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Identification of a Transforming Gene of Human Papillomavirus Type 16

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Previously, we observed sequential two-step alteration, growth stimulation, and progression to a more malignant state in NIH 3T3 cells transfected by human papillomavirus type 16 (HPV-16) DNA. In this study, we prepared a cDNA library from RNA extracted from cells transfected with the HPV-16 DNA and isolated cDNA clones which had growth-stimulating activity. Analysis of these cDNA clones indicated that the E7 open reading frame alone is responsible for inducing both steps of this cell transformation.

Human papillomaviruses (HPVs) have been implicated as etiological agents of human cervical cancer (2, 34). Among several types of HPVs found in human cervical cancer cells, HPV-16 DNA is the most commonly detected in biopsy specimens (8, 12). Recently, HPV-16 DNA has been shown to transform rodent fibroblasts by itself (16, 20, 33) and primary rodent fibroblasts in combination with Ha-*ras* oncogene (19). The HPV-16 DNA has also been shown to immortalize human fibroblasts and keratinocytes (7, 23).

Previously, we analyzed the process of transformation of rodent fibroblast cell lines by the HPV-16 DNA and reported that almost all the NIH 3T3 cells containing HPV-16 DNA were stimulated to grow in monolayer and in soft agar without apparent morphological changes, presumably by the immediate effect of the HPV-16 gene function (20). Upon prolonged incubation of these growth-stimulated cells, a small proportion of the cells became morphologically transformed. Cells at the first stage were nontumorigenic, whereas those at the second stage were tumorigenic in nude mice (T. Noda, S. Showalter, and Y. Ito, unpublished observation). In the rat fibroblast cell line 3Y1 (17), only the first stage was observed.

In this study, we prepared a cDNA library by using RNA extracted from cells transfected with the HPV-16 DNA and obtained cDNA clones functionally active for this transformation. From deletion analysis of these cDNA clones, we identified a gene responsible for both steps of cell transformation.

mRNA was prepared by using oligo(dT)-cellulose chromatography (Pharmacia, Inc.) from total cellular RNA extracted by the method of Chirgwin et al. (3) from 1×10^8 M-3Y1 clone 3 cells, which were transfected with pSVHPV16-M (20) containing a monomer of the HPV-16 DNA and a neomycin resistance gene. The HPV-16 DNA which we used in this experiment has the split E1 open reading frame (ORF) (27). A cDNA library consisting of 1×10^5 independent clones was constructed by using the Okayama-Berg vector (21). Thirty cDNA clones were found to be derived from viral mRNA by colony hybridization as described by Hanahan and Meselson (14) by using the whole genomic DNA of HPV-16 as a probe.

The sizes of the fragments generated from the cDNA clones by *Bam*HI digestion represented the sizes of the cDNA portions shown in Table 1, since, within the Okayama-Berg vector, there are two *Bam*HI cleavage sites near the cloning site, one at each side. The majority of the cDNA clones (27 of 30) were found to represent the early

TABLE 1. Functional analysis of cDNA clones derived from viral transcripts in M-3Y1 clone 3 cells

Clone	Size[a] (kb)	Saturation density (10^6 cells/10-cm dish)	Colony-forming efficiency (%)
Neo[b]		2.4	0.3
52	5.0	2.2	0.1
57	3.4	2.9	0.1
41	3.4	3.2	1.1
17	2.6	3.1	0.1
13	2.4	2.6	0.1
43	2.3	2.6	0.2
5	1.8	2.8	0.4
7	1.8	3.0	0.2
40	1.7	2.8	0.8
46	1.7	2.7	0.7
53	1.7	2.6	0.1
60	1.7	3.0	0.0
92	1.7	2.8	0.1
22	1.7	8.3	42.3
31	1.7	6.4	27.2
61	1.6	6.9	38.8
11	1.6	3.2	0.6
34	1.4	3.0	0.2
18	1.1	3.1	0.2
28	1.1	2.7	1.0
64	1.1	2.8	0.3
67	1.1	2.6	0.5
27	1.0	2.7	0.5
30	1.0	2.3	0.9
44	1.0	2.6	0.8
48	1.0	2.6	0.8
29	1.0	3.2	0.7
56	1.0	3.2	0.4
4	0.8	2.2	0.4
15	0.6	3.8	0.3

[a] The plasmids containing cDNA were digested by a *Bam*HI into two fragments: a cDNA portion and a vector. The size of the fragment corresponding to the cDNA is indicated. In the case of plasmids containing one *Bam*HI site within the cDNA portion (cl-52 and cl-92), the size of each fragment corresponding to the cDNA insert was combined. kb, Kilobase.

