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Involvement of Human Papillomavirus Type 8 Genes E6 and E7 in Transformation and Replication

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We investigated the transforming activity of human papillomavirus type 8 (HPV8) by expressing all early open reading frames from a heterologous promoter in different rodent fibroblast lines. Morphological transformation was observed only in G418-selected mouse C127 and Rat 1 cells containing an intact E6-coding region. E6 of HPV8 did not transform NIH 3T3 cells as did E6 of bovine papillomavirus type 1. C127 cells transformed by E6 were anchorage independent and had a reduced serum requirement but did not form tumors in nude mice. E7 of HPV8 showed no transforming potential, in contrast to E7 of HPV18 and HPV16. It was, however, able to complement an E7 mutant of bovine papillomavirus type 1 with a defect in high-copy-number DNA maintenance. The data indicate that the expression of the HPV8 E6 open reading frame is sufficient to induce morphological transformation in rodent fibroblasts, whereas E7 is involved in the replication of the viral DNA.

Infection with human papillomaviruses (HPVs) is most frequently recognized as resulting in benign epithelial changes. Malignancies may develop on the basis of long persistence, particularly depending on the HPV type and the influence of environmental factors (23). The cell-transforming functions of papillomaviruses have been extensively studied with bovine papillomavirus type 1 (BPV1) and have been assigned to the open reading frames (ORFs) E5 and E6 (27, 28, 38). The E5 gene encodes a 44-amino-acid, hydrophobic, membrane-associated protein which accounts for the major transforming activity (29). The E6 protein was detected both in the nucleus and associated with non-nuclear membranes, and its primary structure reveals possible zinc ion-binding sites (1). The focus-inducing activity of E6 under the control of the BPV1 promoter is rather low. E6 acts synergistically, however, with E5 and is highly active by itself when transcription is initiated from a retroviral long terminal repeat (LTR) (26, 27).

HPVs are usually much less efficient in transforming rodent fibroblasts in vitro. Foci take longer to develop and are smaller in number. In the case of HPV16 and HPV18, which are associated with cervical cancer, transforming activity has been assigned to ORF E7 (14, 36). The E5 ORF may be deleted as a result of integration of viral genomes into host cell DNA, which is frequently observed in malignant cervical tumors (17, 30). In contrast, the E7 ORF is actively transcribed, coding for a 17-kilodalton cytoplasmic phosphoprotein whose amino acid sequence is again reminiscent of zinc finger proteins (34).

A large group of HPVs is associated with epidermodysplasia verruciformis, a lifelong disease characterized by disseminated, flat warts that develop into squamous cell skin carcinomas in 30 to 50% of patients (22). The vast majority of these cancers contain the DNA of HPV5 or HPV8, which were furthermore detected in skin carcinomas of immunosuppressed organ transplant recipients (5). Infection with one of these types therefore seems to imply a high risk for malignant conversion in the course of epidermodysplasia verruciformis. The genomes of HPV5 and HPV8 have been sequenced (11, 39) and showed extensive homology even in the long control regions, which differ considerably between individual HPV types (16). The genome organization of HPV5 and HPV8 was comparable with that of other papillomaviruses, but no ORF with homology to E5 could be identified. Using C127 mouse fibroblasts as the test system, Watts et al. (36) described the transforming activity of HPV5 and HPV1 DNA. The results with HPV1 could not be reproduced by others (8), and G418-resistant C127 cells containing a chimeric HPV8 pSV2 *neo* construct (31) did not appear transformed (P. G. Fuchs, unpublished results). This report describes experiments to identify transforming functions of HPV8 genes driven by a retroviral LTR. ORF E6, but not ORF E7, transformed rodent fibroblasts in vitro. Instead, ORF E7 of HPV8 was able to complement an E7 mutant of BPV1 with a defect in high-copy-number DNA maintenance (19). This points to a direct or indirect role of this ORF in viral DNA replication.

MATERIALS AND METHODS

Cell cultures. Mouse C127 cells (18), NIH 3T3 cells (13), Rat 1 cells (3), and BPV1-transformed C127 B81 cells (15) were maintained in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 10% fetal calf serum, streptomycin (120 μg/ml), and penicillin (120 μg/ml).

