JOURNAL OF VIROLOGY, Apr. 1992, p. 2125–2134
0022-538X/92/042125-10$02.00/0
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Vol. 66, No. 4

The E6 and E7 Genes of Human Papillomavirus Type 6 Have Weak Immortalizing Activity in Human Epithelial Cells

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Received 16 October 1991/Accepted 31 December 1991

Previous studies have shown that the E7 gene of human papillomavirus (HPV) type 16 or 18 alone was sufficient for immortalization of human foreskin epithelial cells (HFE) and that the efficiency was increased in cooperation with the respective E6 gene, whereas the HPV6 E6 or E7 gene was not active in HFE. To detect weak immortalizing activities of the HPV6 genes, cells were infected with recombinant retroviruses containing HPV genes, alone and in homologous and heterologous combinations. The HPV6 genes, alone or together (HPV6 E6 plus HPV6 E7), were not able to immortalize cells. However the HPV6 E6 gene, in concert with HPV16 E7, increased the frequency of immortalization threefold over that obtained with HPV16 E7 alone. Interestingly, 6 of 20 clones containing the HPV16 E6 gene and the HPV6 E7 gene were immortalized, whereas neither gene alone was sufficient. Thus, the HPV6 E6 and E7 genes have weak immortalizing activities which can be detected in cooperation with the more active transforming genes of HPV16. Acute expression of the HPV6 and HPV16 E6 and E7 genes revealed that only HPV16 E7 was able to stimulate the proliferation of cells in organotypic culture, resulting in increased expression of the proliferative cell nuclear antigen and the formation of a disorganized epithelial layer. Additionally, combinations of genes that immortalized HFE cells (HPV16 E6 plus HPV16 E7, HPV16 E6 plus HPV6 E7, and HPV6 E6 plus HPV16 E7) also stimulated proliferation.

Of the more than 60 different types of human papillomaviruses (HPVs), approximately 20 types infect the epithelium of the genital mucosa. The lesions range from condylomas or benign genital warts to premalignant and invasive carcinomas. DNA typing of the HPVs has shown that there is an association of certain types with the clinical diagnosis of the lesion. HPV6 and HPV11 account for the majority of condylomas (19, 50). In contrast, HPV16 and HPV18 are found in the premalignant lesions, and these types comprise over 90% of the cases in invasive carcinoma (6, 8, 30, 56). The HPV16 and HPV18 E6 and E7 genes are transcriptionally active in lesions (12, 28, 50) and in cell lines derived from cervical carcinomas (2, 47, 49). Although HPV6 is extremely rare in cervical tumors, tumors at other sites (vulva and anus) have been reported to harbor HPV6 more frequently (5), suggesting some oncogenic potential for this type. In a few instances in which the HPV6 genome has been cloned from the tumor, alterations in the regulatory sequences suggested that increased transcription of the E6 or E7 gene might be important (9, 25, 42).

The case for the involvement of HPV16 and HPV18 in epithelial malignancy is strengthened by the fact that these types can transform rodent and human cells. HPV16 DNA induced morphologic transformation in NIH 3T3 cells, and the transformed cells were tumorigenic in nude mice (55). Cloned DNAs containing HPV16 or HPV18, in cooperation with an activated *ras* gene, immortalized primary rodent cells (29, 51), and in rare cases HPV6 and HPV11 did also (51). The transformed cells were tumorigenic in syngeneic immunocompetent animals. The E7 gene of HPV16 was sufficient to cooperate with *ras* to produce this transformation as well as cause anchorage-independent growth in established cell lines. Thus, the E7 gene of the oncogenic-associated HPVs has a strong transforming potential in rodent cells.

Studies of human cell transformation have shown that HPV16 and HPV18 DNA immortalized primary human epithelial cells, whereas HPV6 and HPV11 DNA did not (26, 38, 40, 45, 54). The immortalizing functions have been localized to the E6/E7 region of the viral genome (22, 35). Although the HPV16 and HPV18 E6 and E7 genes in cooperation can immortalize efficiently, the E7 gene alone is sufficient (21, 23). In contrast, the E7 gene of HPV6 alone did not immortalize human epithelial cells (4, 21).

Because cooperation between the functions of the E6 and E7 genes of HPV16 has been shown to have a significant effect on the efficiency of immortalization of human foreskin epithelial (HFE) cells, we asked whether there was a similar cooperativity between the E6 and E7 genes of HPV6 in immortalization. Previous studies did not adequately address cooperation between the HPV6 E6 and E7 genes because the expression of the E7 protein was minimal from constructs in which E7 was downstream of E6 (21, 52). In this study, we evaluated the ability of the E6 and E7 genes of HPV6 to immortalize HFE cells when expressed in *trans* from a retrovirus long terminal repeat promoter. Also, cooperation of the viral genes in heterologous combinations was evaluated by examining whether the HPV6 E6 protein could increase the immortalizing frequency of HPV16 E7 and whether the HPV6 E7 gene could cooperate with HPV16 E6. In addition, we tested whether the acute expression of the E6 and E7 genes of HPV16 and HPV6b prior to immortalization could change the morphology or proliferative behavior of the HFE cells in an environment promoting cellular differentiation.

MATERIALS AND METHODS

Plasmids. The retrovirus vector pLXSN, obtained from A. D. Miller, contains a gene conferring resistance to neomy-

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TABLE 1. HPV6b and HPV16 nucleotide sequences contained in the recombinant retrovirus plasmids

Plasmid	HPV type	Nucleotide sequence
pLXSN	None	
pLXSN6E6	6b	48–617[a]
pLXSN6E7	6b	476–848[a]
pLXSN16E6	16	56–562[b]
pLXSN16E7	16	551–875[b]
pLXSN16E6E7	16	56–875[b]

[a] Nucleotide sequences are designated according to Schwarz et al. (46).
[b] Nucleotide sequences are designated according to Seedorf et al. (48).

cin, which is transcribed from a simian virus 40 promoter. The retrovirus vector and the construction of plasmids have been described previously (21). Briefly, HPV16 (48) and HPV6 (46) DNA fragments containing the E6, E7, or E6/E7 open reading frames were inserted downstream of the Moloney murine leukemia virus promoter/enhancer sequences, as described in Table 1.

