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The E7 Gene of Human Papillomavirus Type 16 Is Sufficient for Immortalization of Human Epithelial Cells

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The contribution of the E6 and E7 open reading frames of human papillomavirus type 6b (HPV6b) and HPV16 to immortalization of human keratinocytes was evaluated by using amphotropic recombinant retroviruses. The HPV16 E7 gene could immortalize primary human keratinocytes without the cooperation of the viral E6 gene; however, E6 was able to contribute significantly to the efficiency of the E7 immortalizing function. Infection of HFE cells with retroviruses carrying the 16E6, 6bE6, 6bE7, or 6bE6E7 open reading frame did not result in immortalization.

It is now recognized that the ability of species-specific papillomaviruses to induce epithelial hyperproliferation extends to benign, premalignant, and malignant lesions of the cutaneous and mucosal epithelia (6, 18, 23, 39, 42). The many human papillomavirus (HPV) types identified to date have shown strong specificities for different anatomical sites and for different characteristic lesions. Of the genital papillomaviruses, HPV type 6 (HPV6) and HPV11 are frequently associated with benign condylomas (9, 39), while HPV16 and HPV18 as well as HPV31 and HPV32 are associated with malignant progression and cervical cancers (5, 6, 30, 47). Indeed, more than 90% of cervical carcinomas contain HPV DNA. Analysis of the state of viral DNA in cervical carcinomas and cell lines established from the tumors showed that the viral DNA was usually integrated and that integration often resulted in the loss of expression of some viral genes (6, 32, 34, 35). In most cases, the E6 and E7 genes were transcriptionally active (1, 5, 19, 29, 37, 39) and the E7 protein was abundantly expressed (38). These observations support the hypothesis that HPVs play a critical role in the etiology of cervical cancer.

Many studies have shown that HPVs associated with malignant lesions readily cause transformation in vitro. Although the E7 gene alone was sufficient for the transformation of established rodent cell lines to exhibit anchorage-independent growth (46), cooperation with an activated *ras* oncogene was required for the immortalization and transformation of primary rat cells (3, 14, 20). Introduction of HPV16 and HPV18 DNA into primary human epithelial cells resulted in immortalization of these cells, whereas introduction of HPV6b did not (15, 24, 25, 28, 45). The ability to immortalize human keratinocytes has been localized to the E6E7 region of DNA, and E7 expression has been demonstrated in HPV16 and HPV18 cell lines immortalized in vitro (11, 12, 16, 22, 28).

In this study, we introduced the E6 and E7 genes of HPV6b and HPV16 separately and in combination (designated E6E7) into human foreskin epithelial cells to assess their contribution to immortalization and to compare the efficiencies of the transforming proteins of the two viral types. Since it was important to detect transforming activities of genes that may display low scoring efficiency, a highly efficient gene transfer system was employed. Infection with high-titered amphotropic recombinant retrovirus provided gene transfer that resulted in high viability of epithelial cells and efficient integration and expression of transferred genes.

The retrovirus vector, pLXSN, was kindly provided by A. D. Miller. A diagram of the construction of the recombinant plasmids is shown in Fig. 1. DNA fragments containing the E6 or the E7 gene or the contiguous region encoding E6 and E7 from HPV6b (31) and HPV16 (33) were isolated by using appropriate restriction enzymes and exonuclease III digestions. Transcription of HPV genes within the vector was directed from the Moloney murine leukemia virus promoter-enhancer sequences. The vector also contained a gene conferring neomycin resistance which was transcribed from the simian virus 40 promoter.

Recombinant viruses were generated according to previously described procedures (21). Briefly, plasmid DNA was transfected by calcium phosphate precipitation into the Psi-2 ecotropic packaging cell line, and viruses produced from the Psi-2 cells were used to infect the amphotropic packaging line PA317. Both cell lines are NIH 3T3-derived mouse fibroblast lines obtained from A. D. Miller. Following infection, the PA317 cells were selected in a medium which contained G418 (1 mg/ml). Viruses produced from clonal lines of PA317 were used to infect secondary neonatal human foreskin epithelial cells within 14 days of establishment of the primary culture.

