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Open Reading Frames E6 and E7 of Bovine Papillomavirus Type 1 Are Both Required for Full Transformation of Mouse C127 Cells

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A series of mutations in open reading frames (ORFs) E6 and E7 of bovine papillomavirus type 1 (BPV1) was constructed to analyze the roles of these ORFs in transformation of mouse C127 cells. The mutations were designed to prevent synthesis of specific proteins encoded by these genes. None of the mutations caused a decrease in the focus-forming activity of the full-length viral genome or in the ability of the viral DNA to replicate as a high-copy-number plasmid. Analysis of these mutants in the absence of a functional BPV1 E5 gene revealed a weak focus-forming activity encoded by ORF E6. Mutations preventing synthesis of the E6 protein did cause defects in anchorage-independent growth and tumorigenicity of transfected and transformed cells. However, a frameshift mutation between the first and second ATG codons of ORF E6 did not inhibit induction of colony formation, suggesting that translation from the first methionine codon is not required. Mutations that inactivated ORF E7 or E6/E7 individually did not inhibit induction of colony formation in agarose. However, a defect in this activity was caused by simultaneous disruption of both ORF E7 and ORF E6/E7 when they were expressed from the full-length viral genome but not when they were expressed under the control of a retrovirus long terminal repeat. These results suggest that translation of both ORF E6 and the 3′ end of ORF E7 is required for efficient induction of anchorage-independent growth by the intact BPV1 genome.

Bovine papillomavirus type 1 (BPV1) is a small, double-stranded DNA tumor virus that causes proliferation of both fibroblasts and epithelial cells during the formation of fibropapillomas in its natural host (18). This virus also efficiently induces transformation of rodent cells in culture. The 7,945-base-pair (bp) viral genome contains a number of overlapping translational open reading frames (ORFs) which encode viral proteins (2, 8, 14, 32). A segment which comprises 69% of the genome contains ORFs E1 through E8 and is sufficient to induce transformation of the established line of mouse C127 cells (20). Extensive genetic analysis of this segment of the genome has identified independent transforming genes in the 5′ and 3′ portions of this region. The major transforming gene in the 3′ portion is ORF E5, which encodes a 44-amino-acid protein required for efficient focus formation on mouse C127 cells (7, 10, 11, 27, 31, 36). ORF E6 has been identified as a transforming gene in the 5′ portion of the early region (30, 35). Expression of ORF E6 from a strong promoter can induce C127 cell focus formation and anchorage-independent growth, and mutations within this gene have been reported to reduce the transforming activity of BPV1 (21, 29, 30, 35). ORF E7 partially overlaps with ORF E6, but its role in transformation by BPV1 is unclear. Some mutations located in ORF E7 have been reported to decrease focus formation, evidently as an indirect consequence of decreased replication of the viral genome, whereas other laboratories find that ORF E7 mutations are silent (4, 5, 21, 26). In human papillomavirus type 16, ORF E7 expresses an activity that induces focus formation in established cell lines and cooperates with an activated *ras* gene to transform primary cells (15, 25).

The region of the genome containing the E6 and E7 ORFs is complex and could potentially encode a number of different proteins (see Fig. 1). Analysis of RNA from BPV1-transformed cells indicates that this region is transcribed to produce mRNAs colinear with the genome, as well as mRNAs spliced from nucleotide (nt) 304 to nt 527 or from nt 304 to nt 3224 (1, 34, 35). The first splicing event joins ORFs E6 and E7 in frame (to generate ORF E6/E7), and the second joins ORFs E6 and E4 (ORF E6/E4). Much less abundant spliced mRNAs from this region have also been detected (34). These major mRNAs have the potential to encode a number of proteins, including E6, E6/E7, and E6/E4 proteins translated from either in-frame methionine codon in ORF E6, and an E7 protein. Cells transformed by a BPV1 segment encompassing ORFs E6 and E7 contain an E6 protein of molecular weight 15,000, a size corresponding best to a protein translated from methionine codon 1 in ORF E6 (2). We used directed-mutagenesis techniques to construct mutations designed to prevent translation of specific proteins encoded by this region, and we analyzed the effects of these mutations on transformation by BPV1. The results of this analysis indicate that both the E6 and E7 ORFs are required for complete transformation of mouse C127 cells by BPV1 DNA.

MATERIALS AND METHODS

General methods. Enzymes were purchased from New England BioLabs unless specified otherwise. Bacterial transformations were performed in *Escherichia coli* DH-1, DH-5, or JM101 by standard methods (12, 23).

