Human Papillomavirus Type 16 Transformation of Primary Human Embryonic Fibroblasts Requires Expression of Open Reading Frames E6 and E7

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Human papillomavirus 16 (HPV-16) early genes were tested for their ability to transform primary human embryonic fibroblasts (WI38) or to extend the life span of the fibroblasts. The expression plasmids containing the transcriptional unit for the HPV-16 subgenomic DNA fragments controlled by the simian virus 40 promoter were introduced into WI38 cells with the aid of neomycin selection, and the drug-resistant cells that survived long-term culture were characterized. The fragments containing E6E7 open reading frames (ORFs) intact were found to be capable of extending the life span of WI38 cells. From the results obtained with frameshift mutations in either ORF and from those obtained by cotransfection with the separate plasmids containing E6 and E7 ORFs, it was concluded that HPV-16 transformation (extension of the life span) of human fibroblasts requires expression of genes E6 and E7.

The capabilities of human papillomavirus (HPV) types 16 (6) and 18 (4) (HPV-16 and HPV-18) DNA to transform rodent and human cells cultured in vitro (3, 5, 11-13, 17, 22, 36-38) are believed to constitute partial evidence that these HPVs cause cervical cancers. The cancer cells frequently harbor the HPV DNAs, which are integrated within cell DNA and contain the E6 and E7 open reading frames (ORFs) intact (2, 14, 18, 20, 25, 26), suggesting that either or both of the genes are involved in carcinogenesis. In fact, the human cervical cancer cells contains E6 and E7 proteins, the latter as the most abundant HPV protein (1, 28-30). For rodent cells, the in vitro transforming functions of HPV-16 and HPV-18 have been mapped to the E7 gene (11, 13, 21, 33, 37), but for human cells they have not yet been mapped.

HPV-16 DNA can transform primary human fibroblasts and keratinocytes (5, 22). HPV-16 transformation of these human cells seems to be different from the transformation of rodent cells, because the transformed human cells lack morphological characteristics of fully transformed cells (35) and appear to be less malignant. The HPV-16-transformed fibroblasts have a life span longer than that of normal primary fibroblasts (about 50 population doublings [PDs]) but are not immortalized (22). The HPV-16-transformed keratinocytes, on the other hand, are immortalized and can grow indefinitely, but they are not much different morphologically from the untransformed keratinocytes (5, 22). In this study, we examined the subgenomic HPV-16 DNA fragments with various ORFs (presumably coding for early genes) placed under the control of the simian virus 40 promoter for their capability to transform primary human embryonic lung cells (WI38 fibroblasts [9]). We found that expression of both E6 and E7 ORFs was required for extension of the life span (transformation) of primary human fibroblasts.

HPV-16 DNA ORFs (8, 12, 27) and the DNA fragments tested for human cells are shown in Fig. 1A, and construction of the expression plasmids with and without the neomycin resistance gene was described previously (11). Each of these fragments, isolated and appropriately processed by

standard methods (16), was inserted in a sense orientation into pSV2-0, an expression vector with simian virus 40 transcriptional regulatory elements (34), at the *Hind*III site (Fig. 1B). The constructed plasmids were designated pSV2-E1, pSV2-E2E4E5, pSV2-E6, pSV2-E7P, pSV2-E6E7, and pSV2-E5 (11). From these pSV2 plasmids, the *Pvu*II (or *Acc*II)-*Bam*HI fragments containing the HPV ORFs placed under the control of the simian virus 40 early promoter were isolated and inserted into the second expression vector, pSV2neo (32), at the *Bam*HI site (Fig. 1C). The final product was the expression plasmid composed of three portions: the pBR322-derived portion allowing the plasmid to replicate in *Escherichia coli*, the transcriptional unit for the HPV ORF, and the transcriptional unit for the neomycin resistance gene. The constructed pSVneo expression plasmids containing HPV ORFs were designated pSVneo-E1, pSVneo-E2E4E5, pSVneo-E5, pSVneo-E6, pSVneo-E6E7, and pSVneo-E7P (11). These pSVneo plasmids and their backbone vector (pSV2neo) (2.5 µg of DNA per 10⁵ cells) were transfected to monolayer cultures of WI38 cells (at 22 to 27 PDs), which have a life span of 50 PDs, by the calcium precipitation method (7). Two days after transfection, the cultures were replated at a split ratio of 1:20 and maintained for 15 days with refeeding with Dulbecco modified Eagle medium containing 10% fetal bovine serum and G418 (300 µg/ml for 5 days and 100 µg/ml for 10 days). Thereafter, the cultures were kept without G418. The drug-resistant colonies (2 to 15 colonies per 10-cm dish) were maintained and processed essentially as described by Pirisi et al. (22).