The growth rate was determined in minimal essential medium containing either 5 or 0.5% fetal calf serum. Anchorage-independent growth was tested by seeding 50,000 cells in 2-ml soft agarose overlays (0.3% agarose Typ VII in minimal essential medium containing 10% fetal calf serum maintained at 37°C) onto hardened layers of 0.6% agarose in minimal essential medium containing 10% fetal calf serum (4 ml per 60-mm-diameter plate). The cultures were incubated in a CO_2 incubator at 37°C and fed twice weekly with soft agarose overlay. Colonies were scored 2 to 3 weeks later. Tumorigenicity in nude mice was determined after subcutaneous injection of 0.2-ml suspensions of 5×10^5 trypsinized, well-washed cells in isotonic NaCl solution. Animals were examined twice weekly for the appearance of tumors.

Transfections. Transfections were performed by using the calcium phosphate coprecipitation method (12). Briefly,

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each 75-cm^2 cell culture flask received 1 ml of a precipitate containing 138 mM NaCl, 5 mM KCl, 0.7 mM $Na_2HPO_4 \cdot 2H_2O$, 0.1% glucose, 40 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.05]), 6.25 μg of salmon sperm DNA, and appropriate amounts of the DNA to be transfected. Precipitates were allowed to form for 20 to 30 min at room temperature and were added to the cells after complete removal of the medium. Following 20 min at room temperature, 10 ml of complete medium was added. Four hours later, the cells were treated with 1 ml of 17.5% glycerol for 1 min and then washed. Transfected cells were trypsinized 48 h after transfection and expanded at a split ratio of 1 to 6. For selection of drug-resistant cells, G418 (Geneticin; GIBCO Laboratories; active substance, 48% [wt/wt]) was added at a concentration of 400 μg/ml 24 to 48 h later and resistant colonies were pooled, starting at about 3 weeks. Cells were fixed with ethanol and stained with Giemsa stain.

Cellular DNA and RNA extraction. Total cellular DNA was extracted by the protocol of Ebeling et al. (10) with slight modifications. Cells from confluent monolayers were harvested by trypsinization and lysed with 600 μl (per 10^7 cells) of a solution containing 2% sodium lauroyl sarcosinate, 50 mM Tris hydrochloride (pH 7.5), and 10 mM EDTA. Treatment with 250 μg of RNase A per ml for 1 h at 37°C was followed by incubation with 500 μg of proteinase K per ml for 1 h at 37°C. The DNA was extracted twice with phenol-chloroform (1:1) and sheared by passing through a 0.9-mm-gauge needle. After ethanol precipitation, the DNA was redissolved in distilled water and stored at −20°C. Restriction endonuclease digestion, gel electrophoresis, and Southern blot analysis of the DNAs were performed by standard methods (20). Total RNA was prepared by the guanidium thiocyanate method (7). RNA samples were denatured in 50% formamide, 2.2 M formaldehyde in MOPS buffer (20 mM morpholinepropanesulfonic acid [MOPS] [pH 7.0], 7.5 mM sodium acetate, 1 mM EDTA). After being heated for 10 min at 65°C, samples were electrophoresed on 1% agarose gels containing 2.2 M formaldehyde in MOPS buffer. Northern (RNA) blot analysis was performed by the standard protocols (15). Dot blot analysis of cellular DNA was done as previously described (4).