Cell culture. NIH 3T3-derived mouse fibroblast lines, Psi-2 and PA317, obtained from A. D. Miller, were maintained in Dulbecco's modified minimal essential medium supplemented with 10% fetal bovine serum and antibiotics. Psi-2 and PA317 cells were used as packaging cell lines to make ecotropic and amphotropic viruses, respectively (33). Human primary and secondary epithelial cultures were derived from neonatal human foreskin epithelium. Day-old foreskin biopsies were cut into 1- to 2-mm squares and incubated overnight at 4°C in phosphate-buffered saline (PBS) containing dispase (25 mg/ml). The epidermis was separated from the underlying dermis, trypsinized, and sheared into single-cell suspensions. Cells were pelleted, resuspended in serum-free medium (SFM; GIBCO, Grand Island, N.Y.), and plated onto 100-mm dishes. The epithelial cells were used for infection with recombinant HPV-containing retroviruses within 14 days of establishment of the primary epithelial culture.

Generation of amphotropic retroviruses. Production of recombinant retroviruses was done according to a previously described procedure (33). Plasmid DNA was transfected into Psi-2 cells by calcium phosphate precipitation. Viral supernatants derived from transfected Psi-2 cells were used to infect PA317 cells to generate clonal lines containing one unrearranged proviral copy of the HPV gene. Following infection and drug selection in G418 (1 mg/ml), such clonal lines of PA317 were used to generate viruses used for infection of epithelial cells. Viral titers were approximately 3×10^4 to 5×10^4/ml. All infection media contained Polybrene (4 μg/ml).

Infection of HFE cells. One milliliter of viral supernatant (LXSN, LXSN16E6, or LXSN6E6) was combined with 3 ml of SFM and overlaid onto HFE cells seeded the previous day at 5×10^5 cells per 60-mm dish. After 9 h, the viral supernatant was removed, and the cells were washed twice with PBS and fed SFM. The following day, 50, 10, and 1% of the cells were replated onto 100-mm dishes. After attachment, the cells were drug selected in medium containing G418 (50 μg/ml). Selection was completed on day 7. On day 8, the second virus (LXSN, LXSN16E7, or LXSN6E7) was introduced, and the clones of epithelial cells were isolated when they approached 2 to 4 mm in diameter. The cell lines obtained after the infection procedure are designated according to the order of the introduced recombinant retrovirus.

Immortalization of HFE cells. Both pooled and clonal populations of secondary HFE cells which contained homologous and heterologous combinations of the E6 and E7 genes, as well as control populations, were passaged to assay for immortalization in SFM medium. Cell lines were split approximately 1:6 once a week, and lines which continued to proliferate after 8 months in culture were considered to be immortal. The cooperation of HPV16 E6 and E7 genes (LXSN16E6E7) was previously shown to have high immortalizing efficiency (21); therefore, this virus was used as a control to evaluate the efficiency of the immortalization assay.

Radioimmunoprecipitations. One 100-mm dish which contained cells at 75% confluence was labeled with medium supplemented with [^{35}S]cysteine (200 μCi per plate; NEN) and ^{35}S-express label (100 μCi per plate; Amersham) for 2 h. Rabbit polyclonal antisera generated against HPV16 and HPV6 E6 and E7 bacterial fusion proteins (16) were used to precipitate the HPV proteins. Equivalent trichloroacetic acid-precipitable counts of labeled protein extracts were reacted with antiserum to allow semiquantitation of protein levels. Radioimmunoprecipitation was done according to a previously described procedure (24).

Organotypic cultures (rafts). Primary HFE cells were infected with the recombinant viruses (LXSN, LXSN16E6, LXSN16E7, LXSN16E6E7, LXSN6E6, and LXSN6E7) and selected in medium containing G418. At passage 1 after selection, pooled populations were used to generate rafts. Raft cultures were made by using a modification of previously published procedures (1, 7, 27, 31). Briefly, a dermal-equivalent collagen layer was prepared as described by the manufacturer (Collaborative Research Inc., Bedford, Mass.); 3×10^5 primary human fibroblasts were mixed with collagen, polymerization buffer, and nutrient medium. The primary and secondary foreskin dermal fibroblasts were isolated and maintained as described previously (7). Following polymerization, approximately 5×10^5 cells of retrovirus-infected epithelial cells were seeded on top of the collagen gel. The cultures remained submerged for 7 days and were raised to an air-liquid interphase for 12 days subsequently. Cultures were fed every other day with a modified Rheinwald and Green medium (31). The serum in the growth medium was delipidized as instructed by the manufacturer (International Enzymes Inc., Fullbrook, Calif.). Thereafter, the medium was supplemented with 10^{-8} M retinoic acid. Rafts were fixed in 10% buffered formaldehyde, paraffin embedded, sectioned, and stained with hematoxylin and eosin for morphologic analysis.

Immunohistochemical staining for PCNA. Sections of Formalin-fixed rafts were reacted with a mouse monoclonal antibody to the proliferative cell nuclear antigen (PCNA). Localization of the antibody by the streptavidin-biotin immunoperoxidase method was done by a technique described previously (18, 20).

RESULTS

Immortalization of HFE cells by the E6 and E7 proteins of HPV6 and HPV16. The HFE cells were sequentially infected with recombinant retroviruses. After infection with the first virus and selection in drug-containing medium, 100-mm plates containing approximately 30 to 50 colonies of HFE cells (approximately 8 to 16 cells per colony) were infected with the second virus. Clones as well as pooled populations were passaged for immortalization. The results of the immortalization assay are shown in Table 2. The clones obtained after infection with viruses in combination LXSN+

TABLE. 2. Immortalization efficiency of combinations of HPV genes

Infecting virus combination[a]	Incidence of immortalized cell lines	
	Clonal populations[b]	Pooled populations[c]
LXSN+LXSN	0/12	−
LXSN+LXSN6E7	0/20	−
LXSN+LXSN16E7	3/20	+
LXSN6E6+LXSN	0/12	−
LXSN6E6+LXSN6E7	0/20	−
LXSN6E6+LXSN16E7	10/20	+
LXSN16E6+LXSN	0/12	+
LXSN16E6+LXSN6E7	6/20	+
LXSN16E6+LXSN16E7	12/12	+
LXSN16E6E7	10/12	+

[a] Each infecting virus combination is designed by the recombinant viruses used, listed in the order in which they were introduced into the cells.

[b] Given as the ratio of the number of clones analyzed that achieved immortalization to the total number of clones analyzed.

[c] +, an immortalized cell line resulted from passaging of a pooled population of infected cells; −, passaging of a pooled population yielded no immortalized lines.