Construction of mutations. Oligonucleotide-directed mutagenesis with uracil-substituted template DNA was used to generate mutations E6oc1, E7oc1, E7oc2, E67SA, and E7oc1/E67SA (17, 37). The template was an M13 clone, HS5, which contains the *Hin*dIII-to-*Sma*I small fragment of BPV1 DNA. The *Mlu*I (nt 7351)-to-*Sma*I (nt 945) fragment of mutant clones identified by restriction site analysis was used to replace the analogous fragment in pBPV-142-6, which consists of the full-length BPV1 genome inserted into

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pML2d at the unique *Bam*HI site (28). Plasmid DNA was isolated from individual bacterial transformants and sequenced to confirm the presence of the mutations.

Plasmid pE6fs1 was constructed from pXH731, which consists of pBPV-142-6 with an 8-bp *Xho*I linker substituted for the 8 bp from nt 100 to 107 in the E6 ORF (30; D. Lowy, personal communication). This plasmid was cut with *Xho*I, incubated with the large fragment of DNA polymerase I and all four deoxyribonucleoside triphosphates, and ligated to generate a +4 out-of-frame insertion in ORF E6. Plasmid pE6fs2 was generated by partial digestion of pBPV-142-6 with *Rsa*I in the presence of 30 μg of ethidium bromide per ml and insertion of an 8-bp *Xho*I linker at nt 240, creating an out-of-frame insertion in ORF E6. Plasmid pE67SA/XhoI was created by partial digestion of pE67SA with *Pvu*II, which cuts at the mutated site in this plasmid, and insertion of an 8-bp *Xho*I linker at nt 527.

Plasmids with a frameshift mutation in ORF E5 were constructed by substituting the small *Kpn*I-to-*Sal*I fragment of pE5XL-2 for the analogous fragments of pE6fs1, pE6fs2, pE6oc1, pE7oc1, pE7oc2, and pE67SA. The fragment from pE5XL-2 contains an 8-bp *Xho*I linker inserted at the *Bst*XI site in ORF E5 (10). To construct the long terminal repeat (LTR)-containing plasmids, pBPV-142-6, pE6fs1, pE6fs2, pE6oc1, pE7oc1, or pE7oc2 was digested with *Sma*I, ligated to 8-bp *Xho*I linkers, and then cut with *Hpa*I. The small fragment was used to replace the analogous fragment in pXH800 (30), placing the E6 and E7 ORFs in the transcriptional orientation 3′ to the Moloney murine sarcoma virus LTR promoter.

C127 cell transformation. Transfections and focus-forming assays were performed as described previously (10). Briefly, 50 to 200 ng of plasmid DNA was digested with *Bam*HI and added as a calcium phosphate precipitate to 70% confluent mouse C127 cells. After 8 to 10 h, the cells were subjected to a 20% (vol/vol) dimethyl sulfoxide shock and then incubated for 12 h in medium containing 5 mM sodium butyrate. The cells were passaged 1:3 and incubated at 37°C with medium changes twice weekly. Foci were counted 14 to 18 days after DNA transfer. No foci appeared on plates that did not receive viral DNA. All plasmids were assayed for focus-forming activity in at least three experiments in which they exhibited comparable activities. To assay the E5 double mutants, 1 μg of *Bam*HI-digested plasmid DNA was transfected into cells together with 100 ng of plasmid pKO-NEO, which encodes the neomycin resistance gene. The cells were subsequently selected in medium containing 400 μg of G418 per ml. For transfections with the LTR plasmids, 1 μg of each intact plasmid was used. Cloning cylinders were used to establish cell lines from individual transformed foci, and pooled cell lines were established from plates that contained at least 50 transformed foci.

Colony growth in agarose. (i) Established assay. Cell lines established from pools of induced foci were plated at a density of 40,000 cells per 60-mm-diameter dish in medium containing 0.3% agarose. The plates contained a bottom layer of 0.5% agarose in medium. Cells were fed twice weekly with 2 ml of medium containing 0.3% agarose. Colonies with a diameter greater than 0.20 mm were scored as positive 14 to 18 days after plating. Results are presented as the percentage of the number of colonies formed by cells transformed by BPV-142-6.