Following infection with the various amphotropic viruses (LXSN6bE6, LXSN6bE7, LXSN6bE6E7, LXSN16E6, LXSN16E7, or LXSN16E6E7) and selection in G418 (50 μg/ml), HFE colonies (3×10^4 to 5×10^4 colonies) were pooled and passaged weekly. At passage 6, the cells exhibited differences both in morphology and in proliferative capacity. Pooled cultures containing LXSN6bE6, LXSN6bE7, LXSN6bE6E7, OR LXSN16E6 behaved in a manner similar to that of cells which contained vector. There were low plating efficiency, slow growth, and the appearance of larger cells which often presented extended processes, indicating an aging HFE culture that was close to senescence (Fig. 2; data not shown for LXSN6bE6). The majority of cells which contained HPV16 E7 showed similar features, though clusters of proliferating small cells were found in greater abundance than they were in cells exposed to vector. In contrast, there was a dramatic difference in cells which contained HPV16 E6E7. These cells exhibited a high plating

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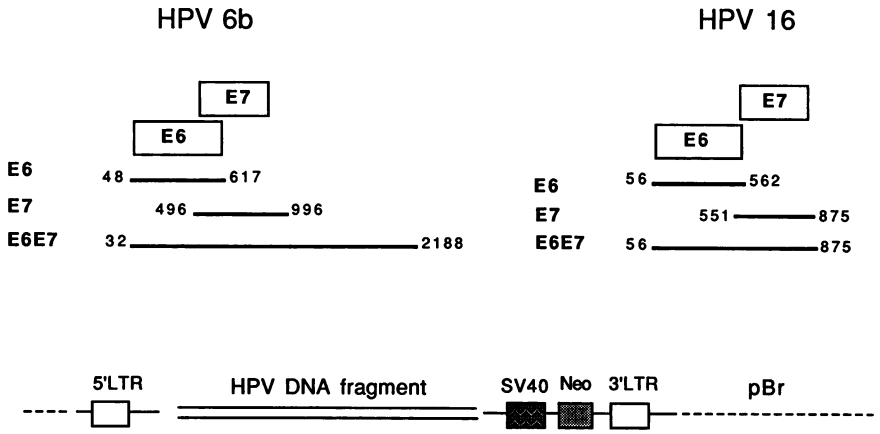

FIG. 1. Construction of recombinant retrovirus plasmids which contain HPV genes. The locations of the HPV6b and HPV16 genes are shown along the DNA fragments which have been isolated by restriction enzyme and/or exonuclease III digestion. The nucleotide positions are indicated at the ends of the fragments. The retrovirus vector, pLXSN, with the putative HPV gene(s) is shown at the bottom. Abbreviations: LTR, Moloney murine promoter-enhancer; SV40, simian virus 40 promoter; Neo, gene conferring neomycin resistance; pBr, pBr 322 plasmid sequence which contains the origin of replication and the gene conferring ampicillin resistance.

efficiency and vigorous growth and were a homogeneous population of small proliferating cells, as were HFE cultures at passages 1 and 2.

Cells at passage 6 were seeded at low density to assay for colony-forming efficiency, and colonies that formed were isolated for further passaging to determine the frequency of immortalization. The decision to maintain the cells as a pooled population until passage 6 rather than pick individual clones directly after selection was based on the assumption that immortalization would be a rare event. Cells were defined as immortalized when they had the capacity to proliferate in culture for a minimum of 8 months or approximately 30 passages. The results are shown in Table 1. Cells which contained vector exhibited low colony-forming efficiency, and colonies could not be passaged further; neither pooled nor clonal populations yielded immortalized lines. Cells containing 6bE6, 6bE7, or both gave similar results, as did cells containing 16E6. Cells which contained 16E7 yielded approximately 100 colonies per 1,000 cells seeded. Fifteen colonies were isolated and continually passaged, and they yielded several immortalized lines. The lines, as analyzed by Southern hybridization, were shown to be derived from three originally infected cells. The 15 clones represented a total of 9 unique clones. Thus, the frequency of immortalization by 16E7 in extended-life-span colonies was three of nine. Cells containing 16E6E7 had a fivefold-higher colony-forming efficiency than cells containing 16E7. Eighteen colonies were picked; all exhibited extended life spans, and all yielded immortalized lines. The 18 clones represented eight originally infected cells, and the frequency of immortalization by 16E6E7 was eight of eight.