[b] Neo, 3Y1 cells transfected only with pSV2-neo as a control.

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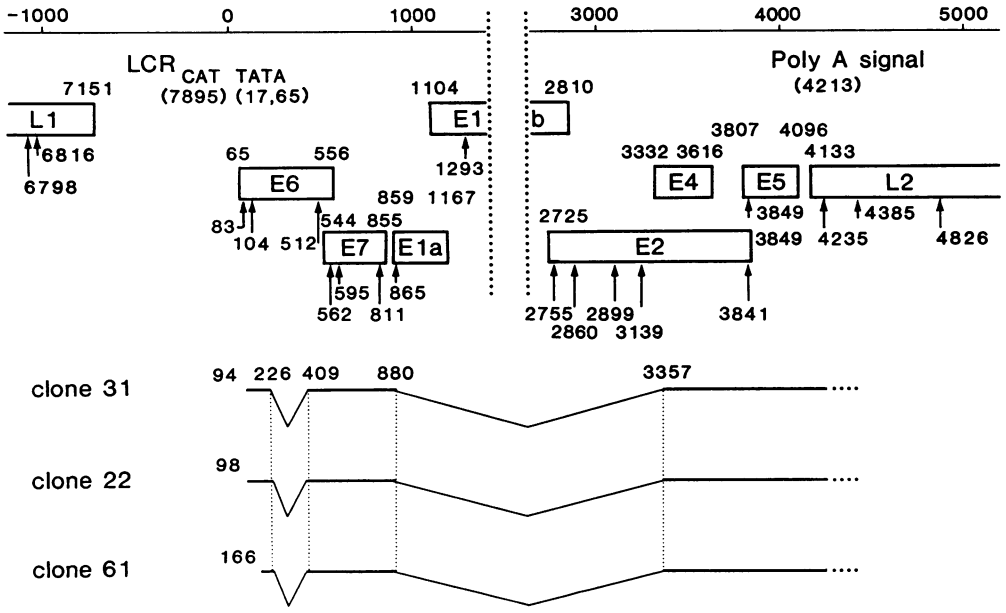

FIG. 1. Structural analysis of cDNA clones that have growth-stimulating activity. The 5′ end and donor and acceptor sites of splicing in cl-31, cl-22, and cl-61 are indicated in terms of the nucleotide number of the HPV-16 genome. Above these, the positions of ORFs are shown as open boxes. The nucleotide number above each ORF indicates the first and last nucleotide in each ORF, and those with arrows indicate the position of a possible translation start signal (ATG) in each ORF. The long control region (LCR) and positions of CAT and TATA boxes are indicated. A possible polyadenylation signal of the early region is also indicated.

region of the HPV-16 genome by Southern blot analysis (data not shown).

To examine the growth-stimulating activity of these cDNA clones, 3Y1 cells were cotransfected with each cDNA clone together with pSV2-neo (31), a plasmid capable of expressing a bacterial neomycin resistance gene that makes eucaryotic cells resistant to the chemical G418, and a G418-resistant population of 3Y1 cells was obtained. DNA transfection was performed by using the calcium phosphate precipitation method (13). Growth-stimulating activity was assessed by the saturation density and colony-forming efficiency of G418-resistant cell populations as described below. 3Y1 cells and cell clones derived from 3Y1 cells after DNA transfection were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (KC Biochemical Inc., Lenexa, Kans.). NIH 3T3 cells and their clonal derivatives were maintained in Dulbecco modified Eagle medium containing 10% calf serum (Colorado Serum Corp., Denver, Colo.). To determine saturation densities of cells, 5×10^5 cells were seeded in a 100-mm dish and incubated for 17 days, with the medium changed twice a week. To determine colony-forming efficiency, 1×10^4 cells were incubated in Dulbecco modified Eagle medium containing 10% fetal calf serum and 0.28% agarose (Seakem; FMC Corp., Rockland, Maine). The number of cells that formed colonies were counted, and the efficiency of colony formation was calculated after a 3-week incubation.

