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Human Papillomavirus Type 16 DNA-Induced Malignant Transformation of NIH 3T3 Cells

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A biological function for human papillomavirus 16 (HPV 16) DNA was demonstrated by transformation of NIH 3T3 cells. HPV 16 DNA has been found frequently in genital cancer and has been classified as a papillomavirus on the basis of DNA homology. A recombinant HPV 16 DNA (pSHPV16d), which contains a head-to-tail dimer of the full-length HPV 16 genome, induced morphologic transformation; the transformed cells were tumorigenic in nude mice. Expression of transforming activity was unique because of the long latency period (more than 4 weeks) required for induction of morphologic transformation and because the transfected DNA existed primarily in a multimeric form with some rearrangements. Furthermore, virus-specific RNAs were expressed in the transformants. The transformation of NIH 3T3 cells provides a model for analyzing the functions of HPV 16, which is associated with cervical carcinomas.

The human papillomaviruses (HPVs), a heterogeneous group of papovaviruses, induce epithelial or fibroepithelial proliferations of skin or mucosa (32). Specific diseases associated with papillomavirus infections, such as common warts, epidermodysplasia verruciformis, and genital warts (condylomas), correlate with specific HPV types (for a review see reference 17). Furthermore, certain HPVs are implicated as etiologic agents in the development of malignant lesions of the genital tract (2, 6, 7). HPV 16 and HPV 18 DNAs, which have been isolated and molecularly cloned from cervical carcinoma cells and which have been classified as HPVs on the basis of DNA sequence homology (2, 6), are strongly associated with cervical carcinomas (6, 7, 22). However, the biological significance of the association of HPV 16 and HPV 18 DNAs with cervical cancer has not been determined.

Transforming potential has been demonstrated with DNAs from several members of the papillomavirus family, including bovine papillomavirus (BPV) type 1 (BPV 1) and BPV 2 (3, 16), Shope papillomavirus (28), and deer papillomavirus (8). The transforming potential and genomic organization of BPV 1 have been extensively analyzed, and putative transforming genes have been identified (16, 21). However, similar analyses of HPVs have been hampered by the relative difficulty of transforming cultured cell lines with HPVs. Even though successful in vitro transformation by human papillomaviruses was reported recently with cloned DNAs of HPV 1 and HPV 5 (29), which are associated with warts and epidermodysplasia verruciformis (17), respectively, it is important to study the biological function and molecular characteristics of other human papillomaviruses in view of the heterogeneity of HPVs and virus type-specific association with malignant conversion.

To investigate the biological activity of HPV 16, NIH 3T3 cells were transfected with molecularly cloned HPV 16 DNA. A recombinant form of HPV 16 DNA (pSHPV16d) induced morphologically transformed foci. The transfected HPV 16 DNA was present within the cells in a multimeric state, and virus-specific transcripts were detected.

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MATERIALS AND METHODS

Recombinant HPV 16 DNA. HPV 16 DNA was originally isolated from cervical carcinoma cells, where it was found as multimeric circular extrachromosomal DNA. This DNA was cloned at its unique *Bam*HI site into pBR322 (6). To investigate the biological activity of HPV 16 DNA, a head-to-tail dimer of full-length HPV 16 DNA was subcloned into the *Bam*HI site of pSV2-neo (25), which contains a selectable marker gene for neomycin (G418) resistance. The recombinant HPV 16 DNA, pSHPV16d (Fig. 1), contained the complete genome of HPV 16 with no interruption of putative functional DNA sequences at the *Bam*HI cloning site.

Transfection. Transfections were performed by using a modification of the calcium phosphate coprecipitation method (30). NIH 3T3 cells (10^5 cells per 60-mm dish) cultured in Dulbecco modified Eagle medium supplemented with 10% calf serum (GIBCO Laboratories) were exposed to 1 μg of pSHPV16d DNA which was coprecipitated with DNA extracted from NIH 3T3 cells as a carrier. After 12 h, the transfected cells were subcultured 1:3. The cells were fed every 2 to 3 days. Foci were isolated by using cloning cylinders and were expanded for further analysis.