All HPV16 E7 extended-life-span colonies exhibited some level of crisis from which emerged the immortalized progeny. The colonies which contained 16E6E7 exhibited less drastic cell crisis, and several did not undergo crisis at all. One possible reason for the difference in the behavior of cells is that HPV16 E6 protein provided additional signals for the cells to proliferate. The function of E6 could be directed at binding tumor suppressor proteins such as p53 (43) as well as transactivating the expression of viral or cellular genes (10). Our finding of the relative importance of E6 and E7 in an in vitro transformation system parallels the observations in vivo. While most anogenital tumors retain and express both the E6 and E7 genes, some tumors retain only E7 (44).

This study demonstrated that HPV16 E7 alone immortalized primary human keratinocytes. Previous studies (11, 22) which concluded that both the E6 and E7 genes of HPV16 were necessary for immortalization detected only extended-life-span activity when E7 alone was introduced. Drug selection allowed the analysis of a pure population of transformants. These transformants would not be under the inhibitory influence of the surrounding normal cells. Also, retrovirus infection allowed the analysis of a large population of target cells which received the HPV genes. Thus, the high titer of virus in combination with the use of a selectable marker resulted in the detection of infrequent events.

Münger et al. defined the contribution of the E6 and E7 genes by using plasmids which contained termination linkers in the E6 and E7 open reading frames (22). Immortalization was scored on the basis of resistance to differentiation in a medium which contained high concentrations of calcium and serum. Münger et al. concluded that both genes were necessary for immortalization of HFE cells. However, resistance to differentiation and immortalization may develop by two distinct processes. We therefore chose to assay for immortalization by culturing cells under proliferation conditions in a medium (keratinocyte growth medium; Clonetics Corp.) which did not stimulate cellular maturation and senescence in an attempt to determine the gene(s) necessary and sufficient for indefinite proliferation of keratinocytes.

In contrast to the HPV16 E7 gene, the HPV6b E7 gene was not able to extend the life span of epithelial cells. Previous studies using the whole HPV6b genome, the early region, or E6E7 sequences have shown that HPV6b did not transform primary rodent (40, 41) or human epithelial cells (24, 45). However, the level of expression of the transforming protein may have accounted for the negative results, since the reported 6E6E7 (40) construct may be deficient in E7 expression. The transcript which encodes the E7 protein is a major transcript in cells which contain HPV16, whereas the transcript which encodes the E7 protein is a minor transcript in cells which contain HPV6 (4, 36). Therefore, to circumvent differences in the level of message, the analogous E7 open reading frames were placed directly down-