(ii) Acute assay. C127 cells were transfected with 200 ng of viral DNA as described above. At 72 h after the 1:3 passage of the cells, one-third of the cells were assayed for focus formation to determine the transfection efficiency. The remaining cells were suspended in medium containing 0.3% agarose and plated onto 60-mm-diameter dishes containing a layer of 0.5% agarose in medium. Plates were fed twice weekly, and colonies with a diameter greater than 0.05 mm were counted 18 to 21 days after plating. The results were normalized for transfection efficiencies and are presented as the percentage of the number of colonies induced by wild-type DNA.

Tumorigenicity. Four- to six-week-old female BALB/c nude mice purchased from Life Sciences were injected subcutaneously with 10^6 cells and observed every 3 to 4 days for 6 weeks for the appearance and size of tumors.

Viral DNA analysis. Total cellular DNA was isolated from cell lines as described by Liskay and Evans (19), gently sheared by repeated passage through a 23-gauge needle, and electrophoresed on a 1% agarose gel. After transfer to nitrocellulose, viral sequences were detected by hybridization to BPV1 DNA labeled with ^{32}P by nick translation or random priming (23).

Primer extension. Total RNA was isolated from cell lines established from pools of foci induced by wild-type DNA or by the viral mutants (9). Twenty nanograms of 5′ end-labeled primer complementary to nt 544 to nt 575 of ORF E7 was hybridized to 20 μg of poly(A)$^+$ RNA and extended at 32°C for 30 min with avian myeloblastosis virus reverse transcriptase (Life Sciences) as previously described (6). The extension products were ethanol precipitated and visualized by polyacrylamide gel electrophoresis and autoradiography.

RESULTS

To determine the roles of the E6 and E7 ORFs of BPV1 in transformation of mouse C127 cells, we constructed mutations designed to prevent the synthesis of each of the putative protein products from this region. The locations of these mutations are diagrammed in Fig. 1, and the specific mutations and their predicted genetic consequences are listed in Table 1. Plasmid pE6fs1 contains a frameshift mutation in ORF E6 at nt 100, immediately 3′ to the first in-frame ATG codon in this ORF. This mutation is predicted to prevent translation of all E6, E6/E7, and E6/E4 proteins that use this ATG as the translation initiation codon. Plasmid pE6fs2 contains a frameshift mutation in ORF E6 at nt 240. This mutation is located 3′ to the second ATG codon in ORF E6 and is predicted to prevent synthesis of all E6, E6/E7, and E6/E4 proteins regardless of which ATG codon is used. Loss of ORF E6/E4 proteins is unlikely to affect transformation, because premature stop codons in ORF E4 (e.g., E4am1 in Fig. 1) are phenotypically silent in C127 cells (13, 24). pE6oc1 contains a premature translation stop codon at nt 315 that is predicted to affect the synthesis of only E6 proteins since it is located 3′ to the splice donor site at nt 304 that is used in the synthesis of spliced E6/E7 and E6/E4 mRNAs. pE7oc1 contains a premature stop codon in ORF E7 at nt 510, 3′ to the sole ATG codon in this ORF and 5′ to the splice acceptor site at nt 527; therefore, it should prevent synthesis of only the E7 protein. The E67SA mutation changes the invariant AG dinucleotide of the splice acceptor site (nt 526 and nt 527) to TG. This change was predicted to prevent synthesis of the major E6/E7 spliced mRNA and, hence, synthesis of the E6/E7 fusion protein. In this mutant, nt 525 was also changed from C to T, thus creating a new *Pvu*II site. Neither substitution resulted in a change in the amino acid sequence encoded by ORF E7. We also constructed three plasmids containing mutations predicted to prevent translation of both E7 and E6/E7. pE67SA/Xho was