Among the pSVneo-expressing plasmids containing the E1 to E7 ORFs, only pSVneo-E6E7 could extend the life span of WI38 cells. During the period of 20 to 30 days after transfection, all of the drug-resistant colonies of WI38 cells transfected with pSV2neo, pSVneo-E2E4E5, and pSVneo-E5 ceased to grow or disappeared from the plastic surface. During the period of 30 to 50 days after transfection, some of the E1, E6, E6E7, and E7 WI38 cells were growing. Thereafter, only E6E7 cells continue to grow and reached confluence in a single dish, when they were replated. The PDs were calculated from the assumption that a single colony had derived from a single cell initially seeded. The G418-resistant

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FIG. 1. Construction of expression plasmids. (A) HPV-16 subgenomic DNA fragments tested for transforming effect on human cells. Nucleotide numbers and ORFs are based on published data (8, 12, 27). The lines numbered at both ends (numbers show the end nucleotides after trimming with Klenow fragment to obtain flush ends) indicate the locations of DNA segments: E6 (from *DdeI* to *DdeI*), E7P (from *PvuII* to *PstI*), E6E7 (from *DdeI* to *PstI*), E1 (from *PvuII* to *HincII*), E2E4E5 (from *Tth1111* to *StuI*), and E5 (from *DdeI* to *StuI*). (B) pSV2 plasmids with HPV-16 early genes. The DNA fragments shown in panel A with *HindIII* linkers added at both ends were each inserted in a sense orientation into pSV2-0 (34) at the *HindIII* site. Arrow indicates the direction of transcription. The products were pSV2-E6, pSV2-E7P, pSV2-E6E7, pSV2-E1, pSV2-E2E4E5, and pSV2-E5 (11). (C) pSVneo plasmids with HPV-16 early genes. The *PvuII* (or *AccII*)-*BamHI* fragments from the pSV2-plasmids shown in panel B were inserted into pSV2neo (32) at the *BamHI* site by using *BamHI* linkers. The products were pSVneo-E6, pSVneo-E7P, pSVneo-E6E7, pSVneo-E1, pSVneo-E2E4E5, and pSVneo-E5 (11). (D) Sites of mutation in pSVneo-E6E7 frameshift mutants. The mutation was introduced at the *Fnu4HI* (F), *NdeI* (N), *TaqI* (T), or *AccI* (A) site in the E6E7 fragment as described in the text. The products were pSVneo-E6(F)E7, pSVneo-E6(N)E7, pSVneo-E6(T)E7, and pSVneo-E6E7(A). (E) pSVneo plasmids with separate transcription units for E6 and E7 ORFs. The E7 or E6 transcription unit from pSV2-E7P or pSV2-E6 was inserted into pSVneo-E6 or pSVneo-E7P at the *EcoRI* site so that the two units had the same orientation for transcription. The products were pSVneo-E6/E7 and pSVneo-E7/E6.

colonies except for E6E7 ceased to grow, usually before they reached 30 PDs, suggesting that exposure of WI38 cells to G418 shortens the life span or calculated PDs. The E6E7 cells, when they exceeded 70 PDs, were frozen and stored. Since extension of the life span is characteristic of the human

TABLE 1. Transformation frequency of WI38 cells induced by expression plasmids with HPV-16 subgenomic DNA fragments

Plasmid ^a	Transformation frequency ^b in expt:				
	1	2	3	4	5
pSV2neo(vector)	0/4	0/4	0/2	0/2	0/2
pSVneo-E1	0/4	NT ^c	NT	NT	NT
pSVneo-E2E4E5	0/4	NT	NT	NT	NT
pSVneo-E5	0/4	NT	NT	NT	NT
pSVneo-E6	0/4	0/4	0/2	0/2	0/2
pSVneo-E7P	0/4	0/4	0/2	0/2	0/2
pSVneo-E6E7	4/4	2/2	2/2	1/2	2/2
pSVneo-E6(F)E7	NT	NT	NT	0/4	0/4
pSVneo-E6(N)E7	NT	NT	NT	0/4	0/4
pSVneo-E6(T)E7	NT	NT	NT	0/4	0/4
pSVneo-E6E7(A)	NT	NT	NT	0/4	0/4
pSVneo-E6/E7	NT	NT	2/2	2/2	2/2
pSVneo-E7/E6	NT	NT	NT	2/2	2/2
pSVneo-E6 and pSV2-E7P ^d	NT	NT	NT	2/2	2/4
pSV2-E6 and pSVneo-E7P ^d	NT	NT	NT	2/2	2/2

^a Construction of plasmids used for transfection is described in the text and the legend to Fig. 1.