Plasmids. The expression vector pZipNeo-SV(X)-1 (6) was a gift from R. C. Mulligan. HPV8-pZipNeo recombinant plasmids, consisting of different restriction fragments of the HPV8 early region (nucleotides 1 through 5111 [11, 24]) and the eucaryotic retroviral expression vector pZipNeo-SV(X)-1, were constructed as follows. HPV8 DNA was digested with two or more endonucleases to generate appropriate blunt-ended restriction fragments. These were ligated to *Bam*HI octamer linkers, cleaved with *Bam*HI, and cloned into the single *Bam*HI site of pZipNeo-SV(X)-1. Clone pI867 harbors the *Hpa*I-*Xmn*I fragment (1 through 1126), clone pI86 harbors a *Hpa*I-*Hin*cII fragment (1 through 725), clone pI87 harbors a *Hae*III-*Rsa*I fragment (586 through 970), clone pI81 harbors the 2.1-kilobase *Pvu*II fragment of HPV8 (826 through 2935), and clone pI84 harbors a *Hpa*I-*Pvu*II fragment (3160 through 4115). Clone pPF82 contains the directly cloned *Bcl*I-*Bam*HI fragment of HPV8 (2681 through 5111). After ligation and transformation of *Escherichia coli* HB101, clones were isolated on medium containing ampicillin (50 μg/ml) and kanamycin (30 μg/ml). This selects for bacteria with plasmids having an intact kanamycin/neomycin expression unit. Clones were screened by colony hybridization with an HPV8 probe, followed by restriction analysis of analytical plasmid preparations to confirm the correct orientation of the inserts. Clone pI86 turned out to contain a head-to-tail dimer of the *Bam*HI-linkered *Hpa*I-*Hin*cII fragment. The mutant expression vector pI867ME was constructed by inserting a *Xba*I octamer linker at the *Eco*RV site of the HPV8 *Hpa*I-*Xmn*I fragment (1 to 1126). The mutation was confirmed by sequence analysis by the method of Maxam and Gilbert (21).

The BPV1 mutant *dl*576 (19) was used together with pSV2neo (35) in cotransfection assays to obtain G418-resistant cell lines. Clone pdBPV1 (25) was obtained from P. Howley.

RESULTS

Detection of transforming activity of HPV8. To study the transforming potential of HPV8, we subcloned all early ORFs into the *Bam*HI site of the retroviral expression vector pZipNeo-SV(X)-1 (Fig. 1). C127 cells were transfected with the resulting plasmids and were split 24 to 48 h after transfection at a ratio of 1 to 6 into 60-mm-diameter dishes. The cells were then fed twice weekly without further subdividing. No foci appeared during an observation period of 8 weeks.

In parallel, cell lines were established with all constructs by selection for G418 resistance. These cell lines were labeled with C (derived from C127), 8 (for HPV8), and the number of the cloned ORF. Southern blot analysis of DNA from all cell lines revealed the presence of integrated HPV8 constructs at low copy numbers. To test for the integrity of the LTR-HPV8 transcription unit, total DNA was cleaved with *Xba*I that cuts in both LTRs. This gave rise to fragments making up the full-length HPV8 insert linked to adjacent vector sequences. To examine the integrity of the HPV8 DNA insert, it was excised by cleavage with *Bam*HI. An autoradiography of a Southern blot is shown in Fig. 2, lanes 2a. The correct sizes of the bands indicated that both the transcription units and the HPV8 inserts had remained intact. Because of the cloning strategy, the HPV8 insert coding for E2-E4 cannot be excised by *Bam*HI. We therefore used *Sma*I for characterization, which cuts 544 base pairs upstream and 340 base pairs downstream from the insertion site *Bam*HI, and in the insert, resulting in two fragments hybridizing to the HPV8 probe.

The transcription of the viral sequences was demonstrated by Northern blot analysis (Fig. 3) with total RNA from C8 cell lines hybridized to a nick-translated probe representing the HPV8 early region. Different classes of RNA became visible, the largest ones corresponding in size to transcripts that are read through from the 5′ to the 3′ LTR. The minor RNAs are probably generated by splicing events.

Cell lines that contained constructs with HPV8 ORFs E2-E4, E7, and E1 did not exhibit any morphological alterations when compared with the parental C127 cells. Neither did C127 cells established after transfection with the expression vector alone [C127(pZipNeo)]. Cultures of C86 and C867, however, showed areas with morphologically transformed fibroblasts when controlled after relief from G418 selection. The proportion of elongated, spindle-shaped cells increased during the next few passages until the entire culture appeared heavily transformed (Fig. 4). This morphological transformation was reproduced in 11 independent assays.