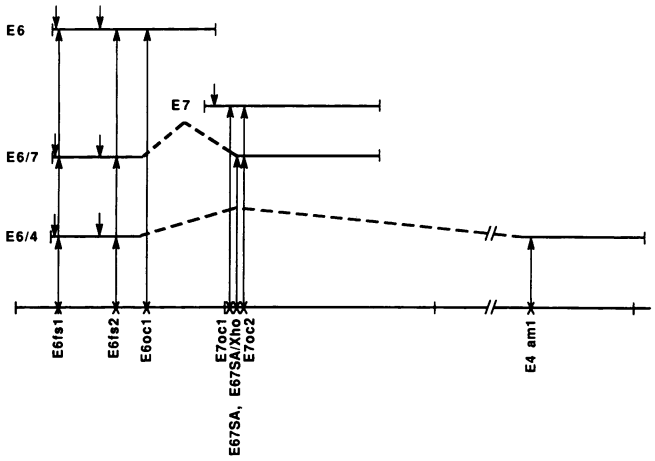

FIG. 1. Positions of mutations in ORFs E6 and E7. The lower line represents a portion of the BPV1 genome extending from the unique *Hpa*I site (nt 1). The short vertical lines are spaced every 500 nt, and transcription proceeds to the right. The horizontal lines above the genome map represent the viral ORFs, and the dashed lines represent sequences spliced out at the RNA level. The portion of the genome between ORFs E7 and E4 has been deleted. The positions of methionine codons in each ORF are indicated by downward-pointing arrows. The Xs represent the positions of the constructed mutations. Upward-pointing arrows indicate the ORFs affected by each mutation. The E67SA mutation affects only ORF E7, as indicated, but the E67SA/Xho mutation affects both ORFs E7 and E6/E7.

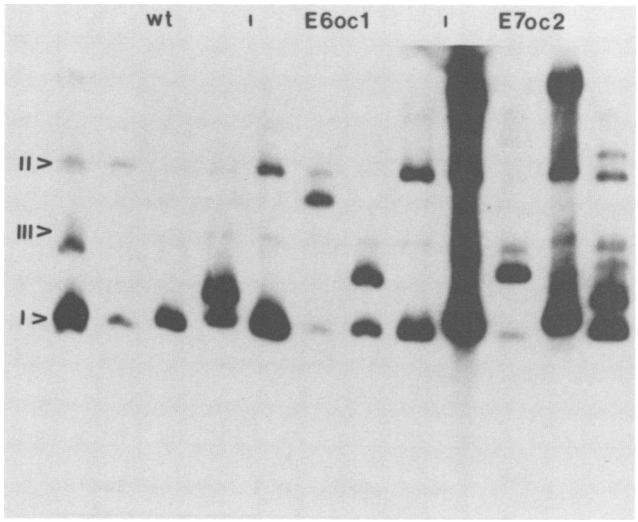

FIG. 2. Viral DNA in transformed cells. Cell lines were established from individual foci induced by the indicated DNAs. After about 20 cell generations, total DNA was prepared from each cell line and electrophoresed on 1% agarose gels. After transfer to nitrocellulose and hybridization to ^{32}P-labeled BPV1 DNA, viral sequences were detected by autoradiography. Positions of form I, II, and III extrachromosomal BPV1 DNAs are indicated. wt, Wild type.

made by inserting a linker at the new *Pvu*II site in pE67SA, creating a frameshift in the E7 ORF. Plasmid pE7oc1/E67SA is a double mutant that contains both the premature stop codon of pE7oc1 and the splice acceptor mutation of pE67SA. pE7oc2 contains a premature stop codon at nt 543, 3′ to the splice acceptor site in ORF E7. These three mutants are predicted to be unable to express the carboxyl-terminal portion of both the intact E7 protein and the major E6/E7 fusion protein. Each of the mutations listed in Table 1 was cloned into the full-length BPV1 genome and assayed for its effects.

Viral DNA and RNA. All of these mutants efficiently induced foci in mouse C127 cells (see below); therefore, we were able to isolate permanent cell lines transformed by each mutant and to examine the state of the viral DNA and RNA in these cells. Individual foci were expanded into cell lines, and total DNA was extracted, transferred to nitrocellulose, and probed with ^{32}P-labeled BPV1 DNA. Figure 2 shows undigested DNA from cell lines transformed by BPV-142-6, E6oc1, or E7oc2. Each cell line contains primarily extrachromosomal BPV1 DNA with a range in copy number among cell lines of about 50 to 200 copies per cell. DNA the size of the input species and some rearranged DNA are present in cell lines generated with wild-type DNA and with the mutants. Similar results were obtained with all of the mutants listed in Table 1. The constructed mutations were retained in the viral DNA in these cells, as documented by the presence of diagnostic restriction endonuclease cleavage sites (Table 1; data not shown). Therefore, under our assay conditions the E6 and E7 ORFs were not required for stable establishment of high-copy-number plasmid replication of BPV1 DNA in C127 cells. In contrast to these results, Botchan and colleagues have reported that BPV1 plasmids containing some ORF E6 and E7 mutations are maintained at a level of only 1 to 5 copies per cell (4, 5, 21). We examined the viral DNA copy number of one of their low-copy-number mutants, *dl*576, which contains a 4-bp deletion at nt 576 in ORF E7. A stable transformed cell line established from a pool of foci induced by *dl*576 contained wild-type levels of monomeric extrachromosomal BPV1 DNA (data not shown). Therefore, we did not observe abnormal replication by a mutant reported to display a low-copy-number phenotype by those investigators.