Of 30 cDNA clones, only 3 clones, cl-22, cl-31, and cl-61, stimulated 3Y1 cells to grow beyond the saturation density of parental 3Y1 cells and to form small colonies efficiently in soft agar (Table 1).

Nucleotide sequencing of these cDNAs, determined by the dideoxy method (24), showed that exactly the same two regions in the genome were spliced out in all three cDNA clones. All three clones appeared to have used polyadenylation signal at nucleotide (nt) 4213, although this was not specifically confirmed by sequencing (Fig. 1). One splicing occurred within the E6 open reading frame (ORF), which deleted 182 nucleotides (from nt 227 to nt 408). Because of this splicing, the E6 ORF in these cDNA clones was split into two ORFs, a long amino-terminal ORF (nt 104 to nt 414), with the potential to encode 43 amino acids and a short ORF (nt 512 to nt 556) with the potential to encode 15 amino acids (Fig. 2). Interestingly, the same type of splicing within the E6 ORF has been reported for transcripts of HPV-18 (1), which is implicated in the formation of cervical cancer cells (25), whereas it has not been observed in transcripts of HPV-1, HPV-6, and HPV-11 genomes (4–6, 10, 11) which are considered to be etiological agents of benign tumors. Although we could not detect any function for the product of this altered E6 ORF in the transformation of rodent fibroblast cell lines in the present studies, this product might play an important role in the process of carcinogenesis. In cl-61, however, only the small ORF has coding potential, because the 5′ end of the clone is at nt 166 and there is no ATG codon between nt 166 and nt 512 and an ATG in the 5′ region of the Okayama-Berg vector was not in frame with the E6 ORF. The entire coding region of the E7 ORF is represented in all three cDNA clones. The region spanning almost the entire E1 ORF and 5′ half of the E2 ORF (nt 881 to nt 3356) was also spliced out, and this splicing generated a fused ORF of E1 and E4, which could encode a polypeptide with 92 amino acids (Fig. 2). This pattern of splicing is commonly observed in transcripts of HPVs (4, 5), although no function of this product has been found. All three positive cDNA clones possessed four ORFs: the short carboxy-terminal E6 ORF, the E7 ORF, the fused ORF of E1 and E4, and the E5 ORF (Fig. 2).

To identify the ORF responsible for the growth-stimulating activity, we introduced deletions into cl-61 and examined the biological activity of these deletion mutants (cl-61 *dl*-1 through *dl*-5) (Fig. 2). *dl*-1, *dl*-2, and *dl*-3 were con-

