Transformation of Human Fibroblasts and Keratinocytes with Human Papillomavirus Type 16 DNA

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Human keratinocytes and fibroblasts isolated from foreskin were transformed by transfection with recombinant human papillomavirus type 16 (HPV16) DNA. The transformed cells exhibited an extended (fibroblasts) or indefinite (keratinocytes) life-span compared with that of normal controls. In addition, HS27, a human fibroblast cell line previously transfected with origin-defective simian virus 40, was successfully transfected. HPV16 sequences were stably maintained in the cells, and extensive amplification and rearrangements occurred with continuous culturing. Moreover, both fibroblasts and keratinocytes expressed several specific HPV16 mRNAs. Because HPV16-transfected cells had viral transcripts and because transfection with the vector alone did not extend the life-span of the cells, it is likely that the virus was responsible for the indefinite life-span. Transfected fibroblast and keratinocyte lines will be useful for investigating the molecular biology of HPV16 and the interactions between the viral DNA and the human genome. Moreover, transfected keratinocytes provide a model for analyzing the effects of HPV16 on the differentiation properties of human epithelial cells.

Papillomaviruses are known etiologic agents of benign proliferation of skin and mucosa histologically classified as papillomas or fibropapillomas (reviewed in references 16, 18, and 30). At least 40 different types of human papillomaviruses (HPV) have been identified (reviewed in reference 16); some are associated with a specific pattern of macroscopic and microscopic lesions (5, 29). HPV are suspected to be involved in the development of genital cancers (30). DNA analysis of these tumors and tumor-derived cell lines has demonstrated the presence of HPV (1, 5, 7–9, 20, 21, 28). Moreover, in an in vivo model, dysplastic changes have been obtained in human normal cervical tissue infected with HPV type 11 (HPV11) and transplanted under a mouse kidney capsule (10).

Different HPV types are associated with specific tumors. In particular, HPV16 and HPV18 (1, 7) are frequently found in cervical carcinomas and in cervical carcinoma-derived cell lines. Thus, circumstantial evidence, but no direct proof, exists for HPV involvement in the pathogenesis of cervical cancer. Supportive evidence for the transforming capacity of HPV16 has been the neoplastic transformation of NIH 3T3 cells transfected with cloned HPV16 DNA (27) or genomic DNA containing HPV16 derived from a human cervical carcinoma (25). To define the role of HPV in the development of human cancer a model must be established to study the interactions between HPV and human cells.

The sequence of events related to HPV infection and proliferation in human tissues is not understood because these viruses proliferate only in fully differentiated cells in vivo (3, 4, 11). Nevertheless, the transformation events are probably related to interactions between the viral DNA and the cellular genome; i.e., viral products could specifically interfere with cellular growth control functions, or integration of the viral sequences into the host DNA could lead to activation of cellular oncogenes or alterations in gene expression. These interactions can also be studied in a system in which recombinant HPV is transfected into human cells.

In the present study, normal human fibroblasts (HFb), human HS27 fibroblasts, a permanent line obtained by transfection with ori⁻ simian virus 40 (17), and normal human keratinocytes (HKc) were transfected with plasmids containing the complete HPV16 genome and, as a selectable marker, a gene for resistance to G418. HS27 fibroblasts were used to determine whether an indefinite life-span per se is a prerequisite for maintenance of HPV in the cells or for malignancy. In these cells, HPV16 sequences were maintained and amplified in the absence of antibiotic selection, and a variety of specific HPV16 mRNAs were expressed. A considerable extension of the life-span is associated with the presence of HPV16 sequences in the transfected cells. The transformed cells provide a model for studying the molecular biology of HPV16 in human cells and for assessing its putative role in carcinogenesis.

MATERIALS AND METHODS

Cell cultures. Two types of media were used for cell culturing: a serum supplemented modified Eagle minimal essential medium (MEM) for HFb and HS27 and a serum-free medium for HKc. The MEM was supplemented with sodium pyruvate (110 mg/liter) L-serine (21 mg/liter), aspartic acid (13.3 mg/liter), penicillin (100 U/ml), streptomycin (100 µg/ml), and fetal bovine serum (10%). The HKc culture medium was a modification of the basal nutrient medium MCDB153 described by Boyce and Ham (2). MCDB153 amino acid concentrations were increased to twofold except for histidine, isoleucine, lysine, methionine, tryptophan, and tyrosine, which were threefold. This basal medium, MCDB153-LB, was supplemented with epidermal growth factor (10 ng/ml), insulin (5 µg/ml), hydrocortisone (1.4 × 10⁻⁷ M), ethanolamine (10⁻⁴ M), phosphoethanolamine (10⁻⁴ M), transferrin (10 µg/ml), bovine pituitary extract (70 µg of protein per ml), and triiodothyronine (10⁻⁸ M). The supplemented medium is referred to as complete HKc medium.