To ascertain that ORF E6 encodes the transforming protein of HPV8, we constructed a translation termination mutant by inserting a stop codon into the E6 gene of pI867 (Fig. 5). After transfection of C127 cells, 14 G418-resistant lines were established and the presence of the E6 mutant construct was confirmed by Southern blot hybridization

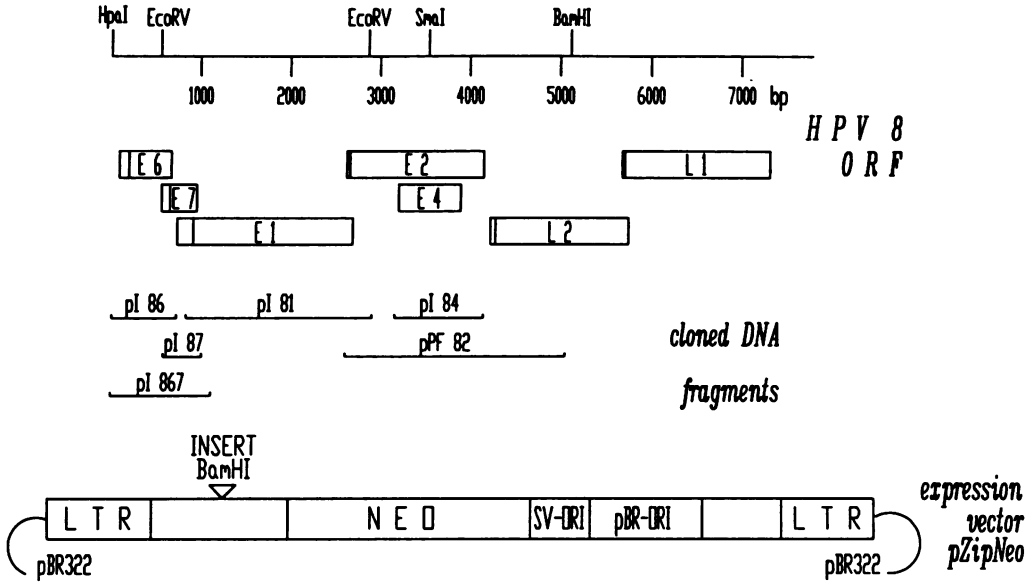

FIG. 1. Construction of expression plasmids containing HPV8 DNA fragments. The positions of the cloned segments are shown beneath the physical map with the ORF of HPV8. Vertical lines within the ORFs represent the first translational start codons. The expression vector pZipNeo-SV(X)-1 is shown at the bottom of the figure. It contains two LTRs of the Moloney murine leukemia virus, a neomycin-phosphotransferase gene (*neo*), and the origins of replication of simian virus 40 (SV-*ori*) and pBR322 (pBR-*ori*). Transcripts of HPV8 DNA inserted at the *Bam*HI site and of the *neo* gene are driven by the LTR promoter and generated by differential splicing. bp, Base pairs.