LXSN, LXSN+LXSN6E7, LXSN16E6+LXSN, or LXSN 6E6+LXSN did not yield any immortalized cell lines. The corresponding pooled populations for each for these clonal lines yielded no immortalized lines except in the pooled population which contained the HPV16 E6 gene (LXSN 16E6+LXSN). As expected from previous studies, 3 of 20 clones which received HPV16 E7 alone (LXSN+LXSN 16E7) became immortalized, whereas all 12 clones chosen after introduction of the HPV16 E6 and E7 genes in *trans* yielded immortalized cell lines (LXSN16E6+LXSN16E7). This was comparable to the frequency achieved by the construct expressing the HPV16 E6 and E7 genes in *cis*, LXSN16E6E7, in which 10 of 12 clones were immortalized.

Ten of twenty clones obtained from infection with LXSN6E6 and LXSN16E7 yielded immortalized lines. This incidence reflects a threefold increase over the number obtained in an infection with the combination LXSN+ LXSN16E7. In addition, LXSN6E6+LXSN16E7 cell lines did not exhibit as drastic a cell crisis as did cell lines which had only HPV16 E7 (LXSN+LXSN16E7) (Fig. 1, C and B, respectively). The cell crisis which occurred was marked by low plating efficiency, slow growth, and a heterogeneous population of large and small cells. Following crisis, the resultant populations stabilized and often became as proliferative as cells that contained both HPV16 E6 and E7 genes (Fig. 1D).

Six of twenty clonal populations obtained from infection with LXSN16E6 and LXSN6E7 yielded immortalized lines. These cell lines behaved similarly to cells which contained LXSN6E6+LXSN16E7, exhibiting cell crisis and subsequent recovery. In contrast, the combination of LXSN6E6+LXSN6E7 yielded no immortalized lines either in the pooled population or among the clones.

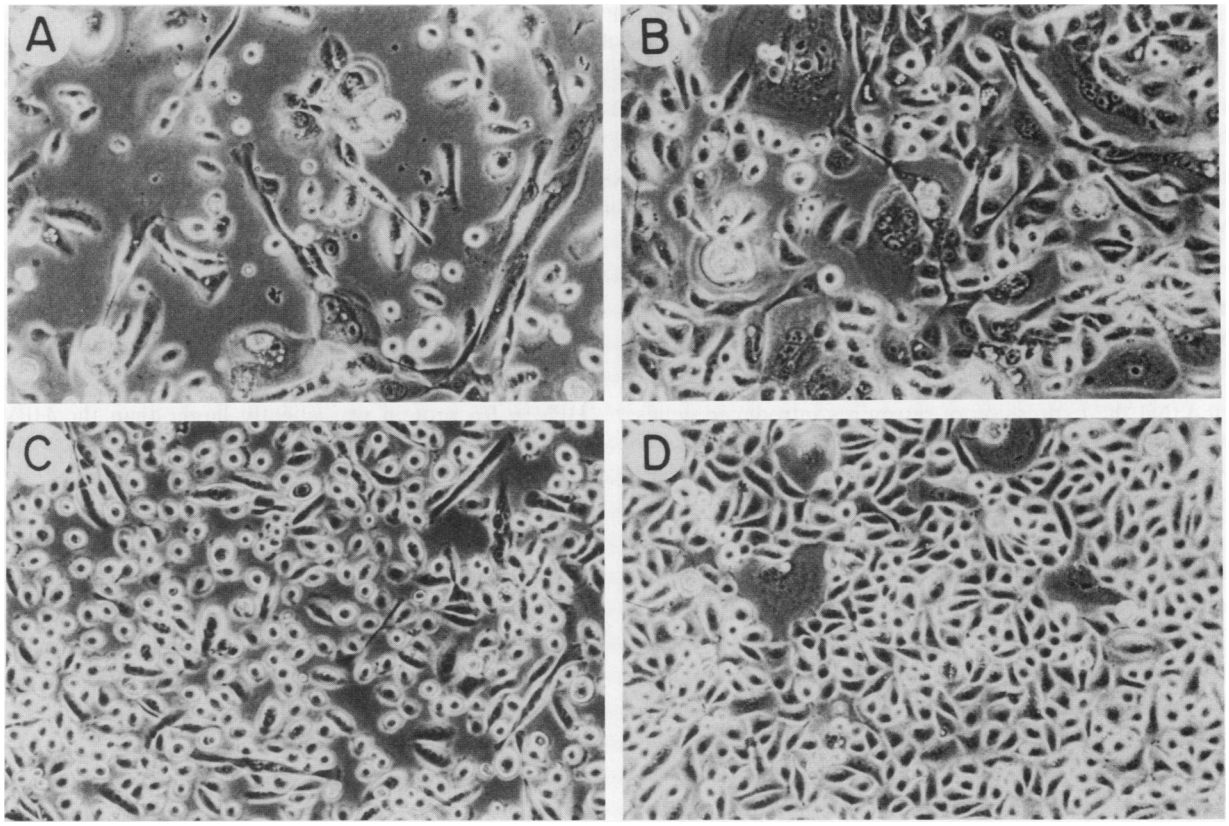

FIG. 1. Phase-contrast photographs of pooled population of HFE cells during cells crisis. (A) Cells containing vector LXSN+LXSN; (B) cells containing LXSN+LXSN16E7; (C) cells containing LXSN6E6+LXSN16E7; (D) cells containing LXSN16E6+LXSN16E7. The LXSN6E6+LXSN16E7 cells have a higher plating efficiency and are more proliferative than cells which contain LXSN16E7 alone.