^b Ratio of cultures with transformed cells (showing a life span longer than that of normal WI38 cells, ca. 50 PDs) to cultures initially seeded.

^c NT, Not tested.

^d Cotransfection.

fibroblasts transformed by the whole HPV-16 genome (22), we concluded that transfection with HPV-16 E6E7 DNA fragments induced transformation of WI38 cells.

The transfection experiments with pSV2neo, pSVneo-E6, pSVneo-E7P, and pSVneo-E6E7 were repeated four times, and the results of experiments 1 through 5 (Table 1) indicate that only the E6E7 fragment (Fig. 1A) could extend the life span of WI38 cells. Because the E6E7 colonies often increased their numbers by two- to threefold during the observation period, probably because of migration of the transformed cells (by floating) from the preexisting colonies, it was difficult to estimate the frequency of transformation (extension of the life span) from the colony number. Thus, the transformation frequency in Table 1 was expressed as the ratio of the cultures that reached confluence (and were passaged) after the long-term cultivation to those seeded initially, although a single positive dish usually contained multiple colonies at the early stage. The transformed cells were allowed to grow until about 70 PDs, with passages. During the passages, some of the cell strains from separated cultures or their clonal derivatives were characterized.

Properties (and morphology) of WI38 cells transformed by the HPV-16 E6E7 ORFs were slightly different among the strains (W67A, W67B, W67C, and W67D) and from those of untransformed WI38 cells (Fig. 2). W67A cells (Fig. 2B) morphologically resembled the untransfected WI38 cells (Fig. 2A), but the W67A cultures showed a lowered saturation density, i.e., approximately half that of WI38 cultures. At a high density, W67A cultures released more cells in the culture medium than WI38 cultures. By contrast, W67B cells (Fig. 2C) were morphologically different from cells of other strains or WI38 cells. They appeared smaller, thicker, and less elongated than WI38 cells. W67C cells (Fig. 2D) appeared thicker and, even after their 69 PDs, grew better than WI38 cells (at 25 PDs) and other E6E7 strains. W67D cells resembled W67A cells. In a soft-agar (0.4% agarose) medium, only W67B cells (Fig. 2G) showed anchorage-independent growth, but the number of large colonies (25 days after

FIG. 2. Human WI38 cells transformed after transfection with the HPV-16 DNA fragment containing E6E7 ORFs: morphology of cells (A through D) and anchorage-independent growth (E through H). (A and E) Untransformed WI38 cells; (B and F) W67A cells; (C and G) W67B cells; (D and H) W67C cells. Phase-contrast photomicrographs (A through D) were taken 5 (A through C) or 4 (D) days after 10^5 cells were seeded in a 6.0-cm dish. Pictures of cells in a 0.4% agarose medium were taken 17 (E) or 25 (F through H) days after 3×10^4 to 10^5 cells were seeded in a 6.0-cm dish. Bar, 100 μ m.

seeding) was less than 1% of the cells initially seeded in an agarose medium. WI38 (Fig. 2E), W67A (Fig. 2F), and W67D cells did not grow at all in an agarose medium, but W67C cells (Fig. 2H) formed small clusters, each consisting of a few cells, 25 days after seeding. Because not all of the E6E7 strains showed anchorage-independent growth, some unknown factor other than HPV proteins probably affects malignancy of the cells.