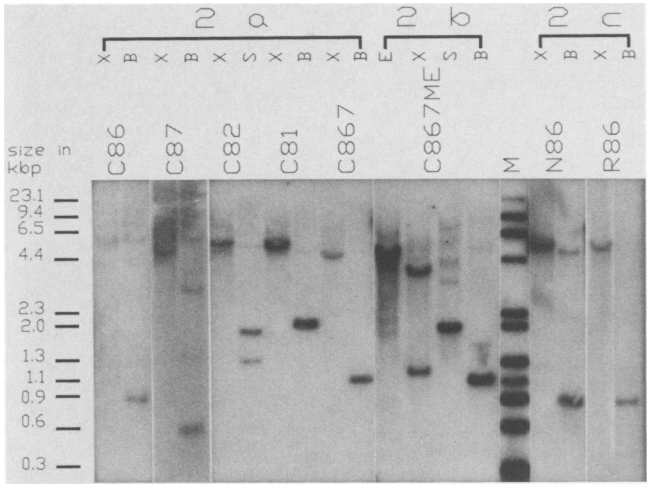

FIG. 2. Southern blot hybridization of ^{32}P-labeled HPV8 DNA (nucleotides 1 to 5111) to total DNA from C127 (C8), NIH 3T3 (N8), and Rat 1 (R8) lines established after transfection with HPV8 expression vectors. Cleavage with *Xba*I (X) releases the entire transcription unit from the 5′ to the 3′ LTR in the range of 4.7 to 6.7 kilobases, depending on the size of the HPV8 insert. *Bam*HI (B) excises the insert DNA with 0.72 (C86, N86, and R86), 0.38 (C87), 2.1 (C81), and 1.1 (C867 and C867ME) kilobases. *Sma*I (S) digestion of C82 generates two HPV8-specific bands with 1.4 and 1.9 kilobases. The identity of the HPV8 E6 mutant line C867ME (lanes 2b) is demonstrated by the existence of only one HPV8-specific *Eco*RV (E) band and one additional *Xba*I (X) cleavage site created by the insertion of a *Xba*I linker at the *Eco*RV site (see Fig. 5). The sizes (in kilobase pairs) of labeled DNA length standards (M) are indicated to the left.

(Fig. 2, lanes 2b). None of these lines showed any signs of morphological transformation. This proves that in vitro transformation is due to E6 and not to E7 and that the transformed phenotype depends on the expression of the E6 protein.

Characteristics of the transformed cells. To investigate additional parameters of transformation in vitro, we examined C86, C867, C81, C87, C127(pZipNeo), and the parental C127 cells and their ability to grow in medium with reduced serum content. The growth curves in medium containing either 5 or 0.5% fetal calf serum are shown in Fig. 6. C86 and C867 cells grew at almost the same rate in both media. In contrast, C81, C87, C127(pZipNeo), and C127 cells had a

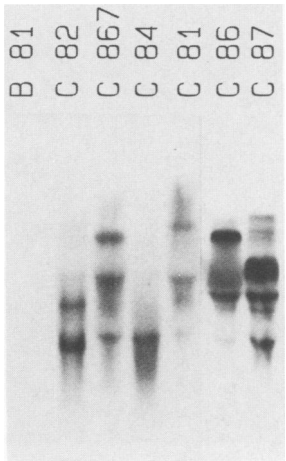

FIG. 3. Northern blot hybridization of ^{32}P-labeled HPV8 DNA (nucleotides 1 to 5111) to total RNA from C127 lines established after transfection with HPV8 expression vectors. BPV1-transformed C127-B81 cells are included as a negative control.