DNA analysis. DNA from transformed cells was digested by using appropriate restriction endonucleases. Reaction mixtures were covered with liquid paraffin to prevent condensation during incubation. The DNA was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and ethanol precipitated. The DNA precipitates were suspended and electrophoresed in an agarose gel. The gel was treated with 0.25 M HCl to partially depurinate the DNA and was subjected to Southern blotting (24). The nitrocellulose filter was hybridized by the method of Wahl et al. (27) with nick-translated ^{32}P-labeled DNA (10^8 cpm/μg) (19) specific for HPV 16 under stringent conditions (50% formamide in the presence of 10% dextran sulfate at 42°C for 16 h). The filter was washed twice in 2× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate, pH 7.0) containing 0.5% sodium dodecyl sulfate at room temperature and twice in 0.1× SSC containing 0.1% sodium dodecyl sulfate at 65°C for 1 h and exposed to Kodak O-mat X-ray film for 24 h.

RNA extraction and analysis. Total cellular RNA was

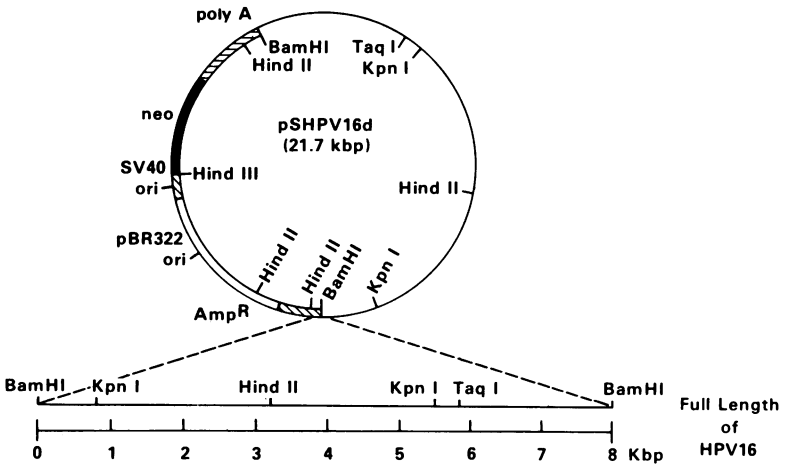

FIG. 1. Molecular structure of the recombinant HPV 16 DNA (pSHPV16d) and restriction map. A head-to-tail dimer of HPV 16 DNA (thin line) was inserted into the *Bam*HI site of pSV2-neo. The pBR322 sequences (open bar), simian virus 40 (SV40) sequences (cross-hatched bar), and Tn*5* neomycin resistance gene (solid bar) are indicated. Ten *Tag*I sites in pSV2-neo are not indicated on the map.

extracted with 8 M guanidine hydrochloric acid by using the method of Adams et al. (1). The polyadenylated RNA fraction was enriched by oligo(dT)-cellulose column chromatography. The polyadenylated RNA was electrophoresed in a 1.2% agarose gel containing 2.2 M formaldehyde (13) and transferred to a nitrocellulose filter by using the method of Thomas (26). Hybridization of the RNA with ^{32}P-labeled HPV 16 DNA was performed as described above for DNA hybridization.

