legend to Fig. 7.

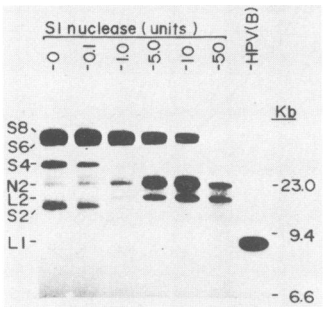

FIG. 9. S1 nuclease analysis of CC7T episomal DNA. Gradient-purified HPV16 episome was treated with increasing amounts of S1 nuclease as indicated. For size reference, *Bam*HI (B)-digested HPV16 DNA (7.9 kb) was also included. The abbreviations indicating the various molecular species are as described in the legends to Fig. 7 and 8.

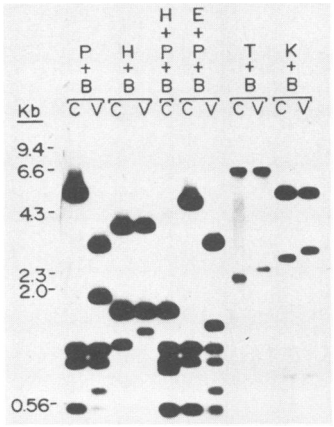

FIG. 10. Restriction analysis of the HPV16 sequences in clone pCC7T-92. DNAs from pCC7T-92 (C) and prototype HPV16 (V) were digested with *Bam*HI (B), *Pst*I (P), *Hha*I (H), *Eco*RI (E), *Taq*I (T), and *Kpn*I (K), and the HPV16 fragments were analyzed in a Southern blot.

7.9 kb (monomer). Stepwise size decreases were not observed (Fig. 8). Instead, increasing *Bam*HI concentrations initially resulted in a reduction of the amount of S6, S8, and S10 species (S4 was not present in this DNA preparation) with a concomitant increase in the amount of supercoiled (S2) and nicked dimeric molecules (N2). The monomeric linear (L1) molecules also appeared rapidly. Eventually, all HPV16 sequences were converted to monomeric linear molecules. The results are best explained by the presence of interlocking HPV16 dimeric circles. This interpretation is supported by analysis with S1 nuclease, which cleaves at random nicks on DNA molecules and thus relaxes supercoiled circles. Under controlled conditions, high concentrations of S1 nuclease induce linearization of nicked circles (44). At high concentrations, S1 nuclease converted the supercoiled circles to nicked (N2) or linearized forms (L2) (Fig. 9). The results are therefore further evidence that the HPV16 oligomers found in CC7T are catenated DNA molecules composed of one to several interlocking circles of dimeric HPV16 genome.

Molecular cloning, restriction, and sequence analysis of the episomal HPV16 genome. To further characterize the HPV16 genome at the nucleotide level, about 50 ng of episomal HPV16 DNA prepared from CsCl gradient-purified Hirt supernatant was linearized with *Bam*HI and cloned into plasmid vector pUC8. Screening of the ampicillin-resistant clones resulting from a bacterial transformation experiment yielded a HPV16-positive clone designated pCC7T-92. The clone contained only a monomeric HPV16 sequence. The restriction pattern of the HPV16 insert in pCC7T-92 was identical to that of the original episome in CC7T cells (Fig. 10). Digestion with *Pst*I confirmed deletion of the *Pst*I(879) site as described above. Cleavage of pCC7T-92 with the double-cutting *Kpn*I indicated retention of both the sites (Fig. 10). Since the recognition sequence of *Pst*I(879) overlaps with the recognition sequence of *Kpn*I(884), abolishment of *Pst*I(879) was most probably due to a point mutation within the *Pst*I recognition sequence without affecting the *Kpn*I recognition sequence. This mutation is located at the 5′ terminus of the E1 ORF (Fig. 11).

Double digestion of pCC7T-92 with *Bam*HI and *Taq*I or *Bam*HI and *Kpn*I further revealed a deletion of about 0.16 kb within the shorter *Bam*HI-*Taq*I and the shorter *Bam*HI-*Kpn*I fragments (Fig. 10). The deletion was more precisely mapped within the 0.64-kb fragment flanked by the *Pst*I(6791) and the unique *Bam*HI(6150) sites, because this fragment was absent in the *Eco*RI-*Pst*I-*Bam*HI triple digest (Fig. 10). The deletion product was expected to have a size of 0.48 kb, equivalent to the 0.48-kb prototype *Pst*I-E fragment and therefore running with the same mobility. Thus, the 0.16-kb deletion mapped within the L1 ORF (Fig. 11). This deletion therefore explains the shortened HPV16 dimeric molecules (0.1 μm less than the expected dimeric size) observed by electron microscopy as described above. Figure 11 summarizes the changes in pCC7T-92 in relation to the prototype HPV16 genome as detected by restriction analysis.