TABLE 1. Constructed mutations

DNA	Mutation	New restriction site	ORFs affected
E6fs1	Frameshift at nt 100	*Pvu*I	E6, E6/E7
E6fs2	Frameshift at nt 240	*Xho*I	E6, E6/E7
E6oc1	Stop codon at nt 315	*Hin*dIII	E6
E7oc1	Stop codon at nt 510	*Dde*I	E7
E67SA	Splice acceptor mutation at nt 527	*Pvu*II	E6/E7
E7oc1/E67SA	Double mutant	*Dde*I, *Pvu*II	E7, E6/E7
E67SA/Xho	Frameshift at nt 527	*Xho*I	E7, E6/E7
E7oc2	Stop codon at nt 543	*Dra*I	E7, E6/E7

BPV1-specific RNA was examined in cell lines established from pools of foci induced by BPV-142-6, E6oc1, E7oc2, or E67SA. There were no apparent differences in the sizes or amounts of BPV1 mRNAs on Northern (RNA) blots probed with a ^{32}P-labeled antisense RNA probe (BPV1 nt 3455 to nt 4450) predicted to hybridize to all BPV1 early mRNAs (data not shown). To examine in more detail the mRNAs from the E6 and E7 ORFs, primer extension analysis was performed on the poly(A)$^+$ RNA from these cell lines with a primer (nt 575 to nt 544) which hybridizes to both the unspliced and major spliced mRNAs from this region. Lane 1 of Fig. 3 shows the wild-type pattern; the 487-nt band corresponds to the unspliced mRNA with a 5′ end at nt 89, and the 264-nt band corresponds to the major spliced E6/E7 mRNA. Cells

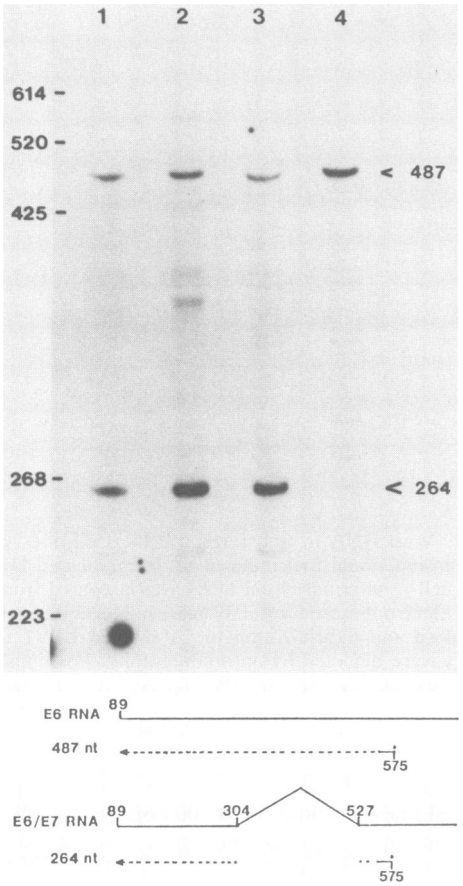

FIG. 3. Primer extension analysis of RNA from transformed cells. Cell lines were established from pools of foci induced by BPV-142-6, E6oc1, E7oc2, or E67SA. A ^{32}P end-labeled antisense oligonucleotide with a 5′ end at nt 575 was hybridized to 20 μg of poly(A)$^+$ RNA from each cell line. After primer extension, the products were electrophoresed on an 8% polyacrylamide–7.8 M urea gel and detected by autoradiography. The lanes contain the products of reactions with RNA isolated from cells transformed by BPV-142-6 (lane 1), E6oc1 (lane 2), E7oc2 (lane 3), and E67SA (lane 4). The solid lines in the diagram indicate the structures of the major E6 and E6/E7 messages. The predicted extension products are represented as broken lines.

transformed by E6oc1 and E7oc2 contained the same transcripts in about the same relative proportions (lanes 2 and 3). As predicted, the spliced E6/E7 mRNA was not detectable in cells transformed by the E67SA mutant, which does not have the AG consensus splice acceptor site at nt 527, whereas the unspliced mRNA was present in amounts comparable to that seen in wild-type-transformed cells (lane 4). Moreover, no prominent novel RNA species were present in cells transformed by these mutants, suggesting that the mutations did not exert major unanticipated effects on RNA metabolism. However, we did not rule out that the mutations have subtle effects on the amounts or structure of BPV1 RNA.