RESULTS

Detection of transforming activity of HPV 16. The transforming activity of pSHPV16d, a recombinant molecule of HPV 16 DNA, was examined in NIH 3T3 cells by using DNA-mediated gene transfer. Morphologically transformed foci were detected 4 or more weeks after transfection (Fig. 2a). The transformed cells were refractive, round cells (Fig. 2c). No foci were observed in control cultures transfected with NIH 3T3 DNA and pSV2-neo DNA (Fig. 2b). The transformation efficiency of pSHPV16d averaged 27 foci per μg of DNA. This value was low compared with BPV 1 DNA and the *ras* transforming gene in pT24 (18), which induced 123 and 135 foci per μg of DNA, respectively. Focus formation occurred with these two positive controls after only 2 weeks of culture, whereas 4 or more weeks was required to detect foci when HPV 16 DNA used in the transfection assay. Because of the long latent period for focus development, transfection results with HPV 16 DNA could be misleading if cultures are evaluated 2 to 3 weeks after transfection (the time period usually used to determine positive transformation with cloned DNAs in the NIH 3T3 cell assay). To estimate the transforming efficiency, transfected NIH 3T3 cells were selected with G418 (400 μg/ml). The number of G418-resistant colonies averaged 644 colonies per μg of pSHPV16d DNA. Therefore, approximately 5% of the cells which incorporated DNA resulted in transformed foci after a relatively long latent period.

HPV 16 DNA in transformed cells. Total cellular DNAs (HMr DNA) from six pSHPV16d-transformed cell lines (SP3T3-1 to SP3T3-6) were purified and subjected to Southern blot analysis by using the ^{32}P-labeled 7.9 kilobase pair (kbp) HPV 16 *Bam*HI fragment. When nondigested HMr DNA was analyzed, DNA sequences homologous to HPV 16 sequences were found in all of the transformed lines (Fig. 3a); no HPV 16 sequences were detected in nontransfected NIH 3T3 cells. Multiple copies of HPV 16 DNA were present in most transformed cell lines. On the basis of a reconstitution experiment in which a known amount of pSHPV16d DNA was mixed with NIH 3T3 cell DNA, the numbers of copies of HPV 16 DNA per cell were estimated to be 1 to 10 copies for SP3T3-3 and SP3T3-6, 50 to 200 copies for SP3T3-2, SP3T3-4, and SP3T3-5, and approximately 1,000 copies for SP3T3-1.

In the Southern blots of nondigested DNA, all detectable pSHPV16d sequences comigrated with high-molecular-weight cellular DNA (Fig. 3a and b). The migration of HPV 16-related sequences was not characteristic of pSHPV16d form I (supercoiled), form II (open circular), or form III (linear) DNA. The association of HPV 16 DNA with HMr cellular DNA might represent integration of the transfected DNA into host chromosomal DNA. Alternatively, extrachromosomal pSHPV16d DNA may comigrate with HMr cellular DNA (longer than 50 kbp) due to the large size of the recombinant pSHPV16d (21.7 kbp). The latter has been observed in studies of cells transfected with BPV 1 DNA; catenated BPV 1 DNA comigrated with HMr chromosomal DNA during agarose gel electrophoresis (11). Yet in the studies with BPV 1, clear monomeric form I and form II input BPV 1 DNAs were also detected. To examine the integrity of HPV 16 sequences, total DNAs from the transformants were digested with *Bam*HI, which excised the unit length of HPV 16 DNA (7.9 kbp) from the flanking pSV2-neo sequences of the donor pSHPV16d DNA. The filters were overexposed to allow detection of minor fragments. A unit-length 7.9-kbp fragment was the predominant product, but additional bands were also observed (Fig. 4a). Fragments larger than 7.9 kbp may have represented integration of HPV 16 sequences into host chromosomal DNA, with additional host flanking sequences linked to the integrated HPV 16 segments. Furthermore, fragments with a unit length less than 7.9 kbp may indicate that there were similar host cell-virus junctions or rearrangements (or deletions) within HPV 16 DNA sequences. To further determine whether pSHPV16 DNA maintained its genetic organization in the transformed cells, HMr DNA was analyzed by using *Kpn*I, *Tag*I, or *Hin*dII. *Kpn*I cleaved two sites within HPV 16 DNA