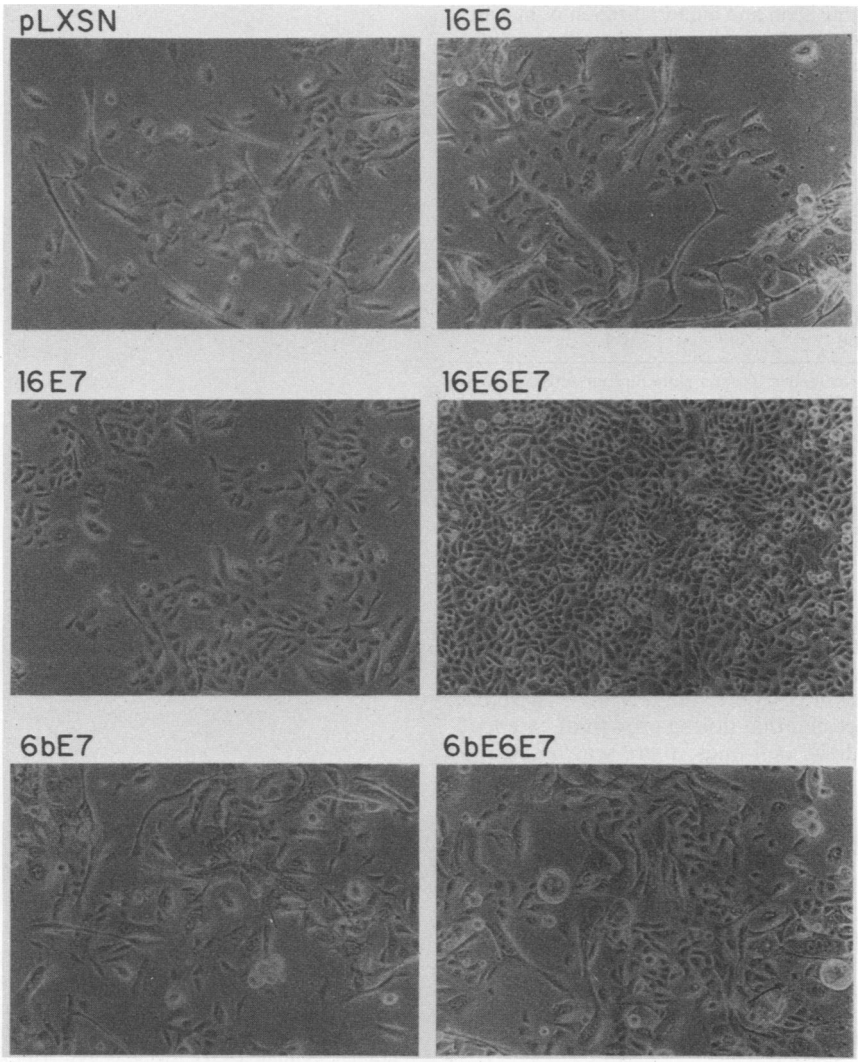

FIG. 2. Morphology of HFE cells infected with recombinant retrovirus. Phase-contrast photographs of HFE cells which contained HPV6b or HPV16 genes or vector at passage 6 are shown. The designation for the HPV genes or vector which had been transferred to the cells is shown on the top left of each panel. Magnification, ×100.

stream of the retrovirus long terminal repeat. Northern (RNA) analysis confirmed similar levels of E7 transcripts, and radioimmunoprecipitation revealed similar amounts of the 6bE7 and 16E7 proteins (data not shown). Even with comparable levels of E7 expression, the results indicated that the E7 protein from the nononcogenic HPV6b did not immortalize or extend the life span of HFE cells, in contrast to the E7 protein of the oncogenic HPV16 virus. We were not able to directly confirm that the E7 protein was expressed in the construct containing the contiguous HPV6 E6E7 sequences, and therefore, we may not have critically evaluated the possibility that 6bE6 and 6bE7 can cooperate to immortalize HFE cells. These results do not explain studies in which some tumors that contain HPV6 have alterations in the regulatory sequences which were thought to increase transcription of the E6E7 genes, thus contributing to the rarely observed tumorigenicity of HPV6 (17, 26, 42).

After infection and selection, the cells were passaged as pooled population until cloning at passage 6; therefore, the clonal lines could be siblings. Analysis of the proviral DNA was performed on all extended-life-span and immortalized lines in order to determine the number of unique clones. Southern hybridizations are shown in Fig. 3. Provirus diagrams below the autoradiograms display the HPV fragments and restriction sites that allowed determination of the number of integrated copies of E7 or E6E7 in the cell lines. Both pLXSN16E7 and pLXSN 16E6E7 contain one *Eco*RI site. Genomic sequences at the site of integration provided the other *Eco*RI site; thus, each fragment represented a unique integrated provirus. All 16E7 cell lines exhibited multiple copies of the provirus. The cell line shown in Fig. 3A, lane 8, was subcloned to determine whether the cell line was not clonal or whether all of the copies of the integrated provirus were contained within each cell. All the subclones examined exhibited the same DNA pattern, indicating a clonal population (data not shown). In contrast to the results obtained with the 16E7 lines, the eight E6E7 cell lines, in general, contained single copies of the provirus. That is, of eight unique clones which contained 16E6E7, only two demon-

TABLE 1. Extended life span and immortalization of human foreskin epithelial cells

Plasmid	CFE[a] (no. of colonies/1,000 cells seeded)	No. of clones with extended life span[b]	Immortalized cell lines	
			No. of clonal populations[c]	Result with pooled populations[d]
pLXSN	5	0/3	0/3	−
pLXSN6bE6	2	0/2	0/2	−
pLXSN6bE7	6	0/6	0/6	−
pLXSN6bE6E7	4	0/4	0/4	−
pLXSN16E6	3	0/3	0/3	−
pLXSN16E7	~100	9/9	3/9	+
pLXSN16E6E7	~500	8/8	8/8	+

[a] Cells were seeded at 10^3 cells per 100-mm plate on four separate plates. The cells were fed keratinocyte growth medium for 14 days. Two plates were fixed and stained to determine the colony-forming efficiency (CFE), and colonies were isolated from the other two plates.