Presence of HPV-16 DNA and mRNA in the representative transformed cells was examined by Southern (DNA) (31) and Northern (RNA) (15) blot methods (Fig. 3A and B). Southern blot analyses showed that HPV-16 DNAs in W67A and W67B cells were integrated within cell DNA, as HPV-16-specific DNA (detected by hybridization to 32 P-labeled [23] subgenomic HPV-16 DNA) moved together with high-molecular-weight DNA after digestion of cellular DNA with *Xho*I (Fig. 3A, lanes 2, 4, 6, and 8). Digestion of cellular DNA with *Bam*HI released the transcriptional unit of the E6E7 fragments (Fig. 3A, lanes 3, 5, 7, and 9), with some rearrangement in W67B cells (Fig. 3A, lanes 7 and 9). To confirm that the transformed cells were of human origin, the human *Alu* DNA (10) probe was allowed to hybridize to DNAs from WI38, W67A, and W67B cells. The hybridization patterns of W67A and W67B cells were not different from that of WI38 cells (data not shown). RNA blot analyses showed that the HPV-16 DNA fragments in the E6E7 cells

(W67A, W67B, W67C, and W67D) were transcriptionally active. Poly(A) RNAs from E6E7 cells contained transcripts hybridizable to HPV-16 subgenomic DNA fragments containing E6 or E7 ORFs (Fig. 3B).

The data presented above indicate that the HPV-16 fragment containing E6E7 ORFs intact induces transformation (extension of the life span) and sometimes anchorage-independent growth of WI38 cells, but it is not clear whether the HPV-16 transformation results from expression of genes E6 and E7 or requires the intact structure (possibly affecting expression of either ORF) of E6E7 ORFs in the HPV genome. We constructed E6E7 frameshift mutants and pSVneo expression plasmids containing two independent transcriptional units for E6 and E7 ORFs and tested their transforming capabilities. We also tested the effects of cotransfection with the separate E6 and E7 expression plasmids on transformation.

The frameshift mutations were introduced into the E6 or E7 ORF in pSVneo-E6E7 by using restriction enzymes *Acc*I, *Fnu*4HI, *Nde*I, and *Taq*I (Fig. 1D). The shorter *Bam*HI fragment was isolated from pSVneo-E6E7 (Fig. 1C); circularized by ligation; digested with *Acc*I, *Nde*I, or *Taq*I; blunt-ended by filling with Klenow fragment; and circularized by blunt-end ligation. The circular mutated E6E7 DNA fragment was digested with *Bam*HI and inserted again in the original orientation into pSV2neo. The resulting plasmids

FIG. 3. Presence of HPV-16 DNA and mRNA in transformed WI38 cells. (A) Southern blot (31) analyses of the WI38 cells transformed by the HPV-16 E6E7 DNA fragment. *Bam*HI-digested pSVneo-E6E7 DNA (30 pg) (lane 1) shows the position of released transcriptional unit of the E6E7 ORFs (arrow). Total cellular DNAs (10 μ g per lane) from W67A cells (lanes 2 and 3), cloned W67A cells (lanes 4 and 5), W67B cells (lanes 6 and 7), and cloned W67B cells (lanes 8 and 9) were digested with *Xho*I (lanes 2, 4, 6, and 8) or *Bam*HI (lanes 3, 5, 7, and 9), electrophoresed in a 1% agarose gel, transferred to a nitrocellulose membrane, and hybridized to 32 P-labeled (23) HPV-16 E6 fragments. (B) RNA blot (15) analyses of RNA from the WI38 cells transformed by the E6E7 fragment. Poly(A) RNAs from W67A (lanes 1 and 3), W67B (lanes 2 and 4), W67C (lanes 5 and 8), cloned W67C (lanes 6 and 9), and W67D (lanes 7 and 10) cells were electrophoresed in 1.3% agarose containing 2.2 M formaldehyde–0.02 M sodium phosphate buffer (pH 7.4), transferred to nitrocellulose membranes, and hybridized to 32 P-labeled E6 (lanes 1, 2, and 5 through 7) or E7 (lanes 3, 4, and 8 through 10) fragments (Fig. 1A). Amount of RNA electrophoresed was 5 μ g per lane (lanes 1 through 4) or 3 μ g per lane (lanes 5 through 10). Molecular sizes of marker RNA are given in kilobases (arrows). (C) Blot analyses of RNA from the WI38 cells cotransformed by the separate plasmids with E6 and E7 ORFs. Cell strains W67K and W67M were obtained after cotransfection with pSV2-E6 and pSVneo-E7P, and strain W67L was obtained after cotransfection with pSVneo-E6 and pSV2-E7P. Poly(A) RNA (lanes 1, 2, 5, and 6) and cytoplasmic RNA (16) (lanes 3, 4, 7, and 8) were examined by the RNA blot method as done for panel B. Amount of RNA was 2 μ g [poly(A) RNA] or 10 μ g (cytoplasmic RNA) per lane. Lanes: 1 and 5, RNA from W67K clone 3; 2 and 6, RNA from W67L; 3 and 7, RNA from W67K clone 2; 4 and 8, RNA from W67M. The 32 P-labeled probes were the E6 fragment (*Dde*I–*Taq*I) from nucleotides 25 to 505 (for lanes 1 through 4) and the E7 fragment (*Pvu*II–*Pst*I) from nucleotides 554 to 879 (for lanes 5 through 8). Molecular sizes of marker RNA are given in kilobases (arrows).