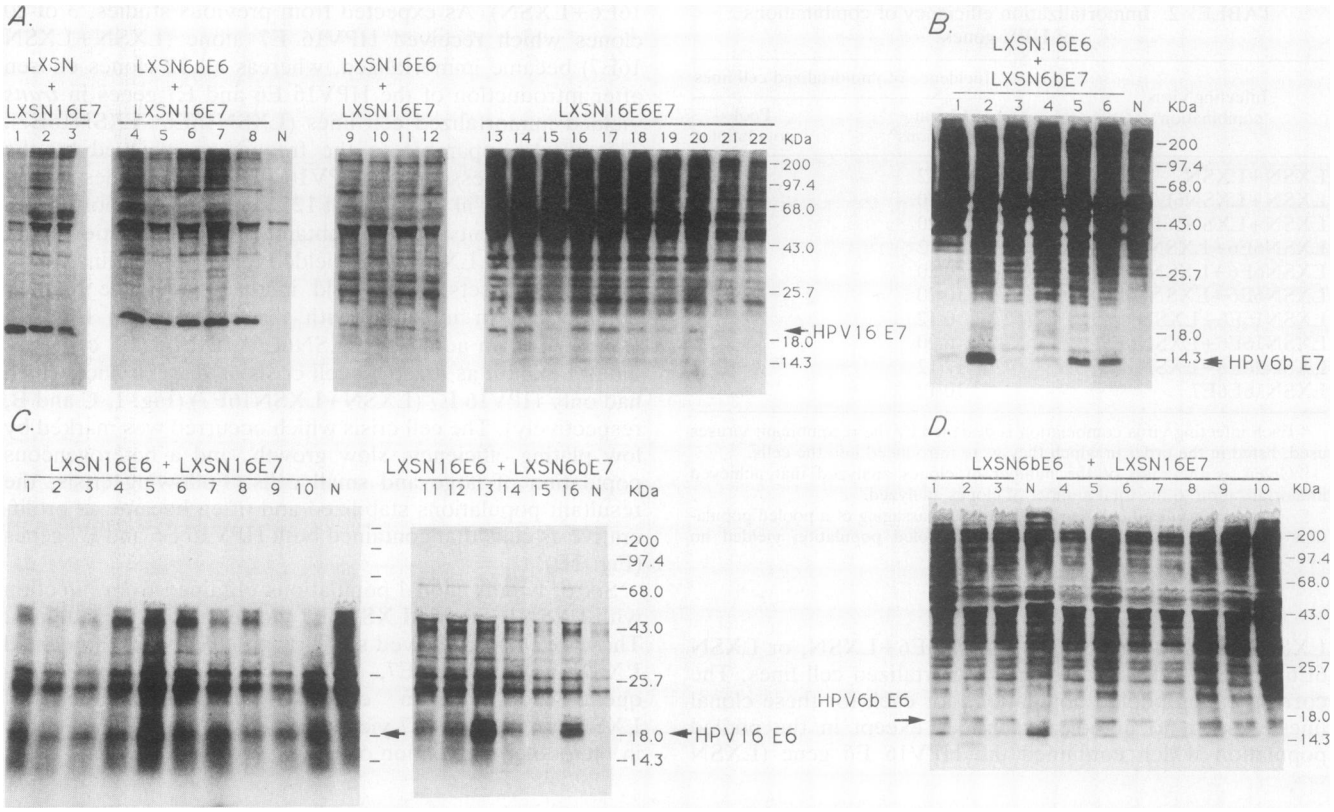

FIG. 2. Immunoprecipitation of the HPV E6 and E7 proteins. The relative levels of the HPV E6 and E7 proteins in HFE cell lines immortalized by homologous and heterologous combinations of the HPV genes are shown. Cells were metabolically labeled in medium which contained [^{35}S]cysteine and [^{35}S]methionine. Cell extracts were incubated with polyclonal rabbit immune serum. (A) Immunoprecipitation of the HPV16 E7 protein in three immortalized cell lines obtained from infection with the combination LXSN+LXSN16E7 (lanes 1 through 3), five cell lines from infection with LXSN6E6+LXSN16E7 (lanes 4 through 8), four cell lines from infection with LXSN16E6+LXSN16E7 (lanes 9 through 12), and 10 cell lines from infection with LXSN16E6E7 (lanes 13 through 22). As a control, normal HFE cells (lane N in all panels) were reacted with the anti-E7 serum. The HPV16 E7 protein is indicated by an arrow. (B) Immunoprecipitation of HPV6 E7 protein in six immortalized cell lines obtained from infection with LXSN16E6+LXSN6E7 (lanes 1 through 6). (C) Immunoprecipitation of HPV16 E6 protein in 10 cell lines immortalized by infection with LXSN16E6+LXSN16E7 (lanes 1 through 10) and in all six cell lines from infection with LXSN16E6+LXSN6E7 (lanes 11 through 16). (D) Immunoprecipitation of HPV6 E6 protein in 10 cell lines immortalized by LXSN6E6+LXSN16E7 (lanes 1 through 10).

Detection of HPV proteins in cell lines. It was necessary to verify that viral genes were expressed in the immortalized cell lines obtained because (i) the introduction of the second virus was not followed by drug selection and (ii) it has been reported that the expression of retrovirus-introduced genes can be repressed after an extended time in the cell (37). All cell lines isolated after infection with the LXSN16E7 virus expressed the HPV16 E7 protein (Fig. 2A). The clones from the combination LXSN+LXSN16E7 (Fig. 2A, lanes 1 to 3) or LXSN6E6+LXSN16E7 (Fig. 2A, lanes 4 through 8) expressed higher levels of the HPV16 E7 protein than did the ones which contained LXSN16E6+LXSN16E7 (Fig. 2A, lanes 9 through 12) or LXSN16E6E7 (Fig. 2A, lanes 13 through 22). The cell lines obtained from infection with LXSN16E6 and LXSN6E7 expressed variable amounts of the HPV6 E7 protein (compare lanes 1 through 6 in Fig. 2B). This protein exhibited at least two migrating forms with molecular sizes of approximately 11 and 13 kDa, as previously reported (17). High levels of expression of HPV6 E7 protein could occur in the absence of immortalization, because similar levels of HPV6 E7 protein were found in clones obtained from infection with LXSN6E7 virus alone (data not shown). The sizes of the HPV16 and HPV6 E6 proteins were approximately 18 kDa (Fig. 2C and D), compared with the more slowly migrating 21-kDa HPV16 E7 protein. When the sizes of the two HPV E6 proteins were compared on the same protein gel in contiguous lanes, the HPV16 E6 protein was slightly larger than the HPV6 E6 protein (data not shown). The respective E6 proteins were expressed in all of the clonal lines analyzed by immunoprecipitation.