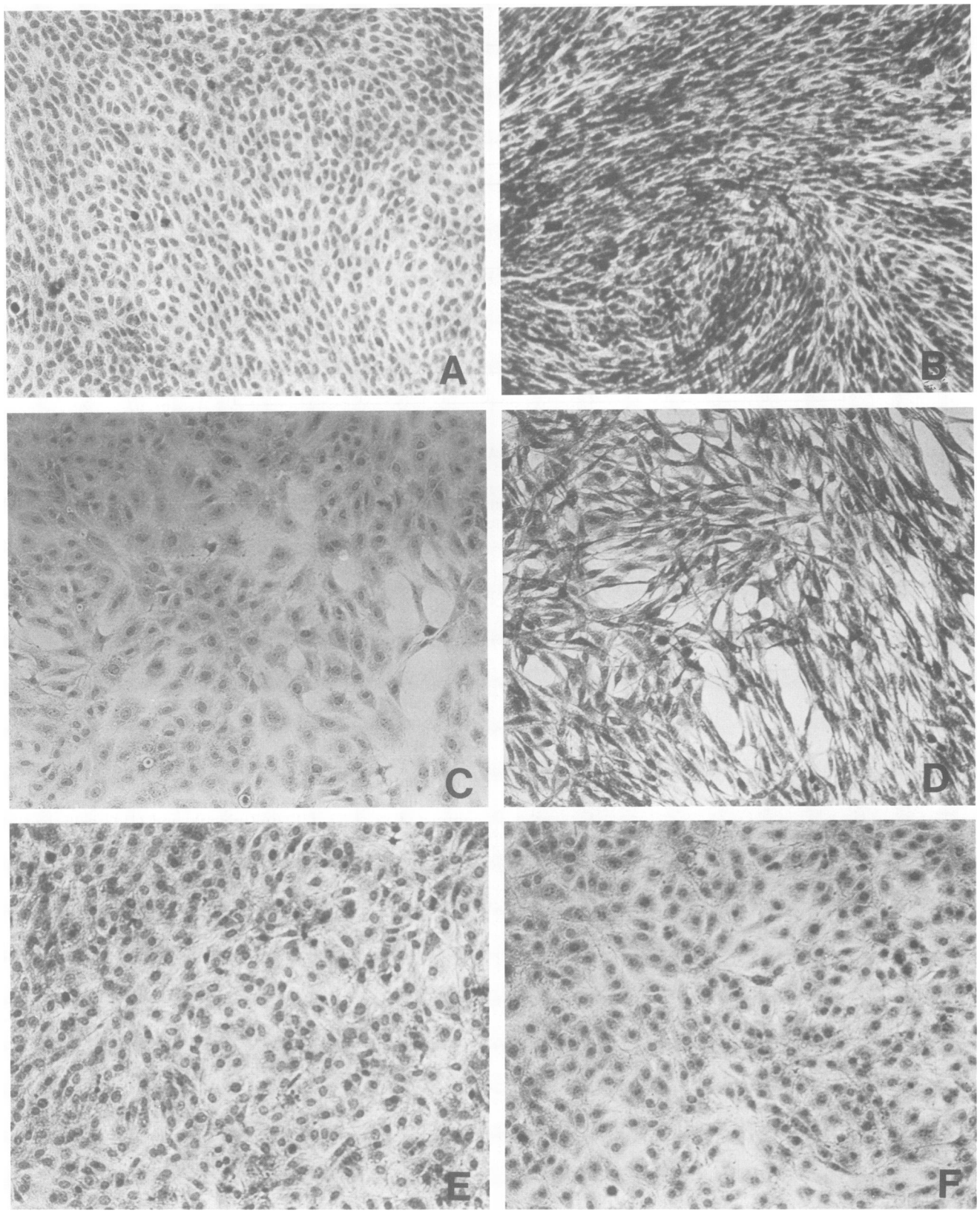

FIG. 4. Morphology of C127 (A and B), Rat 1 (C and D), and NIH 3T3 (E and F) cells established after transfection with pZipNeo-SV(X)-1 (A, C, and E) or pI86 (B, D, and F).

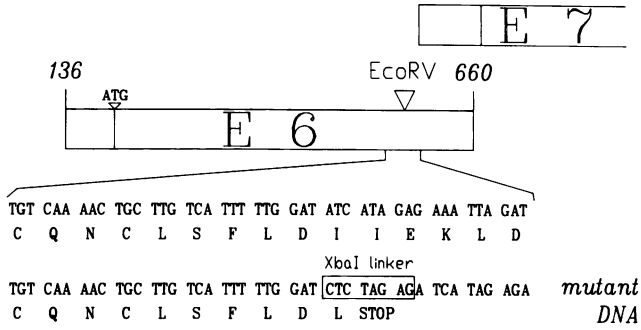

FIG. 5. Construction of an E6 mutant by insertion of a *Xba*I linker at the *Eco*RV site of the *Hpa*I-*Xmn*I fragment of HPV8. The graph shows the position of the *Eco*RV site within ORF E6 (nucleotides 136 through 660). Vertical lines within ORFs E6 and E7 represent the first translational start codon. A part of the deduced amino acid sequence of the wild-type E6 protein and the mutant E6 protein is shown at the bottom of the figure.

more than two times prolonged generation time in medium with 0.5% fetal calf serum.

C867 and C86 cells were also able to grow in soft agarose with cloning efficiencies almost comparable with that of wild-type BPV1-transformed C127 B81 cells (Table 1). The latter formed large colonies within 2 weeks, whereas C867 and C86 clones took about 1 week longer to reach the same size. C81 and C127(pZipNeo) cells did not produce any colonies in soft agarose (Table 1).

Interestingly, C867 and C86 cells were not able to form tumors upon subcutaneous injection in nude mice (0 of 14) during an observation period of 12 weeks, whereas C127 B81 cells produced tumors within 3 weeks.