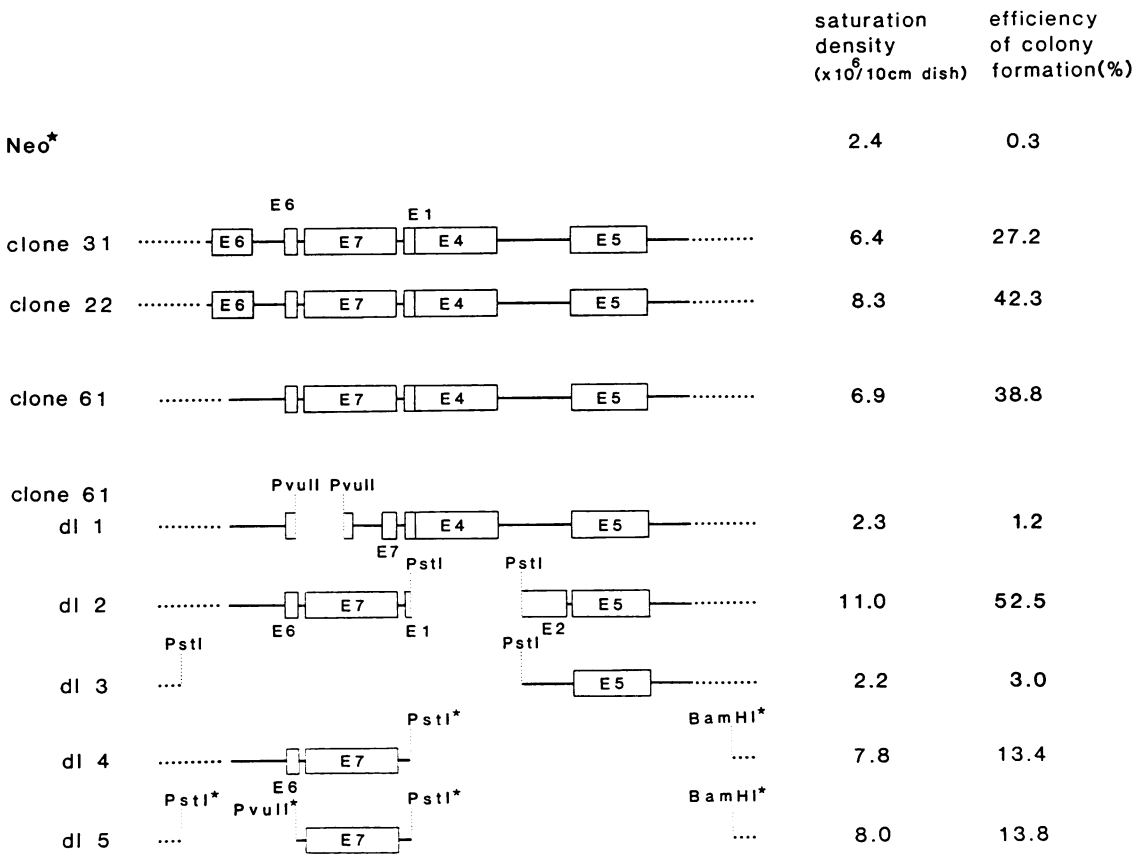

FIG. 2. Growth-stimulating activity of cDNA clones and their deletion mutants. Five different mutant cDNAs were generated by deleting different restriction enzyme fragments from cl-61. ORFs in each clone are indicated as open boxes. Positions of restriction enzyme sites used to generate deletion mutants are indicated. Restriction enzyme sites marked with asterisks indicate that the sites were modified for the purpose of ligation, as described in the text. Saturation density and efficiency of colony formation of 3Y1 cells transfected with each clone are also indicated. Neo*, 3Y1 cells transfected with pSV2-neo as a control.

structed by removing the following fragments, respectively, from cl-61 by using the appropriate restriction enzymes: *dl*-1, the *Pvu*II fragment from nt 553 to nt 683; *dl*-2, the *Pst*I fragment from nt 875 to nt 3692; *dl*-3, the *Pst*I fragment from the *Pst*I site in the vector at the 5′ side of the vector-cDNA junction to nt 3692. *dl*-4 was constructed from *dl*-2. The *Bam*HI site at the 3′ side of the poly(dT) tail in the vector and the *Pst*I site at nt 875 were converted to *Bgl*II sites by use of a linker on two steps. The resultant *Bgl*II fragment was removed, and the plasmid DNA was closed by ligation. *dl*-5 was constructed from *dl*-4. The *Pst*I site in the vector at the 5′ side of the vector-cDNA junction was converted to a blunt end. The fragment from this blunt end to the *Pvu*II site at nt 553 was removed by partial *Pvu*II digestion, and the remaining plasmid was closed by ligation.

3Y1 cells were transfected with these mutants in combination with pSV2-neo, and after G418 selection as described above, saturation density and colony-forming efficiency were examined (Fig. 2). Deletions in *dl*-1 and *dl*-3 largely eliminated the growth-stimulating activity, whereas the other three mutants still retained the growth-stimulating activity in 3Y1 cells, although the efficiency of colony formation of *dl*-4 and *dl*-5 was about 25 to 30% of that of cl-61. It appeared that the decrease in the efficiency of colony formation with *dl*-4 and *dl*-5 was due to removal of the E5 ORF from the cDNA sequence (Fig. 2). However, evaluation of the data was difficult because we could not rule out the possibility that *dl*-4 and *dl*-5 were expressed less well as a result of removal of one of two polyadenylation sites in the plasmid during the construction of *dl*-4 and *dl*-5 plasmids. These three positive mutants shared the E7 ORF, and in cl-61 *dl*-5, it was the only ORF present in the plasmid (Fig. 2). A dense cell sheet and colonies in soft agar formed by cl-61 *dl*-5 are shown in Fig. 3e and j, respectively. They are similar to those induced by the entire HPV-16 genome (Fig. 3c and h) or those induced by cl-61 (Fig. 3d and i). It is clear from the results that the E7 ORF is responsible for the growth-stimulating activity of the HPV-16 genome.