Detection of the HPV16 E7 protein in pooled populations at different passages. Because the level of the HPV16 E7 protein was always low when HPV16 E6 was present and always high in other combinations that resulted in immortalization, expression was examined in the pooled populations at various passages to determine whether a similar pattern occurred. Cells infected with LXSN16E7 and subsequently drug selected expressed higher levels of the E7 protein than did cells infected with LXSN16E6E7 (Fig. 3, lanes under 1 and 2, respectively). The level of E7 expression was similar between passage 1 and passage 8 (approximately 2 months in culture) in either population. The amount of HPV16 E7 protein in cells in which it was introduced as the second virus was low at passage 1 and increased dramatically at passage 8 in the LXSN+LXSN16E7 and LXSN6E6+

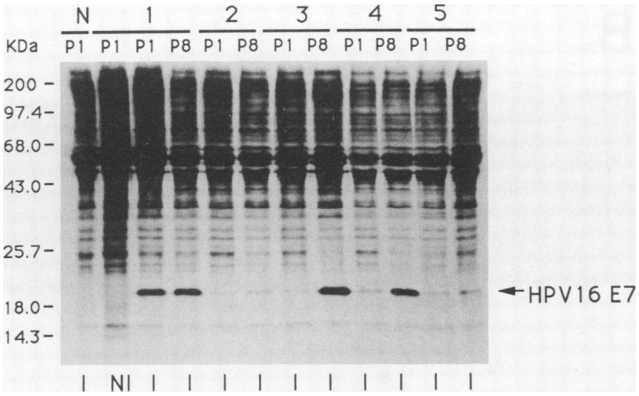

FIG. 3. Immunoprecipitation of the HPV16 E7 protein in pooled populations. The relative levels of the HPV16 E7 protein at passage one (P1) and passage eight (P8) are shown. Pooled populations of HFE cells which contained HPV genes (1, LXSN16E7; 2, LXSN16E6E7; 3, LXSN+LXSN16E7, 4, LXSN6E6+LXSN16E7; 5, LXSN16E6+LXSN16E7) were metabolically labeled, and equivalent amounts of trichloroacetic acid-precipitable counts were reacted with immune (I) and nonimmune (NI) rabbit sera. Normal HFE cells (N) were also reacted with the rabbit immune serum. The E7 protein is indicated by an arrow.

LXSN16E7 populations (Fig. 3, lanes under 3 and 4, respectively) but remained low in the LXSN16E6+LXSN16E7 cell line (Fig. 3, lanes under 5). Thus, the level of the HPV16 E7 protein was similarly low when it was expressed in *trans* as when expressed in *cis* to HPV16 E6. Southern blot hybridization showed that the copy number of the HPV16 E7 gene in the different pooled populations was comparable at passage 8 and thus did not account for the differences seen in the HPV16 E7 protein levels (data not shown).

Effect of the E6 and E7 genes on the organization and proliferation of cells in organotypic culture. To determine the extent to which the E6 and E7 genes of HPV6 and HPV16 could alter the organization of cells in a differentiation-promoting environment, cells were evaluated for the ability to stratify and form an epithelium exhibiting normal cellular architecture at passage 1 following selection in G418. Immunoprecipitation of metabolically labeled lysates demonstrated that each of the transferred viral genes was expressed (data not shown). Cultures containing LXSN, LXSN16E6, LXSN6E6, and LXSN6E7 at passage 1 retained the ability to terminally differentiate, as exhibited by the formation of an organized epithelium consisting of basal, stratified, granulated, and cornified layers (Fig. 4A, C, E, and F, respectively), resembling the normal, orderly process of squamous terminal differentiation of untreated primary HFE cells. The nuclei which were retained in the basal layer were gradually lost as the cells migrated to the upper layers concomitant with accumulation of keratohyalin granules, and the nucleus-to-cytoplasm ratio was low.

In contrast, the epithelium generated by the culture which contained LXSN16E7 was markedly different from normal epithelium, appearing disorganized and exhibiting variable presence of a granulated layer as well as a cornified layer (Fig. 4D). The basal and suprabasal layers seemed exaggerated, and the cornified layer often was a loose aggregate of cells. There was retention of nuclei in the more superficial layers, as well as presence of mitotic cells above the basal layer. The nuclei of cells were often enlarged and hyperchromatic, and the nucleus-to-cytoplasm ratio was high in these cells throughout the layers. These features are characteristics of cervical dysplasias seen in vivo. The epithelium generated by cells containing LXSN16E6E7 exhibited similar features. The addition of HPV16 E6 did not seem to enhance the biological effects seen with HPV16 E7 alone. Indeed, the morphology of the rafts produced with cultures containing LXSN16E6E7 seemed more normal, having a well-defined cornified layer (Fig. 4B).

To assess the proliferative capacity of the cells within the epithelium, cells were stained with an antibody to PCNA, a subunit of DNA polymerase ∂ in proliferating cells (10, 34, 42). Rafts of cultures containing LXSN, LXSN16E6, LXSN6E6, and LXSN6E7 showed staining for PCNA in a few cells of the basal layer (Fig. 5A, C, E, and F). The raft of normal secondary HFE cells also stained only in the proliferation-competent basal cells (result not shown). In contrast, cells containing LXSN16E7 and LXSN16E6E7 showed extensive staining with the PCNA antibody (Fig. 5D and B, respectively). PCNA was expressed in the nuclei of a majority of cells throughout the epithelium.

Because the HPV16 E6, HPV6 E6, and HPV6 E7 genes did not by themselves immortalize HFE, nor did the genes alter the morphology or PCNA staining of cells in organotypic cultures, it was of interest to examine their activity in combinations that lead to immortalization of the HFE cells. Cell lines containing LXSN16E6 and LXSN6E7 produced rafts with an abnormal morphology and exhibited PCNA staining throughout all layers of the epithelium at passage 20 (Fig. 6A and C). The other panels show the morphology and PCNA staining of immortalized cell lines at a similar passage containing LXSN6E6 and LXSN16E7 (Fig. 6B and D). All exhibited a disorganized morphology and aberrant expression of PCNA.

DISCUSSION

Some of the immortalization results presented here are similar to those obtained in previously published studies. Infection with recombinant retroviruses which resulted in introducing only one HPV gene showed that HPV16 E6, HPV6 E6, or HPV6 E7 did not immortalize HFE cells at an incidence detectable on a clonal basis. Additionally, the HPV16 E7 gene alone immortalized HFE cells and was more efficient in the presence of HPV16 E6 and HPV6 E6. Interestingly, the pooled population which contained HPV16 E6 alone (LXSN16E6+LXSN) became immortalized, although no immortalization occurred in the 12 clonal lines selected from this population, suggesting a low immortalizing activity of HPV16 E6 in HFE cells.