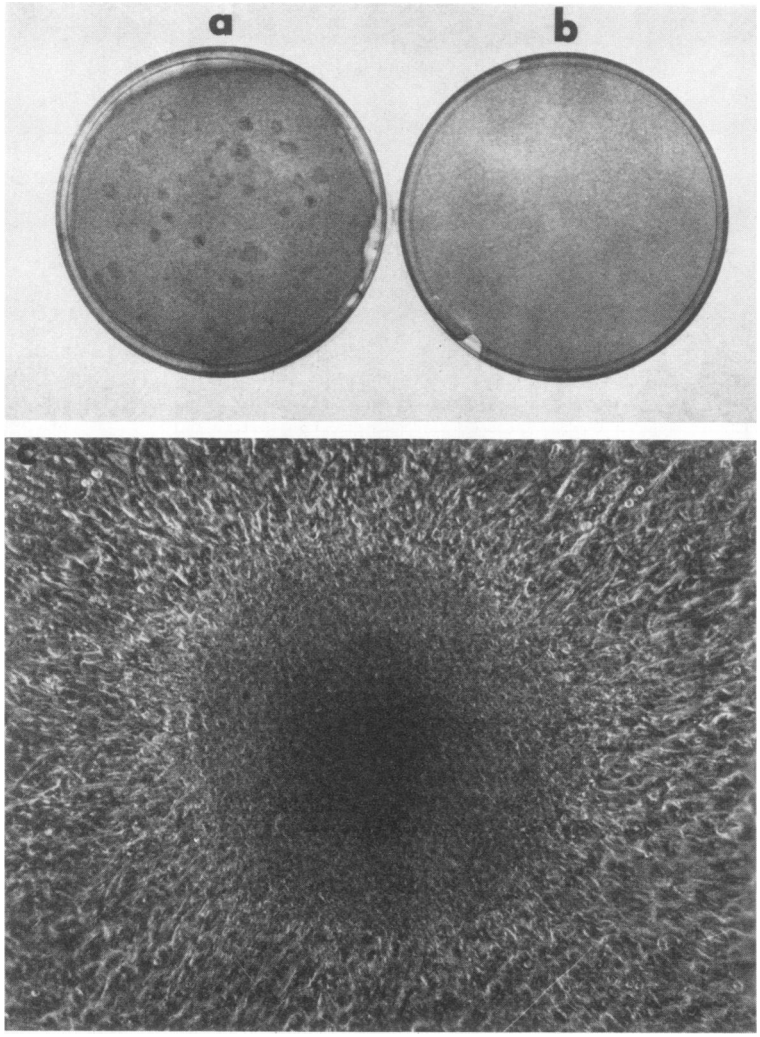

FIG. 2. Morphologically transformed focus induced by pSHPV16d transfection DNA. (a) Dense foci induced by 2 μg of pSHPV16d at 5 weeks after transfection. The cell culture dish was stained with Geimsa stain after cell fixation with methanol. (b) Control NIH 3T3 cell culture at 5 weeks after transfection with pSV2-neo DNA. (c) Typical focus on a monolayer of NIH 3T3 cells as viewed by phase-contrast microscopy (×40) 4 weeks after transfection with pSHPV16d.

but not within pSV2-neo and generated a 9.0-kbp fragment, two identical 4.7-kbp fragments, and one 3.3-kbp fragment. The six cell lines examined contained all three characteristic fragments expected from digestion of recombinant pSHPV16d (data not shown). *Hin*dII or *Taq*I, which cleaved HPV 16 DNA once, also generated patterns characteristic of pSHPV16d (data not shown). The integrity of HPV 16 sequences and their association with HMr chromosomal DNA suggest that the HPV 16 DNA exists in a head-to-tail multimeric state. The stability of HPV 16 DNA sequences in transformed cells was examined by comparing the *Bam*HI digestion patterns of cells that differed in culture age by 20 population doublings. No significant differences in digestion pattern or copy number were observed (Fig. 4a and b). Therefore, most of the rearranged HPV 16 DNA sequences were stable. The possible existence of stable extrachromosomal HPV 16 DNA molecules was investigated by using Hirt extractions (9). Only a small fraction (between 0.05 and 1%) of the HPV 16 DNA sequences in the transformed cell lines was detected in the Hirt supernatants. However, a Southern blot analysis of the DNA in the Hirt supernatants gave equivocal results as to whether this HPV 16 DNA was extrachromosomal or was associated with contaminating genomic DNA.