HKc and HFb were isolated from the foreskin of newborns by a collagenase float technique. A 1% stock solution of collagenase type II (Worthington Diagnostics) was made
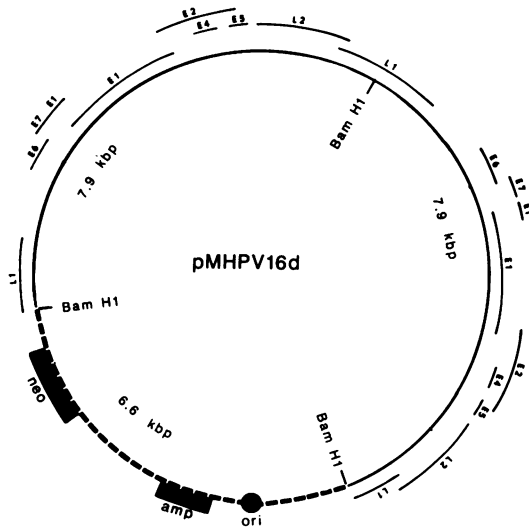
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FIG. 1. Structure of the recombinant HPV16 DNA pMHPV16d.
A head-to-tail dimer of HPV16 DNA (——) was cloned into the
BamHI site of the vector pdMMTneo (- - -). The lines outside the
circle indicate the position of the open reading frames in the HPV16
genome. The molecular size of the viral and the vector sequences
are indicated. kbp, Kilobase pairs; neo, neomycin resistance gene;
amp, ampicillin resistance gene; ori, bacteria replication origin.

in MEM and diluted 1:4 in MCDB153-LB. Individual fore-
skins were suspended dermis side down in 2 to 2.5 ml of
medium in culture dishes (diameter, 35 mm) and incubated
overnight in a humidified, 5% CO_2 incubator at 37°C. The
next morning the epidermis was separated from the dermis,
washed with basal medium, perturbated by pipetting, cen-
trifuged, suspended, and plated in complete medium in a
100-mm culture dish. The plating efficiency of primary HKc
was approximately 1%; after plating 10^6 cells per dish,
confluency occurred in approximately 10 days. Because
there were about 10^7 cells per confluent dish, the primary
HKc achieved 10 population doublings (PD). Subsequently,
the cells were subcultured 1:15 every 6 days; the plating
efficiency of transfected and nontransfected HKc was about
50%. The number of PD per subculture was conservatively
estimated as four.

The dermis in the original culture medium was broken up
if necessary, diluted with MEM to 10 to 15 ml, pipetted again
to break up existing clumps, centrifuged, suspended and
washed in Dulbecco phosphate-buffered saline, and plated in
supplemented MEM. HFb were trypsinized and routinely
subcultured 1:5 every 5 to 6 days. Thus the number of PD
per subculture was taken as 2.2, whereas about 5 PD were
estimated for the primary HFb. HFb were used for transfec-
tion between 10 and 15 PD, and HKc were used for trans-
fection between 14 and 18 PD.

Transfection and selection of transfected cells. HPV16 (23)
was subcloned into the BamHI site of pdMMTneo (12) as a
single insert and as a head-to-tail HPV16 DNA dimer. The
recombinant HPV16 DNAs, pMHPV16s and pMHPV16d,
contained one or two copies, respectively, of the complete
HPV16 genome, with the same orientation with respect to
the vector sequences, and a selectable marker gene for
neomycin (G418) resistance in human cells. The structure of
pMHPV16d is shown in Fig. 1. Both HFb and HKc were
transfected by using modifications of the calcium phosphate
coprecipitation method (26). HFb were transfected 24 h after

plating in 35-mm dishes with 0.5 μg of pMHPV16s,
pMHPV16d, or pdMMTneo only, with normal HFb DNA as
the carrier. Cells were washed 24 h later three times with
phosphate-buffered saline and refed with fresh MEM with
serum. On day 2 after transfection, the cells were trypsinized
and plated for G418 selection (300 μg/ml for HS27 and 100
μg/ml for HFb). The cells were refed with fresh G418-
containing medium every 4 days. G418-resistant colonies
were visible after 20 days in pMHPV16s- and pMHPV16d-
transfected cells, as well as in the cells transfected with
pdMMTneo. No colonies developed in nontransfected cells
treated with G418 for the same time interval.