Expression of genes linked to the long control region of BPV1 is transactivated by expression of an intact ORF E2 (33). The E6 and E7 mutant plasmids transactivated the BPV1 enhancer as efficiently as did wild-type DNA, indicating that these ORFs are not required for this activity and that these mutations do not affect the expression of ORF E2 (data not shown).

TABLE 2. Focus formation by ORF E6 and E7 mutant plasmids

Plasmid	Focus-forming activity (% of wild type)		
	+ORF E5[a]	−ORF E5[b]	LTR constructs[c]
None	0	0, 0	0
pBPV-142-6 (wild type)	100	100, 100	100
pE5XL-2	0.2[d]	20, 53	N.A.
pE6fs1	90 ± 5	0, 0	0
pE6fs2	76 ± 7	0, 0	0
pE6oc1	94 ± 10	0, 0	0
pE7oc1	109 ± 10	20, 74	58 ± 21
pE67SA	91 ± 8	20, 53	N.D.
pE7oc2	85 ± 7	2, 16	100 ± 26

[a] Focus-forming activity of viral genomes containing a wild-type ORF E5. Averaged results from three or more experiments, ± the standard error, are shown as a percentage of the wild-type activity (about 2,000 foci per μg).

[b] Focus-forming activity of viral genomes containing a frameshift mutation in ORF E5. Selection for a cotransferred neomycin resistance gene was also imposed. The results of two independent transfection experiments are shown as percentages of wild-type activity. In these two experiments, 50 and 38% of G418-resistant colonies generated with wild-type DNA were morphologically transformed.

[c] Averaged results from three experiments, ± the standard error, are shown as a percentage of wild-type activity (about 10 foci per μg). N.A., Not applicable; N.D., not determined.

[d] This value is from a previously published report (10).

The BPV1 E7 and E6/E7 proteins have not been detected in cells transformed by BPV1. Using an E6-specific antibody (2), we were unable to detect the E6 protein from cells transformed by either wild-type DNA or the LTR plasmids, and therefore we were unable to confirm the absence of the protein from cells transformed by the E6 mutants. However, the nature of the constructed mutations (frameshifts, premature stop codons), the fact that the viral genomes in the transformed cells retained the mutations, and the RNA analysis of cells transformed by E67SA indicate that the targeted proteins are almost certainly not produced in cells transformed by the mutants.

Focus formation. The mutants were assayed for their focus-forming activity in C127 cells. Table 2, column 1, shows the number of morphologically transformed foci induced by each mutant, expressed as the percentage of foci induced by wild-type DNA. None of the mutants was significantly defective for focus formation in C127 cells. The foci produced by each mutant arose at the same time and were morphologically indistinguishable from foci induced by wild-type BPV1 DNA.

To determine whether the E6 or E7 ORF encodes a transforming activity that is masked by the presence of the E5 transforming gene, we constructed a number of double mutants, each of which combined an ORF E6 or E7 mutation with a frameshift mutation in the coding portion of ORF E5. To clearly visualize foci induced by ORF E5 mutants, we found it necessary to cotransfect each mutant with a plasmid containing a neomycin resistance gene and to select G418-resistant cells. Table 2, column 2, shows the number of morphologically transformed colonies induced in this assay, expressed as the percentage of transformed colonies induced by wild-type DNA. The E5 mutant, E5XL-2, induced morphologic transformation in about one-third as many neomycin-resistant colonies as did the wild type. No transformed colonies were induced by the three mutants with mutations in both E6 and E5, i.e., E6oc1/E5, E6fs1/E5, and E6fs2/E5. Therefore, the E6 ORF appears to encode a weak focus-forming activity that is detectable in the absence of ORF E5. Plasmids with the ORF E5 mutation plus a mutation in either

ORF E7 or E6/E7 produced the same number of transformed colonies as did the E5 mutant. On the other hand, E7oc2/E5, which is predicted to be defective for synthesis of the E7, E6/E7, and E5 proteins, produced about 20% of the number of transformed colonies that pE5XL-2 did. Therefore, while mutations in the E7 and E6/E7 genes separately had no effect, a mutation that disrupts both genes caused a moderate focus-forming defect in this assay. This result suggests that in the context of the full-length genome, the 3′ end of ORF E7 plays some role in focus formation.

