JOURNAL OF VIROLOGY, Feb. 1988, p. 610–613
0022-538X/88/020610-04$02.00/0

Vol. 62, No. 2

Human Papillomavirus Type 16 Open Reading Frame E7 Encodes a Transforming Gene for Rat 3Y1 Cells

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Human papillomavirus type 16 (HPV 16) DNA is capable of morphologically transforming rat 3Y1 cells. The expression plasmids, constructed from the simian virus 40-based expression vector pSV2-0 and specific DNA fragments from the putative early region of the HPV 16 genome, were tested for their transforming capacity. Among the various pSV2 plasmids, only those containing the intact E7 coding region were found to produce foci of the transformed rat cells which could grow in a soft-agar medium. The data indicate that expression of the HPV 16 E7 open reading frame is sufficient to induce focal transformation of rat cells.

Human papillomavirus (HPV) type 16, 18, 31 or 33 DNA has been frequently found in genital cancer biopsies (2, 3, 5, 15, 19, 34) and in cell lines derived from cervical carcinomas (1, 20, 26, 28, 29). Furthermore, HPV-specific mRNAs and proteins have been detected in some of these cells (1, 26, 28–30). These observations imply that the particular types of HPV cause certain genital cancers.

HPV 16 is the most frequent type found in cervical cancer biopsies (5), and its prototype genomic DNA has been molecularly cloned (5) and sequenced (27). Except for its interrupted structure of E1 open reading frame (ORF), the genome organization of HPV 16 is similar to those of bovine papillomavirus type 1 (BPV 1) (4) and HPV 6b (25), and the putative early and late regions can be deduced from comparison of their genomic organizations (27). It was later found that, like other papillomaviruses, HPV 16 has an uninterrupted E1 ORF (1, 19). We have recently reconstructed the presumably nondefective HPV 16 genome from DNAs (10) from the prototype (5) and another defective HPV 16 (19).

Our present understanding of the genomic functions of papillomavirus has been provided mostly by BPV 1, because it can efficiently transform mouse C-127 and NIH 3T3 cells (6, 13, 16). The BPV 1 E1 gene is required for the maintenance of the episomal state of the viral genome in BPV 1-transformed cells (17). The E2 gene *trans*-activates the transcription from the promoter region adjacent to the E6 ORF (9, 32). The E5 and E6 genes transform C-127 cells independently (23, 24, 35, 36). For viral functions in transformation, however, HPVs have not been studied as extensively as has BPV 1, because of the lack of a suitable cell culture system.

HPV 16 DNA has been shown to transform mouse 3T3 cells (34, 37), rat 3Y1 cells (10), human fibroblasts and keratinocytes (21), and, in conjunction with an activated *ras* gene, primary rat cells (18). The transformed cells, even those transformed by HPV 16 DNA with uninterrupted E1 ORF (10), contain viral DNA integrated within the cell DNA (10, 37). HPV 16-transformed mouse 3T3 cells produce tumors in nude mice (37), but HPV 16-transformed rat 3Y1 cells do not (10). HPV 16-transformed 3Y1 cells show anchorage-independent growth (10). In this study, to determine which of the ORFs in the early region of the HPV 16 genome possess a transforming function for rat 3Y1 cells, we constructed six expression plasmids by using specific DNA fragments of HPV 16 genome DNA and a simian virus 40-based expression vector (pSV2-0) system (33) and examined their focus-forming capacities in 3Y1 cell cultures. The transforming function was found to be in E7 ORF.

Figure 1 illustrates the genomic organization of HPV 16, the specific DNA fragments, and the constructed expression plasmids. The source of HPV 16 DNA was a recombinant plasmid, pHPV16K (10), consisting of HPV 16K DNA and pBR322 DNAs ligated at their *Bam*HI sites. HPV 16K DNA is a reconstructed DNA having an uninterrupted E1 ORF, whereas the prototype HPV 16 DNA has a single-base deletion in E1 ORF (1, 19, 27). The fragments shown in Fig. 1b, designated E6 (nucleotides 25 to 657), E7T (509 to 875, containing the entire E7 ORF), E7P (554 to 875, containing a putative E7 coding region), E6E7 (25 to 875), E1 (684 to 3211), and E2E4E5 (2713 to 4469), were each ligated with *Hin*dIII linkers and inserted downstream of the simian virus 40 promoter into pSV-0 (Fig. 1c), which was made from pSV2-Raβg by removal of rabbit β-globin cDNA sequences and addition of the *Hin*dIII linkers to both ends (33). The resulting expression plasmids, pSV2-E6, pSV2-E7T, pSV2-E7P, pSV2-E6E7, pSV2-E1, and pSV2-E2E4E5, were expected to allow the expression of E6, E7, E7, E6E7, E1, and E2E4E5 ORFs, respectively. From these pSV2 constructs, *Pvu*II-*Bam*HI segments containing HPV sequences were isolated, ligated with *Bam*HI linkers, and used for construction of the second expression plasmids with the vector pSV2*neo* (31), which has a neomycin resistance gene (Fig. 1d). The constructs were designated pSVneo-E6, pSVneo-E7P, pSVneo-E6E7, pSVneo-E1, and pSVneo-E2E4E5, respectively. All of the junctions of DNA fragments ligated in this study were sequenced by the dideoxy method (22) for confirmation of the structures.