Because of the low calcium concentration and the high
phosphate content, HKc medium was not suitable for trans-
fection with the calcium phosphate-DNA precipitation
method. Therefore the procedure was modified as suggested
by R. T. Su (personal communication): HKc plated in 35-mm
dishes were refed with diluted Dulbecco MEM (Dulbecco
MEM to water, 6:5.5). Dulbecco MEM was diluted to obtain
a lower osmolarity, more similar to that of HKc complete
medium. After 10 min at room temperature, 0.5 ml of a
suspension of calcium phosphate-DNA precipitate contain-
ing 0.5 μg of plasmid DNA was added to each 35-mm dish.
After incubation for 4 h at 37°C, the cells were washed three
times with complete medium and incubated for 48 h. The
medium was changed again, and complete medium contain-
ing 100 μg of G418 per ml was added. G418 selection was
carried out only for 48 h, because longer exposures to the
drug resulted in complete loss of the cells. Resistant colonies
were visible in the transfected cultures 10 days after removal
of the drug; no survivors were present in the nontransfected
HKc treated with G418 for the same time interval.

DNA and RNA analysis. Restriction endonuclease diges-
tions were performed as recommended by the manufacturer
(Bethesda Research Laboratories). DNA electrophoresis
and Southern blotting were performed according to standard
procedures (15). Hybridization with nick translated ^{32}P-
labeled DNA (10^8 cpm/μg) specific for HPV16 was per-
formed under stringent conditions (50% formamide–10%
dextran sulfate–5× Denhardt solution–1% sodium dodecyl
sulfate at 42°C for 15 h). The filters were washed twice in 2×
SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium
citrate)–0.5% sodium dodecyl sulfate at room temperature
for 30 min and then twice in 0.1× SSC–0.1% sodium dodecyl
sulfate at 65°C for 30 min and exposed to XAR-2 film
(Eastman Kodak Co.) for 6 to 24 h at −72°C with an
enhancing screen.

RNA was extracted by using the guanidinium-cesium
chloride method (15), and the polyadenylated RNA fraction
was enriched by oligo(dT)-cellulose column chromatogra-
phy. RNA electrophoresis was performed in 1.2% agarose
gel containing 2.2 M formaldehyde. RNA was transferred to
a Gene-Screen filter (New England Nuclear) by electroblot-
ting and hybridized as described for DNA.