Expression of viral RNA. Three virus-specific gene messages were expressed in SP3T3-3 cells, as determined by a Northern blot analysis (Fig. 5). These cells contained only a few copies of intact HPV 16 DNA. A 0.47-kilobase message was the major product, and bands at 1.8 and 0.26 kilobase were also detected. No hybridization to HPV 16 DNA was observed in RNA prepared from control NIH 3T3 cells. Virus-specific messages of similar sizes were detected in the other transformed lines (data not shown).

Tumorigenicity of the transformed cells. Because four of six cell lines (SP3T3-1, SP3T3-2, SP3T3-4, and SP3T3-5) harbored many copies of HPV 16 DNA while the other two cell lines (SP3T3-3 and SP3T3-6) harbored only a few copies, the correlation between copy number and transformed phenotype was examined. SP3T3-1, SP3T3-2, SP3T3-3, SP3T3-5, and SP3T3-6 cells were injected into athymic nude

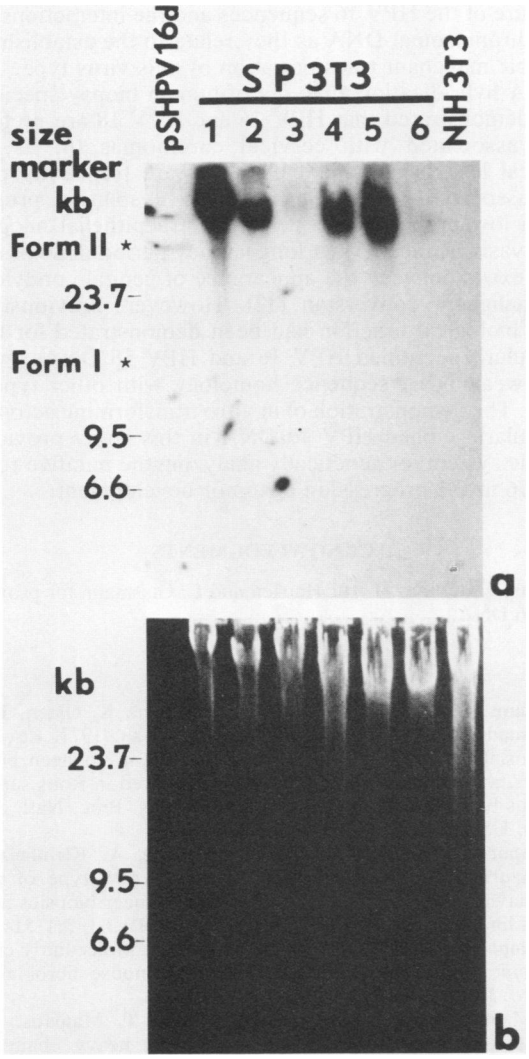

FIG. 3. Detection of HPV 16 DNA sequences in transformed cells. (a) Total cellular HMr DNA was extracted from cultures derived from individual foci of SP3T3-1, SP3T3-2, SP3T3-3, SP3T3-4, SP3T3-5, and SP3T3-6. HMr DNA from NIH 3T3 cells was used as a control; 10 pg of undigested pSHPV16d DNA was electrophoresed in parallel, and lambda phage DNA digested with *Hin*dIII was used as a molecular weight marker. The DNAs (10 μg) were electrophoresed in 0.5% agarose and subjected to Southern blotting. The filters were hybridized with 7.9-kilobase (kb) *Bam*HI HPV 16 DNA probes. (b) Gel before blotting, stained with 0.5 μg of ethidium bromide per ml for 30 min and destained in electrophoresis buffer for 45 min.