The transforming capacities of the HPV 16 DNA fragments (Fig. 1b) in the pSV2 expression vector were examined in rat 3Y1 cells (12), an immortalized normal cell line (Table 1). The pSV2 plasmids (Fig. 1c) were transfected to monolayer 3Y1 cultures by the calcium phosphate precipitation method (8). The transfected cells were replated at a split ratio of 1:40 24 h after transfection and maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum for 4 to 5 weeks before determination of focus number. Whereas plasmids pSV2-E6, pSV2-E1, and pSV2-E2E4E5 failed to produce foci in 3Y1 cell cultures, the plasmids containing the E7 region, i.e., pSV2-E7T, pSV2-E7P, and pSV2-E6E7, induced focus formation. Plasmids

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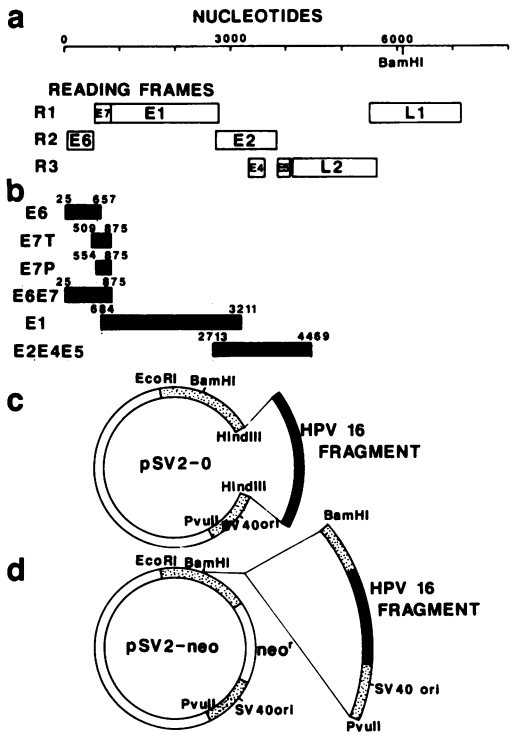

FIG. 1. Construction of expression plasmids containing HPV 16 DNA fragments. (a) Physical and functional maps of HPV 16K DNA, the one revised by insertion of a single base (10). The nucleotide numbering, the *Bam*HI cleavage site, and the locations of ORFs are according to Kanda et al. (10). (b) DNA segments inserted into expression vector pSV2-0. Thick bars indicate the locations of DNA segments; E6 (from *Dde*I to *Dde*I), E7T (from *Taq*I to *Pst*I), E7P (from *Pvu*II to *Pst*I), E6E7 (from *Dde*I to *Pst*I), E1 (from *Pvu*II to *Hin*cII), and E2E4E5 (from *Tth*111I to *Stu*I). The nucleotide numbers above the bars indicate the end nucleotide after trimming with DNA polymerase I to obtain flush ends. (c) Construction of expression plasmids using pSV2-0 vector. Each of DNA segments E6, E7T, E7P, E6E7, E1, and E2E4E5 was inserted into pSV2-0 (33) at the *Hin*dIII site after *Hin*dIII linkers were added at both ends; this process yielded pSV2-E6, pSV2-E7T, pSV2-E7P, pSV2-E6E7, pSV2-E1, and pSV2-E2E4E5, respectively. (d) Construction of expression plasmids carrying a selective marker of neomycin resistance. Each of the *Pvu*II-*Bam*HI fragments from the plasmids described above was inserted into pSV2*neo* (31) at the *Bam*HI site, using *Bam*HI linkers, to produce pSVneo-E6, pSVneo-E7P, pSVneo-E6E7, pSVneo-E1, and pSVneo-E2E4E5.

pSV2-E7T and pSV2-E7P transformed 3Y1 cells almost as efficiently as did the whole HPV 16 genome DNA (10), but pSV2-E6E7 did so less efficiently. The reason for the lower efficiency with pSV2-E6E7 is unclear at present. The foci were composed of densely packed, round cells morphologically similar to those transformed by the whole HPV 16 genome DNA (10) (Fig. 2).