To examine the transforming activity of the E6 and E7 ORFs in the absence of all other BPV1 genes, we used plasmid pHX800, which contains a strong promoter (the Moloney murine sarcoma virus LTR), the E6 and E7 ORFs (nt 1 to nt 945) and no other intact BPV1 ORFs, and a segment of BPV1 DNA that contains the early polyadenylation signal (30). Schiller et al. (30) have shown that this plasmid transforms C127 but not NIH 3T3 cells and have presented evidence that the E6 gene is the active transforming gene in this construct. The mutations in ORFs E6 and E7 were inserted into pHX800 and assayed for focus formation in C127 cells (Table 2, column 3). The three plasmids with mutations in the E6 ORF were markedly defective, whereas premature stop codons in ORF E7 either 5′ or 3′ to the splice acceptor site did not cause defects. Therefore, under the control of a strong heterologous promoter, the E6 ORF exhibits focus-forming activity. The E7 ORF is neither necessary nor sufficient for this activity.

Growth in agarose. The ability of cells harboring the full-length viral genomes to form colonies in agarose was determined by using two different assays. In assay i, C127 cells were transfected with each mutant, and after 4 weeks pools of transformed foci were used to establish cell lines which were subsequently plated into 0.3% agarose and scored for colony formation. In assay ii, C127 cells were transfected with each mutant, but after 3 days they were plated directly into agarose. We refer to assay i as the established assay and assay ii as the acute assay; they were designed to determine whether prior selection for focus formation affected the requirements for anchorage-independent growth. In each experiment, the colony-forming ability of cells harboring wild-type DNA was determined in parallel.

In the acute assay, two mutants with mutations in ORF E6, E6oc1, and E6fs2 produced only about 15% of the number of colonies produced by wild-type DNA, suggesting that an intact ORF E6 is required for efficient colony formation (Table 3). In contrast, cells transfected with E6fs1, which is predicted not to affect synthesis of E6 proteins initiated at ATG 2, formed about five times more colonies than did cells transfected by the other E6 mutants. The results from the established assay were similar, except that there was a greater proportional difference between the two classes of E6 mutants (Table 3): cells transformed by the E6oc1 mutant formed only 1% of the number of colonies formed by the cells transformed by wild-type DNA, whereas the E6fs1 mutant was far more efficient. A genetic mapping experiment was performed on plasmid pE6oc1. By using oligonucleotide-directed mutagenesis with a synthetic oligonucleotide containing the wild-type sequence and a template containing the E6oc1 mutation, the premature stop codon was converted to the wild-type sequence. A small fragment of DNA from two clones containing the regenerated wild-type sequence was used to replace the analogous fragment in pE6oc1. Two independent plasmids constructed in this fashion, pE6MR-1 and pE6MR-2, induced the same number of colonies as did wild-type DNA, demonstrating that the premature stop codon at nt 315 in pE6oc1 caused the defect in anchorage-independent growth (Table 3). These results indicate that translation of ORF E6 is required for efficient colony formation.

TABLE 3. Colony formation in agarose

Plasmid	Avg % (± SE) of no. of colonies induced by pBPV-142-6	
	Acute assay[a]	Established assay[b]
None	1.2 ± 0.6	0
pBPV-142-6	100	100
pE6fs1	67.7 ± 13.0	38.0 ± 11.0
pE6fs2	13.5 ± 3.5	N.D.
pE6oc1	15.5 ± 1.8	0.7 ± 0.7
pE6oc1-MR1	104.0 ± 1.0	N.D.
pE6oc1-MR2	106.0 ± 12.0	N.D.
pE7oc1	45.6 ± 8.5	98.8 ± 4.2
pE67SA	163.5 ± 19.9	228.3 ± 13.0
pE7oc1/E67SA	17.3 ± 1.5	18.8 ± 7.7
pE67SA/Xho	3.2 ± 2.2	16.0 ± 3.5
pE7oc2	6.4 ± 1.4	10.1 ± 2.9
pE7oc2-MR1	93	N.D.

[a] Transfected C127 cells were assayed for colony formation in agarose. The results were normalized for transfection efficiency. Each plasmid was tested at least three times, except for the plasmids used in the mapping experiments, which were tested once (E7oc2-MR1) or twice (E6oc1-MR1 and E6oc1-MR2).

[b] Established cell lines were assayed for colony formation in agarose. The averaged results of at least three separate experiments are presented as a percentage of the number of colonies formed by cells transformed by the wild type. N.D., Not done.