[b] Number of clones that continued to proliferate past 3 months/number of unique clones that were isolated at passage 6.

[c] Number of unique clones that continued to proliferate at passage 30 or past 8 months/number of unique clones that were isolated at passage 6.

[d] −, No immortalized lines obtained; +, immortalized lines obtained.

strated two copies of the 16E6E7 provirus. Multiple integration is an infrequent event in retroviral gene transfers done at a multiplicity of infection of approximately 0.1. Therefore, the DNA analysis suggested that during growth of extended-life-span and immortalized cell lines, there was a selection for multiple copies of the E7 gene in the absence of the E6 gene.

The E7 protein was detected by radioimmunoprecipitation of labeled cell extracts with a polyclonal rabbit serum (8, 13). The level of E7 protein in the pooled population of cells which contained E7 or E6E7 tended to increase at late passage (Fig. 4A). The 16E7 cells exhibited high levels of E7 protein at early passage and more at later passages, and they expressed higher levels of the E7 protein than cells which contained 16E6E7 at comparable passage levels. Expression of the E7 protein in two clonal 16E7 immortalized lines is shown in Fig. 4B. The 16E7 lines expressed higher levels of E7 than the cervical carcinoma cell lines Caski, which contains several hundred copies of HPV16, and Siha, which contains one copy. The results showed that the 16E7 protein was efficiently expressed from a long terminal repeat promoter in HFE cells. Other factors besides promoter usage may have contributed to the overall level of the E7 protein. The results from the DNA analysis, showing multiple integrated copies of the E7 provirus in the HPV16 E7 lines, suggested that the number of copies of the gene was one of these factors.

High levels of the E7 protein appeared to be correlated with immortalization by E7 alone. However, four subclones derived from one originally infected cell were analyzed, and all expressed similar levels of the E7 protein, yet only two subclones yielded immortalized lines. Though all the immortalized lines emerged from clones expressing high or intermediate levels of E7 and no clones emerged from those expressing low levels of E7, not all clones from the highly expressing clones became immortalized (data not shown). Thus, although the amount of E7 protein seemed to be important, other changes in the cells which contributed to the immortalized phenotype occurred.

Transformation by the small DNA tumor viruses likely occurs by modulation of the activity of cellular proteins involved in growth regulation. Whereas the p53 protein is