Three foci of transformed cells, E7T#1, E7P#2, and E6E7#1, were picked up from pSV2-E7T-, pSV2-E7P-, and pSV2-E6E7-transfected cell cultures, respectively, and were examined for the presence of HPV 16 DNA and its transcripts by blotting hybridization methods (Fig. 3). *Bam*HI-digested cell DNA gave rise to several bands of HPV 16 sequences (Fig. 3, lanes 2 to 4). Cytoplasmic poly(A) RNA from E7T#1 and E7P#2 formed one band of about 1.1 kilobases (kb) (lanes 6 and 7), and that from E6E7#1 formed two bands of 2.8 and 1.6 kb (lane 8). The sizes of mRNAs

TABLE 1. Focus formation of rat 3Y1 cells with pSV2 plasmids containing HPV 16 ORFs

Transfected plasmid	No. of foci per 10 μg of DNA in expt no.:		
	1	2	3
pSV2-0	0	0	0
pSV2-E6	0	0	0
pSV2-E7T	ND[a]	16	28
pSV2-E7P	ND	24	32
pSV2-E6E7	2	8	8
pSV2-E1	0	0	0
pSV2-E2E4E5	0	0	0
pSV*neo*-HPV16K2[b]	14	32	44

[a] ND, Not done.
[b] pSV2*neo* (31) ligated with head-to-tail dimer of HPV 16K DNA (10).

expected from the pSV2 constructs used initially for transfection were 1.1 kb for E7T#1 and E7P#2 and 1.6 kb for E6E7#1. These data indicate that HPV 16 DNAs (E7T, E7P, or E6E7) introduced into 3Y1 cells have been rearranged and that HPV 16-specific mRNAs have been produced on such DNAs in the transformed cells.

The three transformed clones, E7T#1, E7P#2, and E6E7#1, could grow anchorage independently and formed colonies in a soft agar medium. Thirty thousand cells cultured in a 0.4% agarose medium in a 50-mm dish were observed for colony formation for 4 weeks. The efficiency of colony formation and the size of the colonies of E6E7#1 were comparable to those of 3Y1HP-1 cells (10), which had been transformed by the whole HPV 16 genome. Colonies of E7T#1 and E7P#2 were smaller and fewer than those of 3Y1HP-1.

For further study of the transforming capacities of HPV 16 ORFs, cell clones presumably expressing various ORFs were prepared and characterized. The pSVneo vectors (Fig. 1d) were transfected to 3Y1 cells, and the transfected cells were cultured in the presence of G418 (400 μg/ml; Sigma Chemical Co., St. Louis, Mo.). Drug-resistant colonies appeared after 2 to 3 weeks. All of the colonies obtained with pSV2*neo*, pSVneo-E6, pSVneo-E1, and pSVneo-E2E4E5 were morphologically indistinguishable from normal 3Y1 cells. The retention of the intact transcription unit of HPV 16

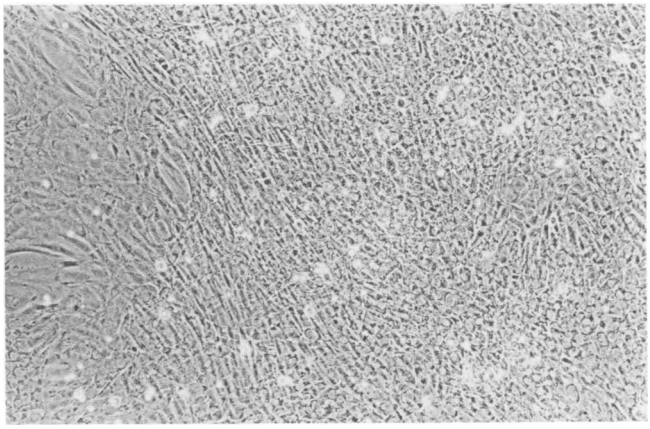

FIG. 2. Focus of HPV 16 E7-transformed rat cells (E7P#2) (magnification, ×60). Photomicrograph was taken under a phase-contrast microscope 28 days after transfection. Normal cells are seen on the left.