The ability of the HPV6 E6 and E7 genes to cooperate with each other to effect immortalization was not detected in the clonal or pooled populations. Cooperation between the E6 and E7 genes of HPV6 in previous studies was inconclusive because of the inability to detect the HPV6 E7 protein expressed from the continuous DNA segment encoding the HPV6 E6 and E7 genes (21, 52). In this study, the expression of both HPV6 E6 and E7 genes was demonstrated by immunoprecipitation. Therefore, the results suggest that the HPV6 proteins, in combination, have little transforming ability.

Two observations suggested that the HPV6 E6 gene enhanced the immortalization potential of the HPV16 E7 gene. First, there was a threefold increase in the incidence of immortalized clones obtained in an infection with the 6E6 and 16E7 in combination, compared with infection with HPV16 E7 alone. Second, the clonal lines containing HPV6

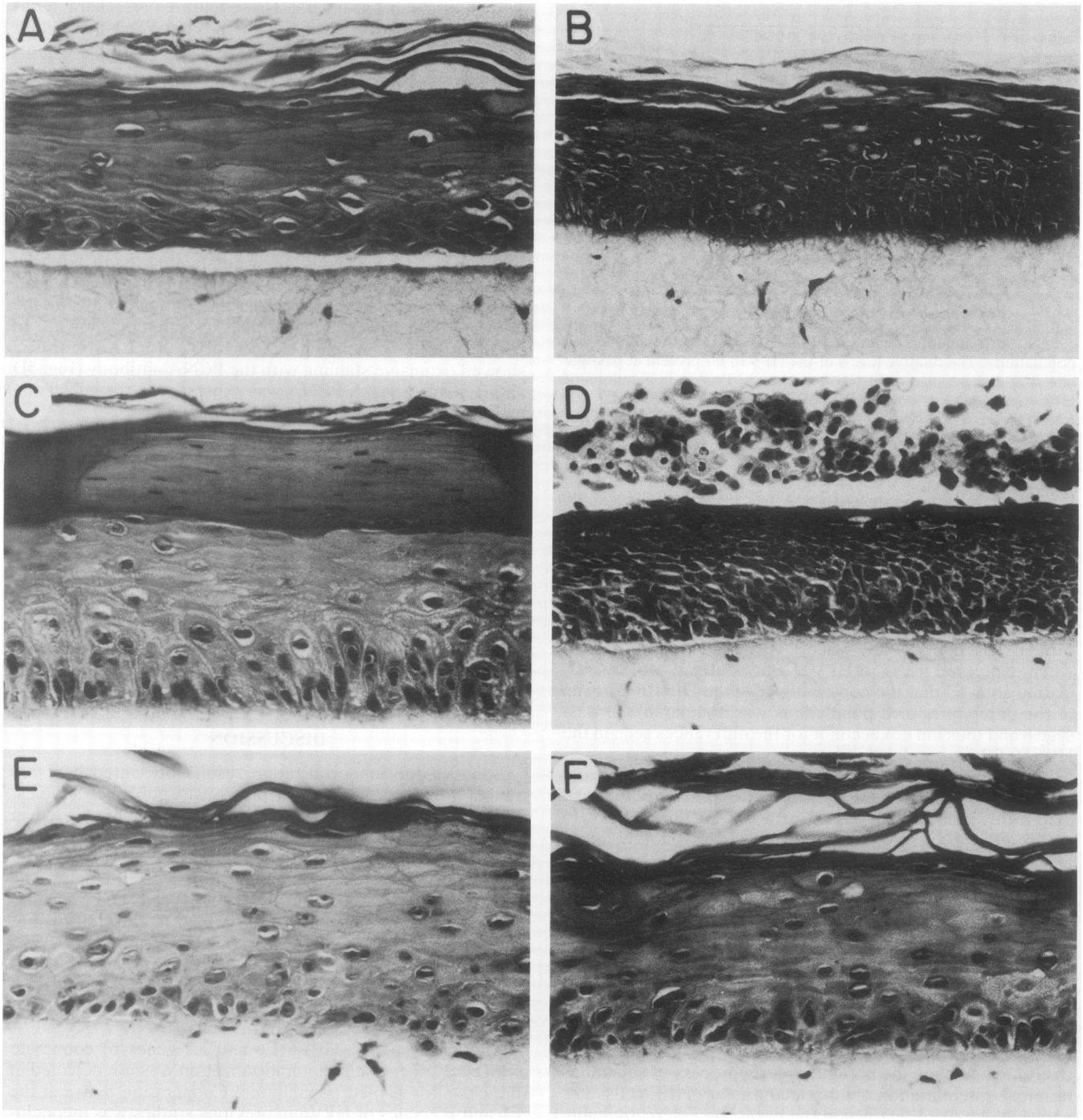

FIG. 4. Morphology of HFE cells on rafts. Cross-sections of rafts were stained with hemotoxylin and eosin. Cells containing LXSN (A), LXSN16E6 (C), LXSN6E6 (E), and LXSN6E7 (F) exhibited a normal epithelium and squamous differentiation. LXSN16E6E7 (B) and LXSN16E7 (D) rafts showed an abnormal morphology. Magnification, ×200.

E6 and HPV16 E7 appeared more proliferative during cell crisis than did lines containing HPV16 E7 only. The E6 proteins of both HPV types have been shown to transactivate transcription (11a). Whether the E6 proteins of both types or only HPV16 E6 binds to p53 is somewhat controversial (11a, 53); however, only the HPV16 E6 protein promotes the degradation of p53. At present, it is unclear which property of E6 is associated with the ability to cooperate with E7 in the immortalization of HFE cells.

The combination of HPV6 E7 and HPV16 E6 clearly showed immortalizing activity. The finding that the HPV6 E7 gene cooperated with the heterologous HPV16 E6 gene was not totally unexpected because the HPV6 E7 protein has been shown to be similar to the HPV16 E7 protein. Both E7