with mutations at *Acc*I, *Nde*I, and *Taq*I sites (Fig. 1D) were designated pSVneo-E6E7(A), pSVneo-E6(N)E7, and pSVneo-E6(T)E7, respectively. For *Fnu*4HI mutation, the *Hind*III E6E7 fragment was isolated from pSV2-E6E7 (Fig. 1B), circularized by ligation, digested with *Fnu*4HI, blunt-ended by Klenow fragment, circularized by ligation, digested by *Hind*III, and returned in the original orientation into pSV2-0. The mutated E6E7 transcriptional unit was isolated by digestion with *Acc*II. The fragment was added with *Bam*HI linkers, digested with *Bam*HI, and inserted into pSV2neo at the *Bam*HI site in the orientation shown in Fig. 1C. The final product with a mutation at the *Fnu*4HI site (Fig. 1D) was designated pSVneo-E6(F)E7. The nucleotide sequences around the sites of mutation were determined, for confirmation of structure, by the M13-dideoxy method (19, 24). Plasmids pSVneo-E6(F)E7, pSVneo-E6(N)E7, and pSVneo-E6(T)E7 were found to induce focal transformation of rat 3Y1 cells, which are transformable by the E7 gene alone (11), but pSVneo-E6E7(A) was not (data not shown). When tested in WI38 cells, none of these mutant plasmids could extend the life span of the cell (Table 1). The data strongly suggest that the transformation of WI38 cells requires the two intact ORFs E6 and E7 in pSVneo-E6E7 to be expressed.

Transformation (extension of the life span) of WI38 cells by independent expression of E6 and E7 ORFs was tested by transfection with single pSVneo plasmids containing separate transcription units for these ORFs and by cotransfection with independent E6 and E7 plasmids. The E7 or E6 transcription unit (*Pvu*II–*Bam*HI fragment) from pSV2-E7P or pSV2-E6 was inserted into pSVneo-E6 or pSVneo-E7P at the *Eco*RI site (Fig. 1E), and the resulting plasmid was designated pSVneo-E6/E7 or pSVneo-E7/E6. Like pSVneo-E6E7, pSVneo-E6/E7 and pSVneo-E7/E6 induced transformation of WI38 cells (Table 1). Cotransfection with pSVneo-E6 and pSV2-E7P or with pSV2-E6 and pSVneo-E7P also yielded transformed WI38 cells (Table 1). Transcription of E6 and E7 ORFs was examined by RNA blot analyses (15) with the WI38 cells transformed by pSVneo-E6/E7 (data not shown) or by cotransfection (Fig. 3C). The cell strains (W67K, W67L, and W67M) and their clonal derivatives contained both E6- and E7-specific mRNAs. The data, together with the results obtained with the E6E7 frameshift mutants, indicate that expression of HPV-16 genes E6 and E7 is required for transformation (extension of the life span) of primary human fibroblasts.

Interestingly, the E7 gene, which is immortalizing and transforming for rat cells (11, 13), failed by itself to transform primary human fibroblasts (Table 1). The E6 gene, which is weakly immortalizing for primary rat cells (13), could be partially effective in extending the life span of WI38 cells. In two of five experiments summarized in Table 1, E6 cells survived much longer than pSV2neo (vector)-transfected WI38 cells, reached confluence, and continued to grow until 45 PDs in one case. However, extension of the life span to longer than that of normal (unexposed to G418) WI38 cells was observed only when genes E6 and E7 were intact (Table 1). The lack of transforming function of pSVE6(N)E7 (Table 1) suggests that if E6* protein, which is the second protein potentially encoded by E6 ORF and generated by splicing (25, 29), is produced in WI38 cells, it cannot substitute for E6 protein in transformation of WI38 cells. It remains to be investigated whether or not other types of human cells, such as keratinocytes, require these two genes for transformation. Possibly, the requirement of the HPV genes for transformation varies with the species and type of cells.

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