RESULTS

The permanent line HS27 and early subpassages of HFb
and HKc were transfected with recombinant HPV16 DNA
(pMHPV16s or pMHPV16d or the vector alone) and selected
for resistance to the antibiotic G418. Within 3 weeks of
selection, approximately 10 to 20 G418-resistant HS27 colo-
nies transfected with pMHPV16s were observed. Cells from
approximately 10 colonies of a single dish were pooled and
maintained under G418 selection for eight PD. This culture
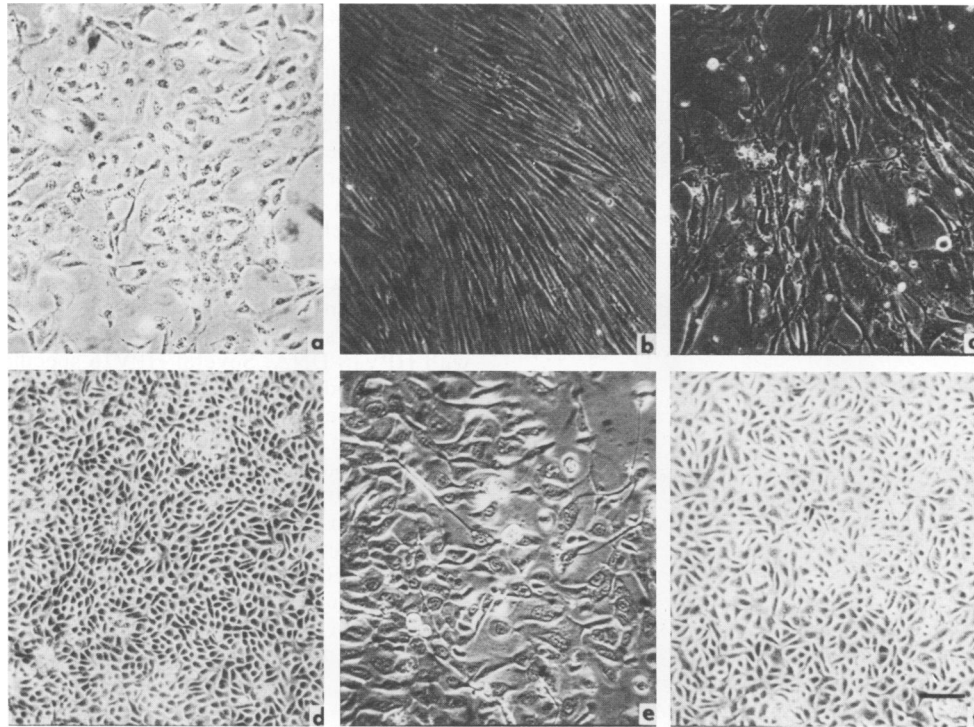was subcultivated to two dishes; only one was maintained on

FIG. 2. Morphology of normal and HPV16-transfected human cells. (a) Nontransfected HS27; (b) normal HFb at 25 PD; (c) HFb/HPV16d-1 at 90 PD; (d) normal HKc at 10 PD; (e) normal HKc after 40 PD; (f) HKc/HPV16d-1 after 65 PD. Bar, 100 μm.

G418. No obvious differences in either morphology (Fig. 2a) or growth rate between HPV16-transfected HS27 cells and nontransfected parental cells were observed.

HFb cells were transfected with pMHPV16d. Approximately 5 to 10 G418-resistant colonies were observed in 3 weeks. HFb/HPV16d-1 cells, pooled from five colonies in one dish, were maintained in selective medium until they reached 50 PD. Subsequently, they were grown without G418. After PD 100, cell growth was not measurable, and in about 30 days most cells had degenerated. However, a few growing colonies remained which were pooled; these reached 145 PD. The cell morphology at confluency was more random and irregular than that of nontransfected HFb (Fig. 2b and c).

Transfection of HFb was repeated with cells from two

TABLE 1. Extended life-span of HPV16-transfected human cells

Transfected cells	PD[a]
HFb/pdMMTneo-1	55
HFb/pdMMtneo-2	55
HKc/pdMMTneo-1	40
HFb/HPV16s-1	85
HFb/HPV16s-2	85
HFb/HPV16d-1	145
HFb/HPV16d-2	85
HFb/HPV16d-3	85
HKc/HPV16d-1	200

[a] Maximum number of PD reached by the pdMMTneo-transfected controls. Fibroblasts were frozen at the indicated number of doublings. HKc were still growing as of November 1986.

other individuals. Both pMHPV16d and pMPHV16s recombinant DNAs were used, as well as the vector sequence pdMMTneo. In all cases, 10 to 20 G418-resistant colonies per μg of transfecting DNA were observed, and cells from individual dishes were collected and grown continuously. Cells transfected with pdMMTneo (HFb/pdMMTneo-1 and HFb/pdMMTneo-2) senesced at 55 PD as did nontransfected HFb, whereas cells transfected with HPV16 plasmids (HFb/HPV16s and HFb/HPV16d) continued to grow (Table 1).

HKc were transfected with either pMHPV16d or pdMMTneo. At 10 days after G418 selection (100 μg/ml of medium for 48 h), 6 to 12 colonies per μg of DNA were observed. HKc transfected with pdMMTneo did not survive beyond three PD postselection, whereas pMHPV16d-transfected HKc continued to multiply (Table 1) beyond 200 PD. Moreover, the normal HKc continued to differentiate until senescence (PD 40), whereas the HPV16-transfected HKc maintained a morphology similar to that of undifferentiated normal HKc (Fig. 2d to f). Thus, HPV16-transfected normal HFb and Hkc acquired an extended life-span.