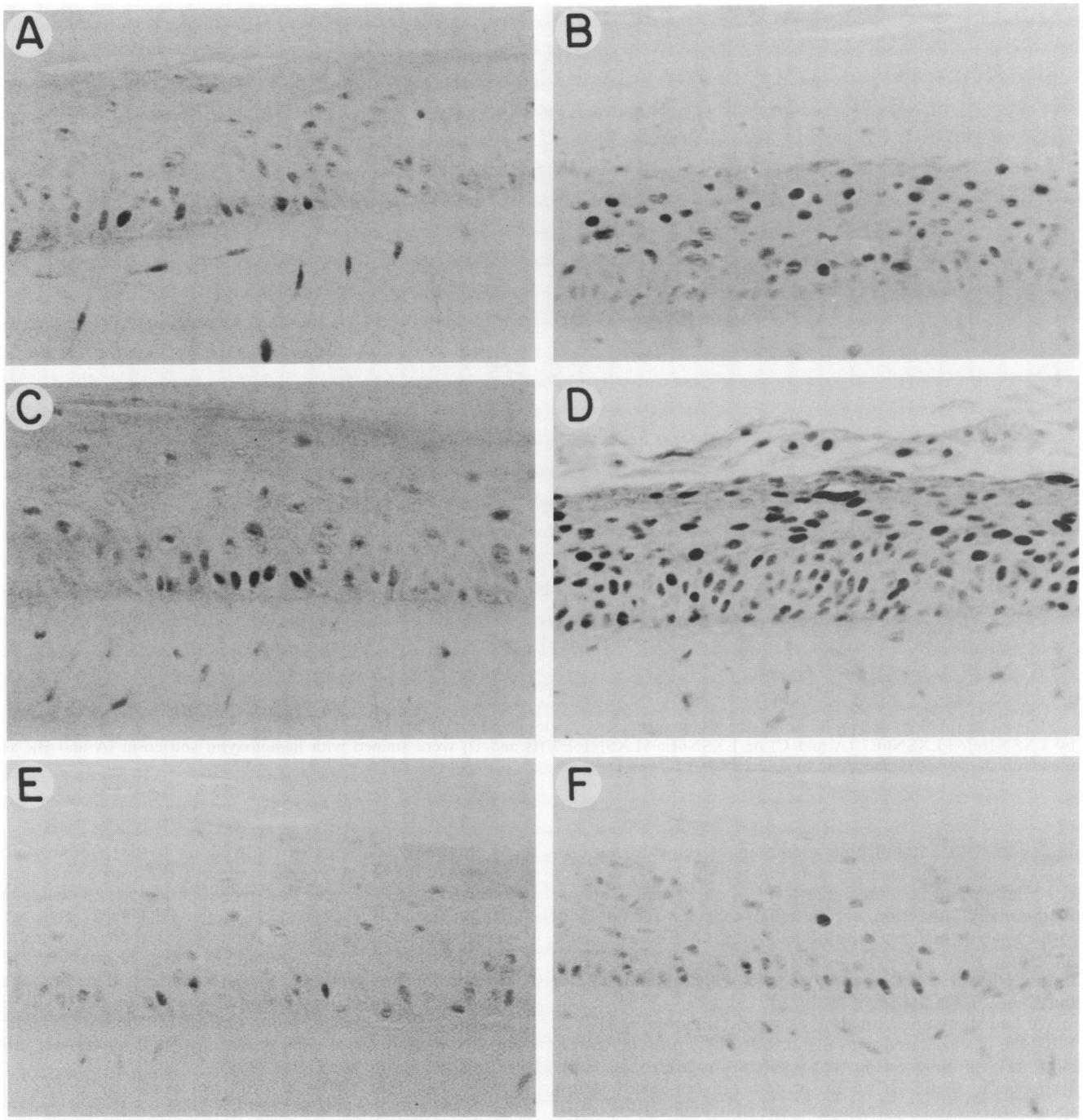

FIG. 5. Proliferation of HFE cells on rafts. Cross-sections of rafts were stained with a monoclonal antibody to PCNA and counterstained with methyl green. HFE cells containing LXSN (A), LXSN16E6 (C), LXSN6E6 (E), and LXSN6E7 (F) exhibited antigen-positive cells in the basal layer, whereas cells which contained LXSN16E6E7 (B) or LXSN16E7 (D) showed staining throughout all layers of the epithelium.

proteins can be phosphorylated by casein kinase II and contain cysteine-X-X-cysteine motifs (3, 15, 17) that have been found to be important for transformation and transactivation by the HPV16 E7 protein (11, 14). Both E7 proteins complex with the retinoblastoma protein (Rb), although the HPV6 E7 protein has been reported to bind with a lesser affinity than that exhibited by HPV16 E7 (13, 17, 36).

Barbosa and coworkers (4) have also analyzed the contribution of the E6 and E7 proteins of HPV6 in inducing anchorage independence in NIH 3T3 cells and in immortalizing human epithelial cells. They concluded that the HPV6 E7 gene had weak transforming activity in rodent fibroblasts but could not detect any immortalizing activity in human cells, using the HPV6 genes alone or in combination with the HPV16 genes. Our ability to detect immortalizing activities in human cells is likely due to the gene transfer method.