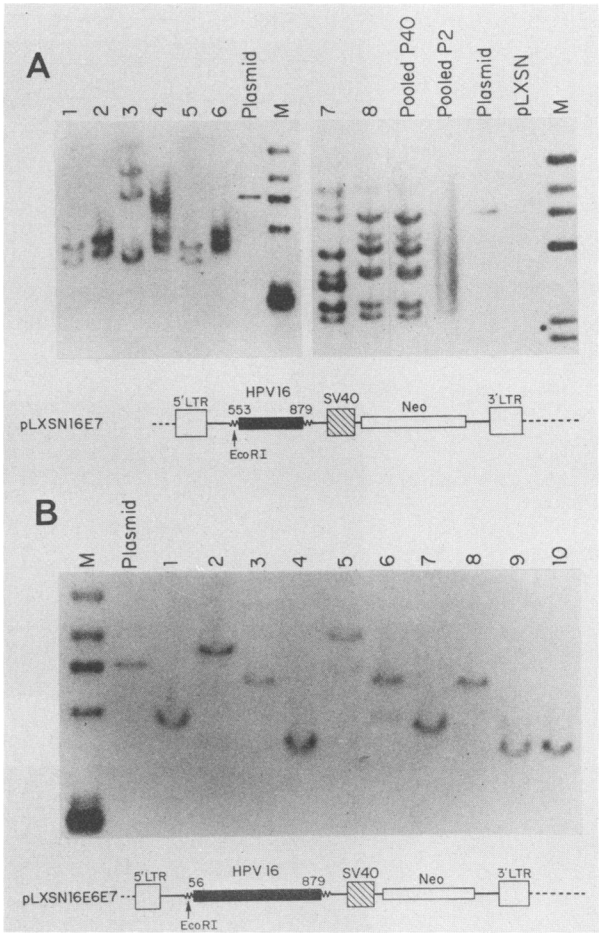

FIG. 3. Analysis of provirus DNA. Southern blot analyses of extended-life-span and immortalized HFE clones which contained the HPV16 E7 (A) or E6E7 (B) provirus are shown. Diagrams of the proviruses with the *Eco*RI sites indicated are below the autoradiograms. Cellular DNA was digested with *Eco*RI, electrophoresed in an agarose gel, and transferred to nitrocellulose. The blot was hybridized to a ^{32}P-labeled DNA fragment which contained the HPV16 E6E7 genes (nucleotides 56 to 875). (A) Lanes 1 through 8, Clonal lines; Pooled P40, pooled populations at passage 40; Pooled P2, pooled population at passage 2; Plasmid, pLXSN16E7; pLXSN, control vector; and M, DNA molecular weight markers. (B) Lanes 1 through 10, Clonal lines; Plasmid, pLXSN16E6E7; and M, molecular weight markers.

stabilized and presumably altered by binding to the polyomavirus T antigens and adenovirus E1B 55K protein (27), HeLa cells and HPV16-transformed human keratinocytes contain low or undetectable levels of p53, suggesting that E6 might in fact facilitate degradation of p53 (43). The findings that human keratinocytes can be transformed without the participation of the E6 protein can be explained several ways. First, inactivation of p53 may not be an obligatory step in transformation. Perhaps the overexpression of E7 more completely inactivated the retinoblastoma gene product (2, 7) or perhaps other cellular proteins such as p107 and p300 bind to E7, thus disrupting growth regulation. Other negative or positive regulators may be affected in the cells immortalized by HPV16 E7. Alternatively, the p53 gene may be altered in the cells which lack E6. Experiments to test these hypothesis are in progress.

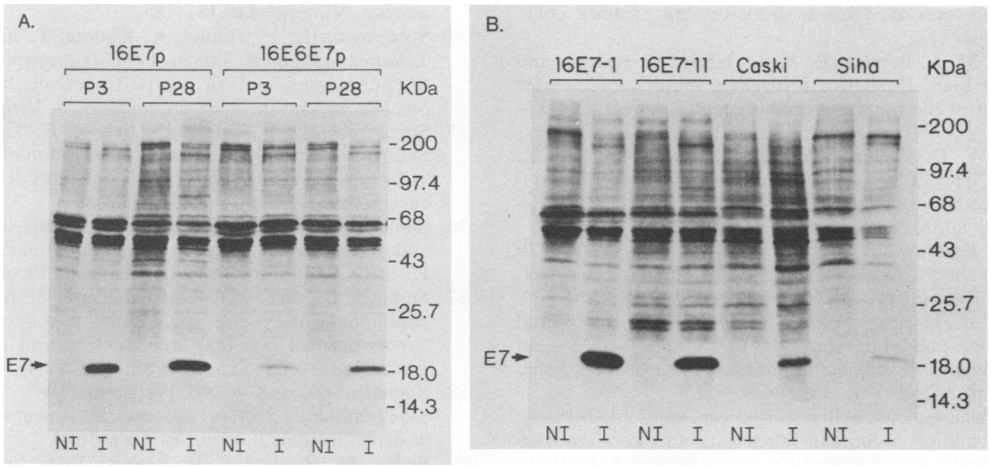

FIG. 4. (A) Immunoprecipitation of the E7 protein. The immunoprecipitation of HPV16 E7 protein from pooled populations of HFE cells which contained HPV16 E7 or E6E7 genes at passage 3 and at passage 28 is shown. Cells were labeled in keratinocyte growth medium which contained [^{35}S]cysteine and [^{35}S]methionine. Cell extracts were incubated with polyclonal rabbit nonimmune (NI) or immune (I) serum. Immunoprecipitated proteins were electrophoresed on a sodium dodecyl sulfate–17.5% polyacrylamide gel. The E7 protein is indicated by an arrow. (B) Immunoprecipitation of clonal lines of HFE cells immortalized by HPV16 E7 and of cell lines derived from cervical carcinomas (Caski and Siha).

In summary, this study showed that the E6 and E7 genes of HPV6b did not have any activity in prolonging the life span of HFE cells in culture. The HPV16 E7 gene was sufficient to immortalize primary HFE cells; however, the addition of HPV16 E6 increased the efficiency of immortalization. All 16E7 lines exhibited multiple copies of the E7 gene and expressed high levels of the E7 protein.

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