To determine whether HPV16 sequences were lost in the absence of selection, HPV16-transfected HS27 cells were grown with or without G418 for five PD. DNA isolated from these cells was digested with BamHI, which released the HPV16 sequences from the vector sequences. DNA was analyzed by Southern blot analysis after gel electrophoresis, and the filters were probed with the entire HPV16 genome. The expected 7.9-kilobase-pair (kbp) BamHI fragment was observed in cells grown in either the presence or absence of G418 (Fig. 3). Moreover, some amplification of HPV16 sequences occurred in the absence of G418 selection.

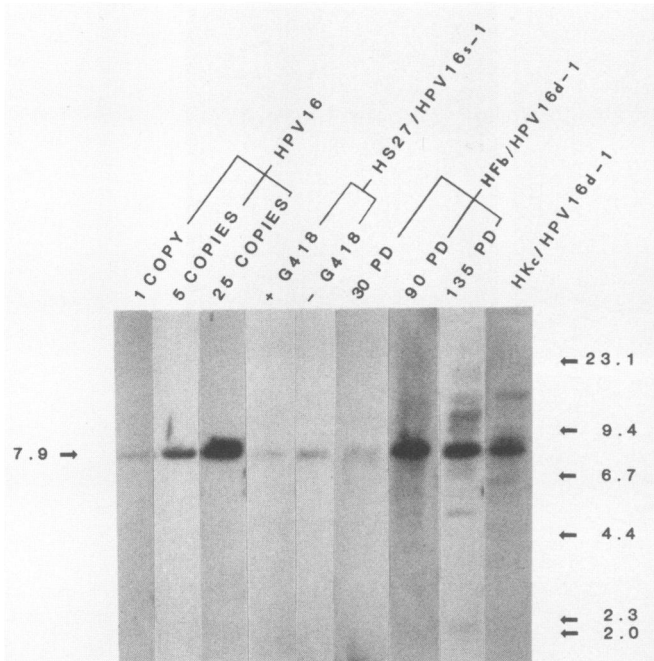DNA from HFb/HPV16d-1 cells isolated at different times

FIG. 3. Amplification and rearrangements of HPV16 as a result of culture in the absence of G418. Cellular DNA (10 µg) was digested with BamHI, elecrophoresed in 0.7% agarose, and electroblotted on a Gene-Screen. The filter was hybridized with the 7.9-kbp HPV16 BamHI fragment. The position of the molecular size markers (in kilobase pairs) is on the right. The arrow at the left side indicates the predicted position of the 7.9-kbp HPV16 fragment. The first three lanes show the results of the reconstruction experiment, performed by digesting with BamHI amounts of plasmid DNA representing 1, 5, and 25 copies per genome mixed with DNA from normal HFb (10 µg).

postselection was analyzed for the stability of HPV16 DNA sequences with respect to the increased life-span of the cells. DNA was extracted from cells at PD 30 which were still under G418 selection, from cells at PD 90 which were cultured without G418 for approximately 40 PD, and from cells at PD 135 which had survived the senescence of the general population. To estimate the number of HPV16 DNA copies in the transfected lines, a reconstruction experiment was performed concurrently by mixing amounts of the plasmid DNA equivalent to 1, 5, or 25 HPV16 copies per cell with DNA from nontransfected HFb. The DNA was digested with BamHI. The lack of additional HPV16 bands in the reconstructed samples demonstrates the completeness of BamHI digestion (Fig. 3). At 30 PD, approximately one to two HPV16 copies were observed as intact 7.9-kbp BamHI fragments (Fig. 3). The same HPV16 DNA species were observed in DNA extracted from cells at PD 90; however, the number of copies of HPV16 DNA increased to 10 to 20. With longer exposures, a few rearranged bands also appeared (data not shown). Thus, amplification of HPV16 sequences occurred in the absence of continued G418 selection. Finally, HPV16-related sequences were also observed in the cells from PD 135. However, extensive rearrangements occurred, and many BamHI fragments had molecular weights higher than that of the original intact HPV16 sequence (Fig. 3). It is likely that the HPV16 sequences integrated into the genomic DNA.