When sequence analysis was performed on the *Pst*I(879) region (Fig. 12), two base changes were noted in reference to the published sequence (37). The first base change was at nucleotide position 878 within the *Pst*I(879) recognition sequence, as predicted by restriction analysis. The adjacent *Kpn*I site was unaffected. This base change resulted in an alanine-to-glutamate amino acid substitution in the fifth codon of the E1 ORF. At nucleotide position 790, at the 3′ end of the E7 ORF, a C-to-T transition had also resulted in an amino acid substitution from arginine to cysteine. The sequence of the deletion junction in the L1 ORF was also determined. The deleted sequence mapped between nucleotides 6296 and 6460, with a deletion of 163 base pairs (Fig. 13a). An insertion of an A · C doublet (Fig. 13b), and thus

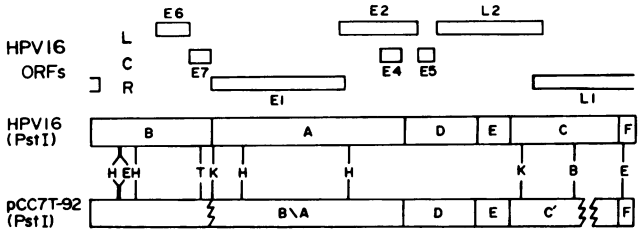

FIG. 11. Restriction map of clone pCC7T-92. The ORFs of the viral genome, the *Pst*I fragments of the prototype, and the pCC7T-92 genomes are indicated by blocks. The *Pst*I-A through -F fragments are indicated by letters A through F within the blocks. B/A and C′ of pCC7T-92 represent fusion and deletion *Pst*I fragments of the cloned plasmid, respectively. LCR represents the long control region of the HPV16 genome. The restriction sites (see legend to Fig. 10) are shown between the HPV16 and pCC7T-92 maps.

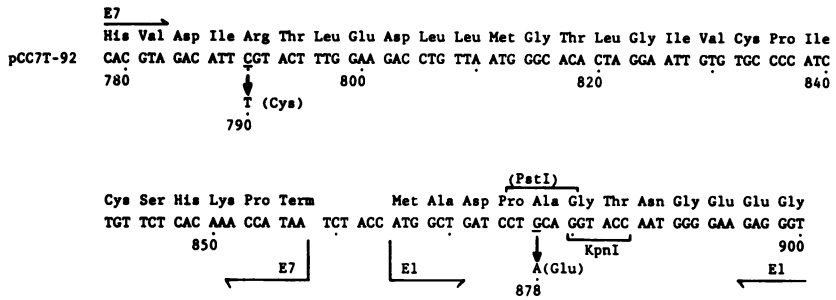

FIG. 12. Nucleotide sequence of pCC7T-92 at the junction of *Pst*I-A and -B. The base changes at nucleotide positions 790 and 878 are indicated by arrows, and the resulting amino acid substitutions are shown in brackets. The base change at 878 also abolishes a *Pst*I site (shown in brackets) which is normally present. The E7 and the E1 ORFs are also indicated.

amino acids valine and histatine, at the deletion site had restored an otherwise shifted translation frame. Also, the guanosine at nucleotide position 1138, which is missing in the published sequence of Seedorf et al. (37), was present in pCC7T-92 (data not shown). This insertion therefore provides the HPV16 genome in CC7T a continuous E1 ORF.

DISCUSSION

Episomal HPV genomes are frequently found in carcinoma tissues from the female genital tract, often coexisting with integrated viral sequences. Although many cervical carcinoma cell lines contain integrated HPV16 or HPV18 sequences, none of the cell lines contains replicating extrachromosomal HPV DNA. This may be explained by the fact that carcinoma tissues often consist of mixtures of benign cells that may contain extrachromosomal HPV DNA and malignant cells with only integrated HPV sequences. Cells from the benign areas might have failed to grow in cultures that had therefore preferentially selected for carcinoma cells with HPV integration. Another possibility is that viral episomes may have been present in the original carcinoma samples, but the culturing process had resulted in the loss of the episomes in the cells. Transfection experiments of cloned HPV16 into murine and rodent cells have similarly failed to establish episomal replication (19, 40, 45). In such cases, expression of some *trans*-acting and/or *cis*-acting genes essential for the establishment and maintenance of an episomal state might not have been initiated or might have been shut down, thus leading to gradual or sudden replication failure. Transient replication of HPV1 in cultured epidermal keratinocytes was described previously (23), but the HPV DNA was lost on further passaging of the cells because of underreplication as a result of cellular heterogeneity (35). In contrast, BPV DNA is able to maintain transient or steady-state episomal replication on transfection. This paper is therefore the first description of the molecular characteristics of autonomously replicating HPV16 molecules in a stable cell line, CC7T/VGH, which is derived from a cervical carcinoma and contains integrated HPV16 sequences. Since no clonal lines have been established from CC7T, it is not known whether a heterogeneous population of cells with distinct HPV16 forms is present in the culture.