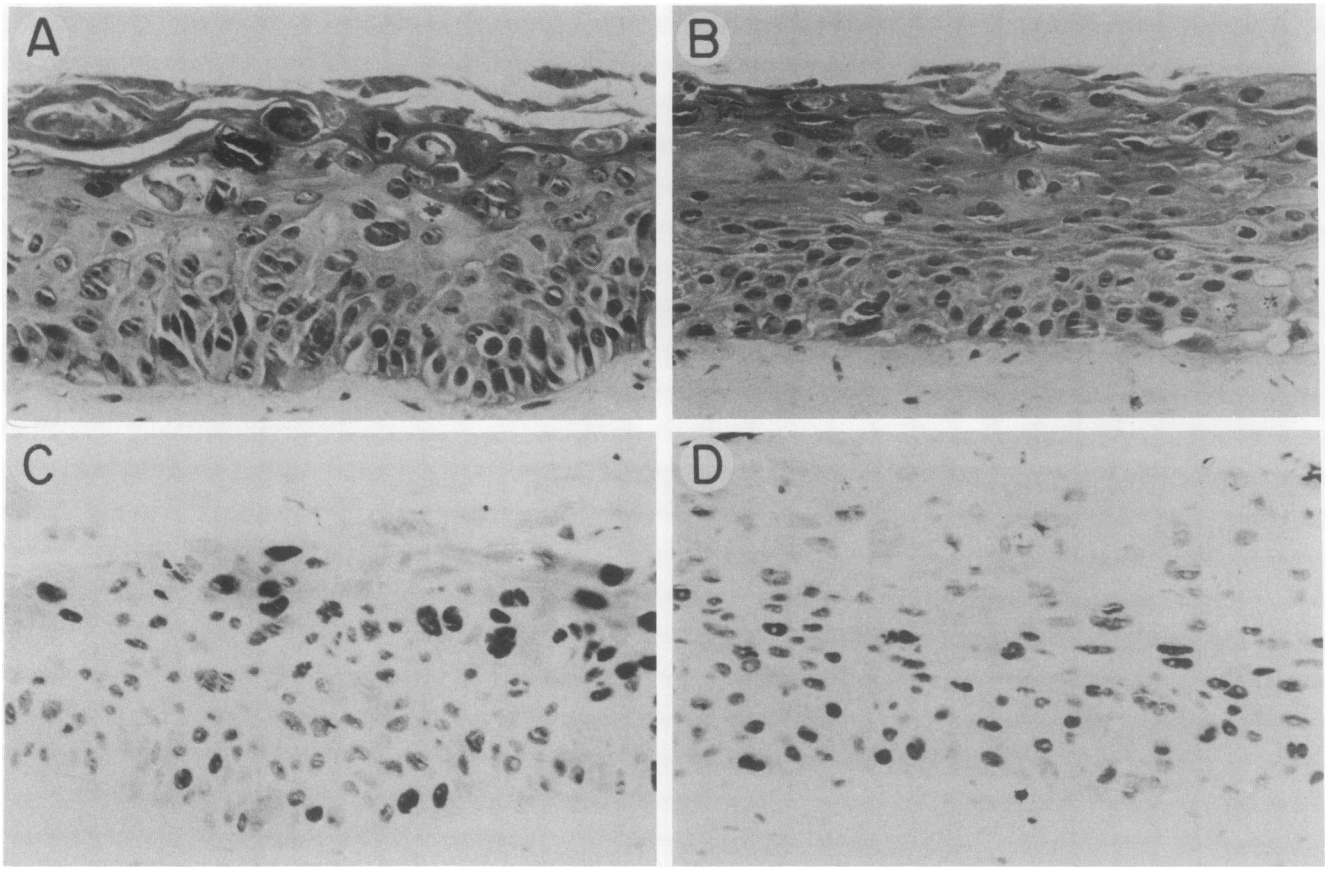

FIG. 6. Morphology and proliferation of cells immortalized by heterologous combinations of HPV genes. Cross-sections of rafts generated by LXSN16E6+LXSN6E7 (A and C) or LXSN6E6+LXSN16E7 (B and D) were stained with hemotoxylin and eosin (A and B), and immunohistochemistry was done to detect PCNA (C and D).

Infection with amphotropic recombinant retroviruses provides for efficient transfer and integration of HPV genes concomitant with a detectable level of expressed proteins. Additionally, infection of epithelial cells by recombinant viruses yields high viability of cells; i.e., after gene transfer and drug selection, cells proliferate as well as do normal untreated HFE cells, even those cells which contain vector only.

We had shown previously that immortalization by HPV16 E7 alone is associated with high levels of E7 protein expression (21), an observation that was confirmed in this study. Expression of the HPV6 E6 protein did not result in low levels of HPV16 E7 protein. One possible explanation is that there is a requirement for high levels of the HPV16 E7 protein in the absence of strong E6 activity. The fact that clones expressing HPV16 E6 uniformly exhibited low levels of the HPV16 E7 protein might indicate that high levels of both HPV16 E6 and E7 proteins are deleterious for the cell. An alternative explanation is that the HPV16 but not the HPV6 E6 protein may suppress the level of HPV16 E7 protein, either at a transcriptional or a posttranslational level.

Growth of HPV-immortalized cell lines in organotypic cultures produces an epithelial layer exhibiting an abnormal morphology with phenotypes similar to that observed in premalignant lesions and in carcinomas found in vivo (7, 31). The acute expression of HPV genes following infection with recombinant retroviruses allowed us to determine which aspects of the phenotype were directly due to the viral genes and which aspects were due to cellular changes. Our results demonstrated that expression of the HPV16 E7 gene was correlated with a dyplastic morphology and the proliferation of cells in areas of the epithelium which normally do not express the proliferation-associated antigen. This finding is consistent with the observation that induction of HPV16 E7 message level increased incorporation of tritiated thymidine or DNA synthesis in cells growth arrested by contact inhibition and serum starvation (43).

The fact that HPV6 E7 did not result in a dyplastic morphology or aberrant PCNA staining indicates that the binding of Rb may not be sufficient for this behavior because HPV6 E7 is also capable of binding Rb, although with less affinity then HPV16 E7 (13, 17, 36). Alternatively, a sufficient amount of Rb must be bound and inactivated for this effect, and the E7 protein of HPV6 is incapable of doing so.

The addition of HPV16 E6 did not enhance the biological effects exhibited by HPV16 E7 because of the morphology of the rafts produced with cultures containing LXSN16E6E7 was not more abnormal than in cultures containing LXSN 16E7. In some cases, the E6/E7-expressing cultures were less disorganized, which may be because LXSN16E6E7 expressed less E7 protein than did LXSN16E7. Thus, although HPV16 E6 contributes to immortalization of human epithelial cells, expression of HPV16 E6 did not result in an

abnormal phenotype on rafts. Furthermore, although the HPV16 E6 protein leads to degradation of the p53 gene product, this does not result in a stimulation in cellular proliferation. Interestingly, the combination of HPV16 E6 and HPV6 E7 resulted in the ability to immortalize HFE cells, and the two proteins in concert signal the cells to proliferate continually, resulting in an abnormal phenotype when the cells were placed in an environment which stimulated normal HFE cells to cease proliferation and terminally differentiate.

Recent work by Merrick and coworkers (32) showed that differentiation markers such as keratins were not consistently altered in late-passage HPV-immortalized cells, whereas proliferation markers were. Our analysis of epithelial cells directly after gene transfer is consistent with their results. Thus, the direct result of the expression of HPV-immortalizing genes is an alteration in proliferation.

In conclusion, this study demonstrated that the E6 and E7 genes of HPV6 have activities which contribute to immortalization of human epithelial cells when used in cooperation with the strong transforming proteins of HPV16. Second, the E7 gene of HPV16 and combinations of HPV genes that result in immortalization of HFE cells, such as HPV16 E6 and HPV6 E7, promote proliferation of cells, resulting in the appearance of a disorganized epithelium and the aberrant expression of PCNA.

ACKNOWLEDGMENTS

We thank R. A. Blanton for invaluable instructions in organotypic culture techniques, J. K. McDougall for critical advice throughout this work, N. Perez-Reyes and D. Myerson for comments concerning histology, and M. Skellie for performing the PCNA staining.

This work was funded by grants CA35568 and CA42792 from the National Cancer Institute. G.W.D. is supported by PHS fellowship CA08847-02.

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