mice (nu/nu; 8×10^6 cells per animal) 1 day after the mice had been X-irradiated (350 R). Progressive tumor formation was observed in all of the transformants. The rapid development of spindle cell fibrosarcomas, reaching an average size of 1.5 to 2.5 cm within 10 days, indicated that the injected cells possessed the fully transformed state. Moreover, the tumor growth rates were similar and independent of the number of copies of HPV 16 DNA, demonstrating that copy number is unimportant for tumorigenic potential. When cells from G418-resistant flat colonies obtained by transfection with pSHPV16d were inoculated into nude mice, no tumors were observed at 3 months postinjection. This indi-

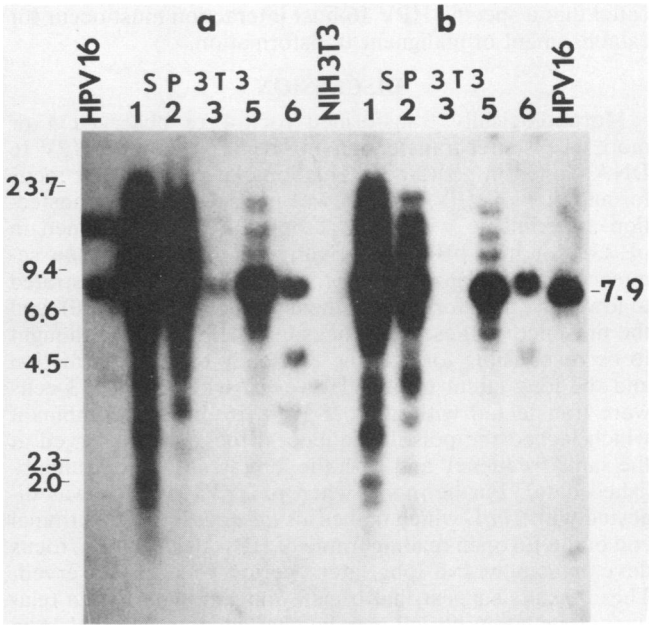

FIG. 4. Stability of HPV 16 DNA in the transformed cell lines. Total cellular DNA was digested and probed for the full length of 7.9-kilobase HPV 16 DNA after 0.7% agarose gel electrophoresis and Southern blot analysis. (a) Total DNA extracted from cultures of transformed lines at 20 population doublings after focus isolation. (b) Total DNA extracted from cultures at 40 population doublings after focus isolation.

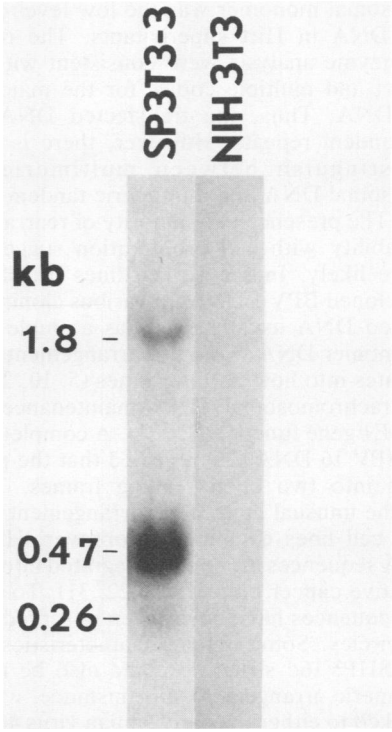

FIG. 5. Blot hybridization of polyadenylated RNA (5 μg per lane) prepared from transformed cell line SP3T3-3 and non-transfected NIH 3T3 cells by using ^{32}P-labeled 7.9-kbp HPV 16 DNA. kb, Kilobase. RNA sizes were determined by using denatured λ-*Hin*dIII and 123-base pair ladder DNA fragments (13).

cated that a specific HPV 16-host interaction must occur for establishment of malignant transformation.