The state of HPV16 sequences in HKc/HPV16d-1 cells was also analyzed at 60 PD. As with HFb in the absence of

continued G418 selection, multiple copies of HPV16, about 10 per cell, were observed. The majority maintained the same structure as that of the original plasmid DNA, but some rearrangements also occurred (Fig. 3). Moreover, the predominance of these rearrangements as higher-molecular-weight species suggests the presence of a subpopulation of integrated forms in these cells.

RNA was extracted from HFb/HPV16d-1 and HKc/HPV16d-1 cells to determine whether the HPV16 sequences were being expressed. For comparison, RNA from NIH 3T3 cells transfected with the same plasmid and selected for G418 resistance was also analyzed. [Poly(A)$^+$]mRNA was isolated and subjected to Northern blot analysis and probed with HPV16 DNA. Several HPV16-specific mRNA species were detected in all of the cell types (Fig. 4). HFb/HPV16d-1 cells contained five major mRNA species: 1.5, 1.8, 3.5, 4.2, and 5.5 kb. Less HPV16 mRNA was detected in HKc/HPV16d-1 cells, but the same mRNA species except for the 1.5-kb band were found. Similar mRNA species were also found in HPV16-transfected NIH 3T3 cells. However, there was less expression of 3.5-kb species relative to that of the other mRNA species. Moreover, mRNA species were also observed in HS27-transfected cells, but in a much lower amount (data not shown). Therefore, the transfected human cells stably maintained and expressed HPV16-specific sequences.

DISCUSSION

Evidence to date indicates that the carcinogenesis process involves multiple stages. One stage must involve indefinite survival to express the cancer phenotype.
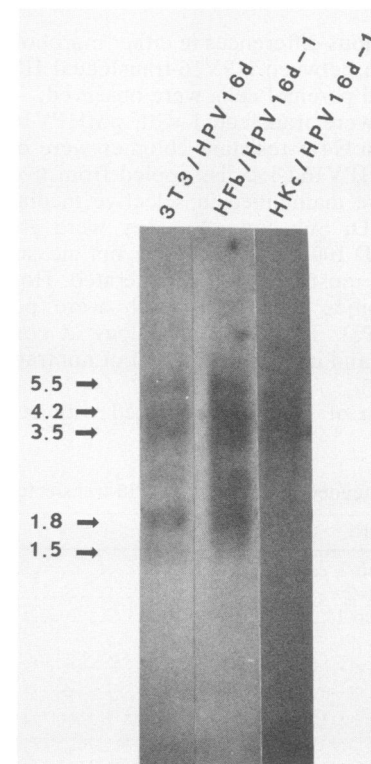

FIG. 4. Expression of HPV16 in transfected cells. [Poly(A)$^+$] RNA was isolated, subjected to Northern blot analysis, and hybridized with the 7.9-kbp HPV16 BamHI fragment. The size (in kilobases) of the detected bands, as indicated on the left, was determined relative to the RNA markers (Bethesda Research Laboratories).

The outstanding characteristic of normal human cells transfected with HPV16 DNA is an extended life-span. HFb/HPV16d lines also developed the ability to grow in soft agarose with a 2% efficiency and produced transitory nodules when injected subcutaneously in nude mice (data not shown). The acquisition of an extended or indefinite life-span induced by HPV16 may be an important step in the process of transformation in human cells. Ordinarily, the life-span of carcinogen-initiated human cells fails to extend to cell lines. Even though they may have certain characteristics of neoplasia, their life-span is usually within twofold of nontreated cells (6, 19). Because HPV16-transfected HKc continued to multiply after more than 200 PD, they can be defined as a continuous cell line with an indefinite life-span.

An extended life-span after transfection with HPV16 has particular importance because this is not a general property of all HPV. Normal HKc are not transformed by HPV1 (11). Furthermore, infection of carcinomas with HPV1 or cotransfection with HPV1 and origin-defective simian virus 40 results in either eventual loss of the HPV1 DNA or in a level of transcription of HPV sequences that is lower than that of simian virus 40 (3). Transfection of HS27 (a simian virus 40 line) with HPV16 did not make these cells tumorigenic, although the cells did maintain and express HPV16.