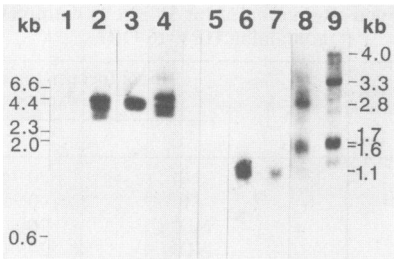

FIG. 3. Presence of HPV 16 DNA and mRNA in transformed rat cell clones E7T#1, E7P#2, and E6E7#1. HPV 16 DNA bands were detected by Southern blotting. Total cellular DNA was extracted by treatment of cells with *N*-lauryl sarcosine and proteinase K, followed by phenol extraction. A 10-μg sample of DNA digested with *Bam*HI, which cuts pSV2 plasmids at one site, was electrophoresed through a 1% agarose gel and immobilized on a membrane filter as described previously (11). The probes were pSV2-E6E7 DNA labeled with [^{32}P]dCTP (6,000 Ci/mmol) by the method of Feinberg and Vogelstein (7) (5×10^8 cpm/μg of DNA). DNA from: lane 1, normal 3Y1 cells; lane 2, E7T#1 cells; lane 3, E7P#2 cells; lane 4, E6E7#1 cells. HPV 16 mRNAs were analyzed by Northern blotting. Poly(A) RNA was selected from cytoplasmic RNA by oligo(dT)-cellulose column chromatography. A 5-μg sample of poly(A) RNA was electrophoresed through a 1.3% agarose gel containing 2.2 M formaldehyde–0.02 M sodium phosphate buffer (pH 7.4) at 60 V for 9 h and then transferred to a nitrocellulose membrane (14). The probe was HPV 16 genomic DNA (isolated from vectors) labeled with ^{32}P as described above. RNAs from: lane 5, normal 3Y1 cells; lane 6, E7T#1 cells; lane 7, E7P#2 cells; lane 8, E6E7#1 cells; lane 9, 3Y1HP-1 cells (10). Molecular size in kilobases is given on the left and right.

(the *Pvu*II-*Bam*HI fragments containing HPV 16 sequences) and the occurrence of HPV 16-specific mRNA transcription were verified in some of these clones by Southern and Northern (RNA) blotting methods using ^{32}P-labeled HPV 16 DNA as a probe (data not shown). By contrast, 65% (41 out of 63) of colonies containing pSVneo-E7P and 49% (30 out of 61) of those containing pSVneo-E6E7 morphologically resembled those transformed by the entire HPV 16 genome. Eight well-isolated, morphologically transformed colonies containing pSVneo-E7P or pSVneo-E6E7 were picked up and examined for their growth in a soft-agar medium. They showed different degrees of anchorage-independent growth; two clones grew well, four clones grew less efficiently, and two clones did not grow at all in an agarose medium.

In this study we attempted to determine which part of the HPV 16 genome is responsible for its transforming capacity for rat 3Y1 cells, using specific fragments inserted into pSV2 expression vectors. The specific HPV 16 fragments were chosen from the putative early region deduced from comparison of the genomic organization between HPV 16 and BPV 1 (23) (Fig. 1a). Fragments containing the E6, E7, E6E7, E1, and E2E4E5 regions (Fig. 1b) were placed under the control of the simian virus 40 promoter (Fig. 1c and d) and introduced into rat 3Y1 cells. Among the pSV2 expression vectors containing the HPV 16 fragments, only those containing the E7 coding region induced foci of transformed cells (Table 1, Fig. 2). We conclude, therefore, that the E7 ORF encodes a transforming gene.

This conclusion is consistent with previous findings with human cervical cancer biopsies and cell lines derived from cervical cancers (19, 20, 26, 28, 29). The HPV 16 DNA in cancer cells is mostly integrated within cell DNA and often has rearrangements including large deletions. The common feature of the integrated, rearranged HPV 16 genomes is the retention of the intact E6E7 and the transcriptional control regions (19, 20, 26). Recently, it was shown that two cell lines derived from cervical cancer, SiHa and CaSKi, have the intact E6E7 region and that E7 protein is the most abundant viral protein in those cells (28–30). It is likely, therefore, that the E7 ORF of HPV 16 is involved in generation or maintenance of human cervical cancers.

The present data do not rule out the possibility that HPV 16 has another transforming gene(s). Use of site-directed mutagenesis to each HPV 16 early gene may answer the question. Furthermore, it is possible that the other early genes of HPV 16 are transforming for cells from other species, since BPV 1 E5 and E6 ORFs encode transforming genes for mouse cells (23, 24, 35, 36). The expression plasmids containing various HPV 16 ORFs remain to be tested for cells from various origins.

Anchorage-independent growth of 3Y1 cells transformed with pSVneo-E7P or pSVneo-E6E7 varied from clone to clone. The capacity of 3Y1 cells transformed by the whole HPV 16 genome to grow in a soft-agar medium also varied with the clone (10). Probably the expression of the E7 ORF is sufficient to transform rat cells morphologically, but additional factors may be necessary to change the morphological transformants to malignant. Such factors may enhance the level of HPV 16 transcription or the susceptibility of cells to the HPV gene product. Studies of these factors will be important for analyses of carcinogenesis by HPV.

This work was supported in part by a Grant-in-Aid from the Ministry of Health and Welfare for the Comprehensive 10-Year Strategy for Cancer Control, Japan.

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