The next question was whether this E7 ORF was also responsible for the progression into morphological transformation of growth-stimulated NIH 3T3 cells. To test this possibility, we introduced into NIH 3T3 cells cl-61 *dl*-5 DNA, which expresses only the E7 ORF together with pSV2-neo, and compared its effect on the growth properties of cells with that of pSVHPV16-M, a plasmid containing the entire HPV-16 genome (Fig. 4). Growth of the G418-resistant cell population was strongly stimulated in both cases as expected (data not shown). When we incubated these growth-stimulated cells for 3 weeks as a cell sheet, numerous foci consisting of morphologically transformed cells appeared (Fig. 4d). There was no difference in focus formation between cells that were transfected with cl-61 *dl*-5 or those transfected with pSVHPV16-M (compare Fig. 4d with Fig. 4c). The results indicated that the E7 ORF alone is capable of inducing both the growth stimulation and progression to a more transformed phenotype in NIH 3T3 cells.

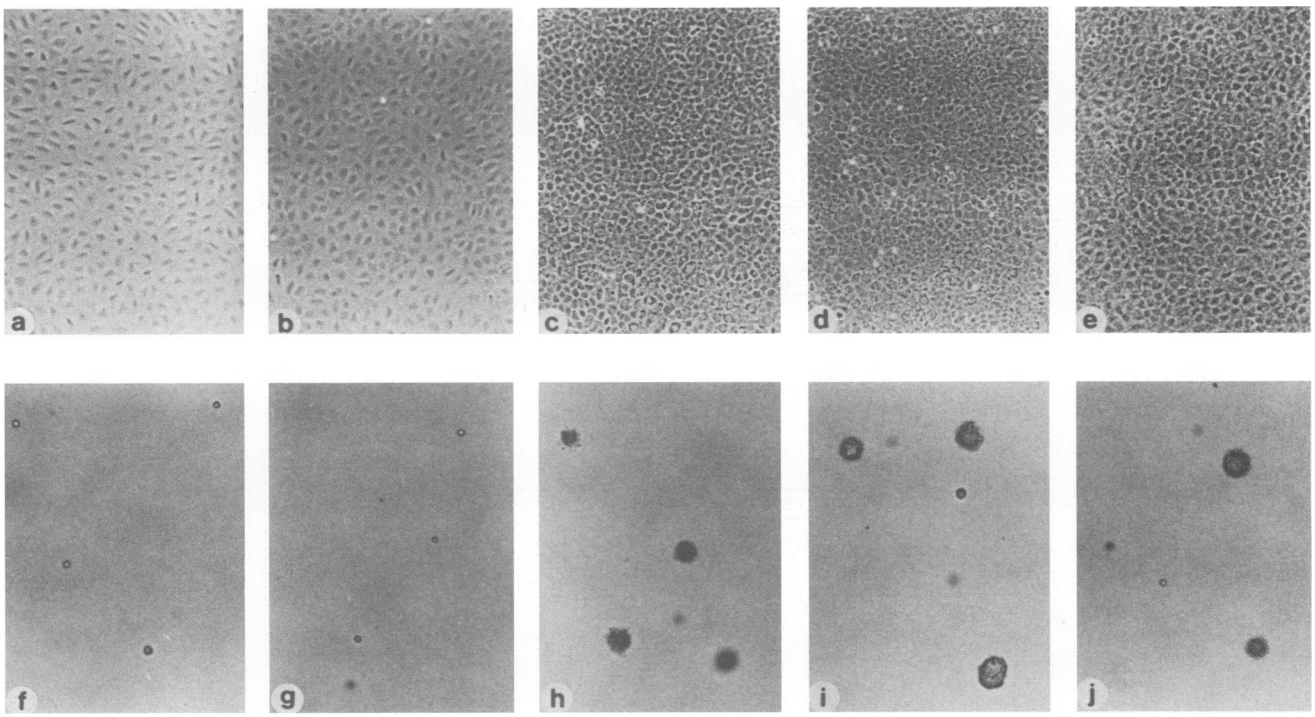

FIG. 3. Growth characteristics of 3Y1 cells transfected with a cDNA clone or its deletion mutant. G418-resistant populations of 3Y1 cells mock transfected (a and f) or transfected with pSV2-neo (b and g), pSVHPV16-M (c and h), cDNA cl-61 (d or i) or cDNA cl-61 cl-5 (e and j) were isolated. One set of cells (5×10^5 per 100-mm plate each) (a to e) was incubated in monolayers for 10 days and photomicrographed with phase contrast. Another set of cells (1×10^4 per 50 mm plate each) (f to j) was suspended in 0.28% agarose in the presence of Dulbecco modified Eagle medium containing 10% fetal calf serum, incubated for 3 weeks, and photomicrographed.