The presence or expression of HPV16 sequences is a requirement for the observed extended life-span of normal human cells transfected with HPV16. Vector sequences alone are ineffective. Maintenance of HPV16 sequences did not depend only on G418 selection of adjoining vector sequences. For example, 50% of the foci derived after transfection of NIH 3T3 cells with the same plasmid lacked the vector sequences (S. Yasumoto et al., unpublished data). Furthermore, amplification and rearrangements of the HPV16 sequences occurred more readily in the absence of G418 in human cells that were cultured in the presence of the drug only for a short interval.

NIH 3T3 cells have been transformed to malignancy by transfection with recombinant HPV16 DNA (27). In this model, transfected nontumorigenic lines were obtained that contain HPV16 sequences, express HPV16 mRNA species, and reveal altered growth properties; malignant subpopulations developed in some of these lines (Yasumoto et al., unpublished data). HPV16-transfected human cells could be compared with the premalignant NIH 3T3 cells. The failure of HPV16 to induce malignancy in human cells with the protocol used compared with NIH 3T3 results reflects the different characteristics of the target cells. Human cells are more resistant than are rodent cells to malignant transformation by carcinogens in vitro (6). Furthermore, biochemical and epidemiological results are consistent with the concept that carcinomas associated with papillomaviruses result from additional carcinogenic insults (16, 24, 30).

HPV16 or HPV18 has been found in the majority of malignant genital tumors and in premalignant cervical dysplastic lesions (7, 16). In benign tumors (condylomas), HPV16 is present only rarely but always as extrachromosomal viral DNA; in carcinomas, HPV16 is usually integrated into host DNA, even though extrachromosomal viral DNA can persist concurrently (8). Because cervical carcinoma tissue and cell lines derived from cervical cancer contain integrated HPV16 or HPV18 sequences, integration could play a role in the transformation process. In HeLa cell chromosomes, four specific sites of integration of HPV18 have been identified by in situ hybridization. Two of them are on normal chromosomes 8 and 9 and two are on abnormal chromosomes deriving from chromosomes 5 and

22. Three of these sites, on chromosomes 8, 9, and 22, correspond to the location of cellular proto-oncogenes c-myc, c-abl, and c-sis, respectively, and occur at or near chromosome fragile sites (N. C. Popescu, S. C. Amsbaugh, and J. A. DiPaolo, Cytogenet. Cell Genet., in press). These results further support the hypothesis that integration of HPV sequences is an important step in the carcinogenesis process.

Extensive rearrangements of HPV16 sequences occur in HPV16-transfected human cells; the presence of bands greater than 7.9-kbp in the BamHI-digested DNA from HPV16-transfected human cell lines could indicate either integration of the viral sequences into the host genome or the persistence of rearranged multimeric extrachromosomal forms.

The expression of HPV sequences is generally found in carcinomas or in carcinoma-derived cell lines positive for HPV DNA; occasionally, however, expression was not detected (13, 14, 22, 28). The requirement for expression of specific HPV16 messages to induce an extended life-span in transfected cells or cervical cancer is not clear. However, HPV16-transfected human cells express a pattern of HPV16 mRNA species very similar to that of transfected NIH 3T3 cells. All the mRNA species observed in NIH 3T3 cells were detected in HFb/HPV16d-1; however, the HKc/HPV16d-1 line had lower amounts and lacked one species. In the NIH 3T3 cells, malignancy is associated with a decreased expression of HPV-specific messages (Yasumoto et al., unpublished data). These observations support the hypothesis that neoplastic transformation might require a specific interaction between the viral DNA and the host genome. Further analysis of RNA from transfected human cells at different stages of their growth will be useful to better understand the effects of changes in the HPV16 expression on the biological properties of these cells.

HKc/HPV16d-1 cultured in serum-free medium differed from normal HKc because they did not undergo differentiation with subculturing. However, as with nontransfected HKc, differentiation was induced by prolonged confluency, lack of growth factors (epidermal growth factor and bovine pituitary extract), or presence of calcium (greater than 0.3 mM) or serum in the medium. However, serum-resistant cells and cultures able to proliferate in the absence of epidermal growth factor and bovine pituitary extract can be obtained by selection in serum-supplemented or in growth factor-free medium at more than 100 PD after transfection. The lack of sensitivity to differentiation stimuli in these selected subpopulations may represent a further step in the transformation process. HPV16-transfected human cell lines provide a suitable model for investigating specific interactions between HPV16 and the host genome and for assessing the role of cocarcinogenic mechanisms in the transformation of human cells.

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