DISCUSSION

Morphologically transformed foci were observed 4 or more weeks after transfection of NIH 3T3 cells with HPV 16 DNA cloned in pSV2-neo. This long latent period of focus formation with HPV 16 DNA was not necessary in transfection experiments with HPV 1 or HPV 5 DNA cloned in pBR322 or in a pBR322 derivative lacking the poison sequences (14, 29). Poison sequences have been demonstrated to lower the transformation efficiencies of BPV 1 (5, 20), and the presence of these sequences in pSHPV16d was thought to be responsible for the low efficiency of focus formation and the long latent period. However, when NIH 3T3 cells were transfected with another HPV 16 dimer recombinant which lacked the poison sequences, foci were observed at the same frequency and with the long latent period (unpublished data). Furthermore, when pSHPV16d DNA was digested with *Taq*I, which makes a single cut in the C-terminal end of the E6 open reading frame of HPV 16 DNA (23), focus development with a long latent period was still observed. These results suggest that transforming activity with a relatively long latent period may be characteristic of HPV 16 in rodent cell lines.

Southern and Northern blot analyses demonstrated that the HPV 16 sequence was present within the cells and expressed at the RNA level. An analysis of undigested DNA from HPV 16-transformed lines showed that the HPV DNA sequences comigrated with high-molecular-weight chromosomal DNA and not as form I or form II plasmid DNA (where extrachromosomal monomeric plasmids are expected). A further indication that HPV 16 is not an extrachromosomal monomer was the low level of recovery of HPV 16 DNA in Hirt supernatants. The results of a restriction enzyme analysis were consistent with the presence of intact and multiple copies for the majority of the pSHPV16d DNA. Thus, the transfected DNA exists as multimeric tandem repeats. However, there is insufficient data to distinguish between multimeric circular extrachromosomal DNA and multimeric tandem integration of the DNA. The presence of a minority of rearranged forms and their stability with cell proliferation suggest that the latter is more likely. In mouse cell lines transfected with molecularly cloned BPV 1 DNA (in various cloning vectors), the transfected DNA usually exists as a stable extrachromosomal monomer DNA without rearrangement (5, 20) and rarely integrates into host chromosomes (5, 10, 20). In BPV 1 stable extrachromosomal DNA maintenance has been attributed to E1 gene function (15, 21). A complete sequence analysis of HPV 16 DNA (23) revealed that the putative E1 gene is split into two open reading frames. This might account for the unusual multimeric arrangement of HPV 16 DNA in the cell lines examined. Moreover, HPV 16 and HPV 18 DNA sequences are found integrated into host DNA in many positive cancer biopsies (6, 22, 31). To our knowledge, these sequences have never been observed to exist as monomeric circles. Some unique characteristics of the recombinant pSHPV16d structure could also be responsible for the multimeric arrangement. For instance, when BPV 1 DNA was linked to either the early simian virus 40 gene (12), PBR322 (5, 10, 11, 20, 21), or the neomycin resistance gene (10), extrachromosomal DNA was found in the transformants, whereas when it was linked to the HLA gene, integration occurred after transfection (4). Further analysis will be required to identify the possible relationship between the structure of the HPV 16 sequences and the interactions with host chromosomal DNA as they relate to the establishment of stable malignant transformation by this virus type.

DNA hybridization analyses of human biopsy specimens have demonstrated that HPV 16 and HPV 18 are preferentially associated with cervical carcinomas (6, 22, 31). Cervical lesions with mild dysplasia are frequently papilloma-associated condylomas. These dysplasias progress with a low probability to cervical intraepithelial neoplasia and invasive cancer (7). A long latency period of 10 or more years exists between the appearance of genital condylomas and malignant conversion (32). However, previously no direct biological function had been demonstrated for these molecularly identified HPV 16 and HPV 18 DNAs, which show weak DNA sequence homology with other types of HPVs. The demonstration of in vitro transforming activity of molecularly cloned HPV 16 DNA in this study provides a suitable system for genetically analyzing the putative role of HPV 16 in the progression of tumor development.

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