By introducing into NIH 3T3 cells the cDNA clone capable of expressing only the E7 gene, we were able to reproduce the entire process of cell transformation observed previously by transfection of the complete HPV-16 genome into the cells. We would like to suggest, therefore, that the main transforming gene of HPV-16 is E7. The E6 and E7 regions of HPV-16 and HPV-18 have been shown to be predominantly retained in the viral genome integrated in the chromosomal DNA of cervical cancer tissue or cell lines derived from cervical cancer (18, 26). It appears that the presence of one or both of these ORFs is required or advantageous for the sequence to be maintained in cancer cells. Our results may suggest that the E7 ORF product plays an important role in carcinogenesis of human cervical cancer. The product of the E7 ORF is relatively the most abundant of all the HPV gene products detected in cancer cells (28).

Very recently, the E7 ORF has been shown by others to have transforming activity on NIH 3T3 (22) or 3Y1 (15) cells. In these cases, however, functions of the E7 ORF have been identified by deletion analysis of the HPV-16 genomic DNA. In the present study, we first identified cDNA clones which had the transforming activity. Analysis of the clones which contained ORFs other than E7 also led us to conclude that the E7 ORF alone was responsible for the transforming activity. We therefore not only showed the transforming activity of the E7 ORF but also determined the exact structure of the coding region of the E7 gene encoded in mRNA expressed in cells in natural infection. There are three possible translation initiation signals (ATG) in the E7 ORF: the first at nt 562, the second at nt 595, and the third at nt 811. Therefore, polypeptides with 98, 87, or 15 amino

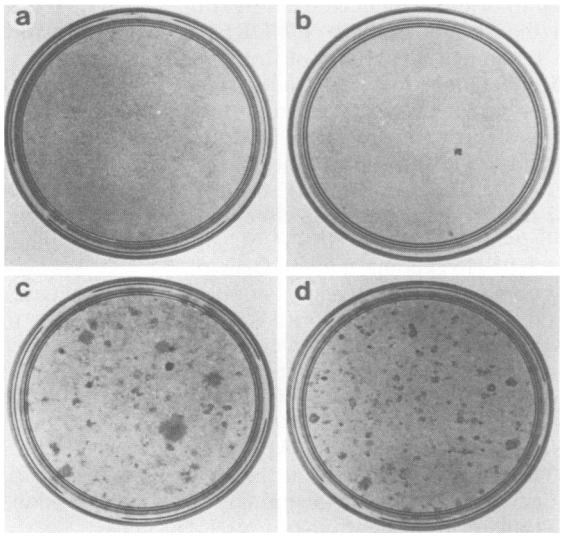

FIG. 4. Focus formation in NIH 3T3 cells transfected with cDNA cl-61 *dl*-5. Monolayers of a G418-resistant population of NIH 3T3 cells (5×10^5 per 100-mm plate each) mock-transfected (a), or transfected with pSV2-neo (b), pSVHPV16-M (c), or cDNA cl-61 *dl*-5 (d) were incubated for 3 weeks, fixed with methanol, stained with Giemsa solution, and photographed.

acids could be encoded by this E7 ORF. A protein of 15 to 20 kilodaltons encoded by the E7 ORF of HPV-16 has been detected in Caski and SiHa cell lines which were derived from cervical cancer (28, 29). This protein is reported to be a cytoplasmic phosphoprotein (30).

Although we have shown that the E7 ORF alone could reproduce the entire process of the transformation of NIH 3T3 cells that was induced by the whole HPV-16 genome, we could not exclude the possibility that other ORFs such as E6 or E5 have other biological activities relevant to cell transformation. In the case of bovine papillomavirus type 1 (BPV-1), the E5 and E6 ORFs have been shown to contain transforming activities (32). Therefore, the transforming potential of these ORFs of HPV-16 needs to be examined. Some other cells, such as human keratinocytes, may have to be used to elucidated the biological function of the E6 or E5 proteins.

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