It remains to be determined whether successful HPV16 replication in CC7T is due to the presence of some unique cellular factors present in this cell line which are absent in other episomeless lines. Alternatively, or in combination, some changes in the nucleotide sequence in the CC7T episome may have removed some constraints on viral replication which could have existed in the HPV sequences found in other cell lines. On transfection into some cervical carcinoma cell lines, we have reproducibly observed transient extrachromosomal replication of pCC7T-92 DNA, although we have so far failed to obtain a stable cell line able to

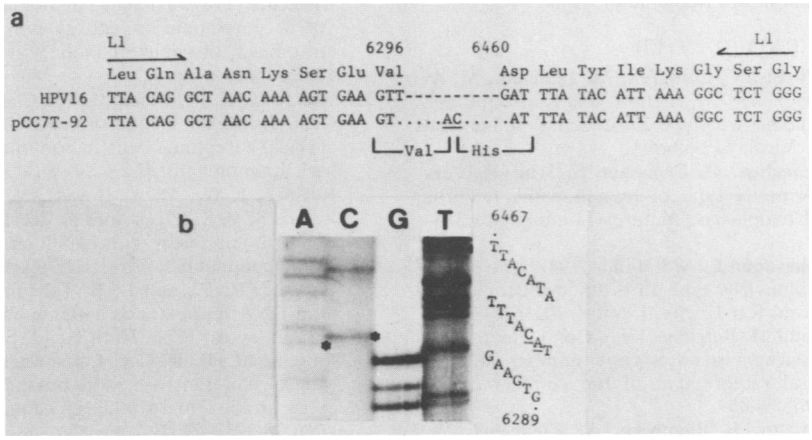

FIG. 13. Nucleotide sequence depicting the deletion junction in the L1 ORF. (a) The upper nucleotide sequence is taken from Seedorf et al. (37). In the sequence of pCC7T-92, the extraneous A · C doublet is underlined, and the resulting amino acids are shown at the bottom. (b) Autoradiograph of a sequencing gel indicating the presence of the extraneous A · C doublet (in asterisks).

maintain such a mode of replication (W.-F. Chou and K.-B. Choo, unpublished data).

Partial sequencing of the CC7T genome revealed a region of deletion of 163 base pairs in the L1 ORF with an insertion of an A · C doublet, thus keeping the L1 ORF in frame. Internal deletions in various regions of integrated HPV16 have been reported (1, 8, 12, 20). In CC7T, two base changes were also detected affecting the E7 and the E1 ORFs, both of which are involved in viral replication in the case of BPV (4, 27, 28). The biological significance of these base changes in relation to HPV16 replication in CC7T remains to be elucidated. Base transition and additions have been reported in many cases of integrated HPV16 sequences (1, 8, 12). Thus, it seems that different isolates of HPV16 frequently contain sequence and size polymorphisms.

On analysis of HPV sequences in surgical tissues of both benign lesions and in carcinomas of the cervix, oligomeric genomes are often noted (5, 11, 20). In view of the presence of catenated HPV16 circles found in CC7T, the HPV oligomers detected in these primary lesions need closer examination for catenation. Similarly, cervical carcinoma cell lines known to carry HPV sequences in high copy number and a complete set of HPV restriction fragments but which have previously been thought to contain only HPV integrations also deserve reexamination. In CC7T, HPV16 catenation may be a consequence of a partially defective cellular topoisomerase system or other protein factors. On the other hand, the HPV16 genome in CC7T may contain mutated catalytic site(s) for cellular topoisomerases or other cellular factors required for the decatenation process. In short, any model proposed for HPV replication will have to account for such a mode of genome oligomerization. The description of episomal HPV in this paper thus opens a way for studies on HPV replication. Analysis of the gene expression program of extrachromosomal HPV16 is similarly facilitated by the use of this cell line, complementing studies of the expression of the integrated HPV genes. It may also represent a step toward the establishment of an in vitro culture of HPV virions.

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