Transformation of cells of different origin. Both NIH 3T3 and Rat 1 cells were transfected with pZipNeo-SV(X)-1 or with pI86. The cells were tested for the appearance of foci, and G418-resistant lines were established in parallel. Southern blot hybridization revealed the presence of unrecombined HPV8 constructs (Fig. 2, lanes 2c). Foci were detectable neither in NIH 3T3 cells nor in Rat 1 cells. Rat 1-derived R86 cells appeared clearly transformed, whereas NIH 3T3-derived N86 cells did not morphologically differ from pZipNeo-SV(X)-1-transfected controls. The E6 specificity of Rat 1 transformation was proven by the normal morphology of G418-resistant cell cultures established with the E6 termination mutant pI867ME.

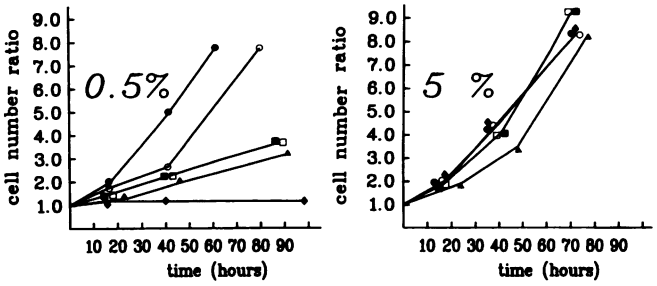

FIG. 6. Growth curves of C127 cells established after transfection with HPV8 expression vectors or with pZipNeo-SV(X)-1 in medium containing either 5 or 0.5% fetal calf serum. The cell lines C81 (◆), C86 (○), C867 (●), C87 (□), C127 pZipNeo (■), and untransfected C127 cells (▲) were plated at 5×10^5 cells per 60-mm-diameter dish and counted directly at distinct time intervals after seeding.

TABLE 1. Anchorage-independent growth of C127 cells established after transfection with HPV8 expression vectors

Cell line	Cloning efficiency	Colony size (mm)
B81(BPV1)[a]	5.3	0.3
C86	6.4	0.25
C867	2.6	0.25
C87	<0.1	0.1
C81	0	
C127(pZipNeo)	0	

[a] Carrying wild-type BPV1.

Complementation of a low-copy-number mutant of BPV1 by E7 of HPV8. Having observed that ORF E7 of HPV8 had no transforming capacity in contrast to E7 of HPV16 and HPV18, we asked for an activity comparable with the E7 function of BPV1. To this end, we performed complementation assays with the BPV1 mutant *dl*576, which carries a 4-base-pair deletion in ORF E7. This mutant replicates extrachromosomally with one to five copies per cell, as described previously by Lusky and Botchan (19). We cotransfected C127 cells with BPV1 *dl*576 and the constructs pI86, pI87, or pI867 and established cell lines after selection for G418 resistance. For control purposes, G418-resistant cell lines were obtained after cotransfection with BPV1 *dl*576 and pSV2neo. Total cellular DNA was extracted at different passages, and the state and copy number of the BPV1 mutant genome were determined by Southern and dot blot analyses. The copy number of extrachromosomal *dl*576 DNA in HPV8 ORF E7 and E6-E7-expressing cells was in the range of 25 to 45 per cell, which significantly exceeds the average of seven copies in cells expressing E6 or no HPV8 gene at all (Fig. 7). The copy number was still lower than in wild-type BPV1-transfected C127 cells (150 to 200 pdBPV1 genome equivalents per cell), indicating that HPV8 E7 achieves only a partial complementation of the high-copy-number defect of *dl*576.

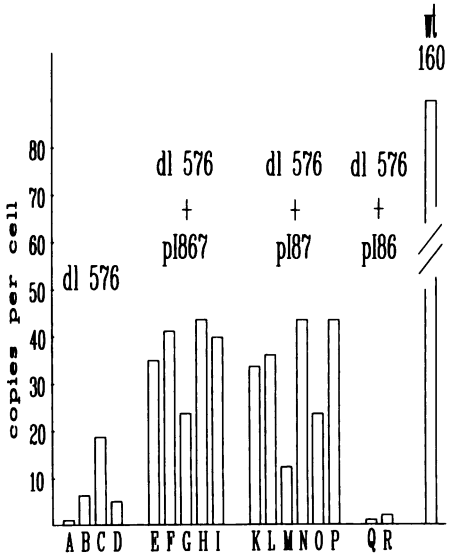

FIG. 7. Quantitation of the DNA copy number of the BPV1 mutant *dl*576. Each vertical bar represents the genome copy number of *dl*576 in a cell line derived from pooled G418-resistant clones. C127 cells were either transfected with *dl*576 alone or cotransfected with *dl*576 and pI867, pI87, or pI86. With wild-type BPV1 plasmid (pdBPV1), transfected C127 cells served as a control. wt, Wild type.

DISCUSSION

Previous reports on transforming functions of HPVs focused on HPV16 and HPV18, which both affect the genital mucosa. This study of HPV8 expands on papillomaviruses infecting the keratinizing epithelium of epidermodysplasia verruciformis patients. As with HPV16 and HPV18, only one gene could be identified in HPV8 that induces morphological transformation of rodent fibroblasts. No activity was detected within the 3′ moiety of the early genome part in accordance with the lack of an ORF similar to BPV1 E5.

Expression of HPV8 ORF E6 under the control of a retroviral LTR turned out to be sufficient to achieve morphological transformation, anchorage independence, and reduced serum requirement of C127 fibroblasts. A mutation that led to a premature translation stop of the presumptive E6 protein rendered the LTR-E6 construct nontransforming. The inactive mutant encodes a protein lacking the carboxy-terminal 34 amino acids of E6, including one cysteine-X-X-cysteine motif. These data indicate that ORF E6 of HPV8 represents a transforming gene as in the case of BPV1 (27). Similar to BPV1 E6, HPV8 E6 cannot transform NIH 3T3 cells. Unlike BPV1, HPV8 E6 constructs induced no foci after transfection of C127 cells and in vitro-transformed cells did not form tumors in nude mice. This may suggest a lower efficiency in initiating and promoting the transformation process, although we cannot exclude that the differences reflect levels of expression rather than characteristics of the transforming proteins.

The role of HPV8 E6 in naturally occurring infections remains to be established. It is noteworthy, however, that transcripts were detected in skin cancers persistently infected with the closely related HPV5, which hybridized with a subgenomic DNA fragment corresponding to ORFs E6 and E7 (22). One may speculate from this that continuous expression of E6 is required in carcinoma cells.

ORF E7 of HPV8 showed no transforming activity either in pI87 or in pI867ME. The negative results on transformation of rodent fibroblasts do not exclude a possible role of HPV8 E7 during in vivo oncogenesis but are in contrast to findings with HPV16 and HPV18, in which ORF E7 encodes a major transforming activity (14, 35a). HPV8 E7 seems to be involved in the replication of the viral genome. A low-copy-number mutant of BPV1, which usually persists with one to five copies per cell (19), replicated much more efficiently in C87 cells. This activity of HPV8 E7 corresponds to that of BPV1 genes E6 and E6-E7 (4).

The discrepancies between the functions of ORFs E6 and E7 of HPV8, HPV16, HPV18, and BPV1 should be discussed in view of the similarities of ORFs E6 and E7. Both encode amino acid sequences with cysteine-X-X-cysteine motifs, which could form zinc ion-binding sites. The presumptive E6 proteins contain four motifs, and the E7 proteins contain two. Such proteins were indeed detected in the case of HPV16 and HPV18, but there is no evidence for a BPV1 E7 protein (2, 32, 33). The splicing pattern in the E6-E7 region of this virus rather suggests the existence of an E6-E7 fusion protein containing four cysteine-X-X-cysteine motifs like E6 (4, 37). The E6 and E7 genes were interpreted as having originated from a 33-amino-acid module by successive duplications and drifts (9). Differences in transcription initiation and splicing generate various proteins from considerably diverged modules, and it is easy to imagine that functions may switch from E6 to E7 as observed with HPV8, HPV16, HPV18, and BPV1. It will be interesting to see if the assignment of transforming activity to ORFs E6 and E7 consistently correlates with the tropism of HPVs. It may be possible to establish crucial elements of transforming modules by nucleotide or amino acid sequence comparisons based on an increasing number of identified transforming genes.

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