E7oc1 and E67SA were not defective for induction of anchorage-independent growth (Table 3). The colonies formed by the cells transfected or transformed with E67SA were actually larger than those formed by wild-type DNA and more numerous. Therefore, individually the E7 and the major E6/E7 proteins were not required for colony formation. However, mutants E67SA/Xho, E7oc2, and E7oc1/E67SA each induced less than 20% of the number of colonies induced by wild-type DNA (Table 3). Therefore, all of the mutations predicted to disrupt both ORF E7 and ORF E6/E7 simultaneously and thus prevent translation of the 3′ end of the E7 ORF resulted in a defect in inducing anchorage-independent growth. A genetic mapping experiment suggested that the stop codon in E7oc2 was responsible for the defect in colony formation. We replaced the mutant *Bsm*I-to-*Dra*III fragment (nt 520 to nt 825) of pE7oc2 with the analogous wild-type DNA fragment. The resulting plasmid, pE7oc2MR-1, induced colonies as well as did the wild type, indicating that the mutation in pE7oc2 causing the loss of colony-forming ability was confined to ORF E7 (nt 449 to nt 856). Therefore, efficient anchorage-independent growth induced by the full-length genome requires both an intact E6 ORF and translation of the 3′ end of the E7 ORF.

We also determined the colony-forming ability of cells transformed by the LTR plasmids. Cell lines established from individual foci induced by LTR WT (wild type; containing intact E6 and E7 ORFs) or LTR E7oc2 (containing a mutation in the 3′ end of ORF E7) efficiently induced colony formation (data not shown). Therefore, under the control of the LTR, expression of the E6 ORF by itself is sufficient to induce anchorage-independent growth.

Tumorigenicity. Cell lines established from pools of foci induced by representative mutants were injected into nude mice (Table 4). None of the mice inoculated with C127 cells developed tumors, whereas cells transformed by wild-type DNA formed visible tumors at 3 to 4 weeks. Cells trans-

TABLE 4. Tumor formation in nude mice

Cell line[a]	No. of tumors[b]/injections			
	Expt 1	Expt 2	Expt 3	Expt 4
C127	0/5	0/4	0/4	0/1
142-6	5/5	3/4	2/4	3/4
E6oc1[c]	0/5	0/4		
E67SA[d]		4/4	3/4	
E7oc2-1	5/5	0/4	0/4	
E7oc2-2				4/6
E7oc2-CL1				0/4
E7oc2-CL2				3/4
E7oc2-CL3				0/2

[a] E7oc2-CL1, E7oc2-CL2, and E7oc2-CL3 were derived from individual foci. All other cell lines were established from pools of greater than 50 foci.

[b] Tumors were scored as positive if they were greater than 1 cm in diameter at 5 weeks after injection.

[c] Cells transformed by E6oc1 formed small tumors (less than 3 mm in diameter) by 5 weeks after injection.

[d] Tumors induced by this line appeared earlier (1 week versus 2 to 3 weeks) and were larger (1 to 2 cm by 3 weeks) than tumors induced by BPV-142-6-transformed cells.

formed by E6oc1 formed markedly smaller tumors that were visible only after 5 to 6 weeks, indicating that this mutant was partially defective for tumor induction. Cells transformed by E67SA induced tumors that were easily visible at 2 weeks after injection and were considerably larger than tumors induced by wild-type-transformed cells. Thus, the major E6/E7 protein was not required for efficient tumorigenicity. The tumorigenicity of cells transformed by E7oc2 was determined for two independent pooled cell lines and three cell lines established from independent individual foci. The tumorigenicity of these cells varied from cell line to cell line and during passage, but about 50% of the cell lines tested were able to induce tumors. Thus, ORF E6 appears to be required for efficient tumorigenicity, and the 3′ end of the E7 ORF may also play a role in this activity.

DISCUSSION

We have assigned transforming functions to BPV1 ORFs E6 and E7 by determining the effects of mutations in these genes on transformation of mouse C127 cells (summarized in Table 5). We did not detect unanticipated changes in viral RNA synthesis in transformed cells, and replication, transactivation, and focus formation by the full-length genome were unimpaired, suggesting that the mutations do not indirectly affect the expression of ORF E1, E2, or E5, which are required for extrachromosomal replication, transactivation, and efficient focus formation. Mutations predicted to prevent translation of the E6 protein, including one that leaves the E7 and E6/E7 proteins intact, cause defects in anchorage-independent growth and tumorigenicity. Mutations that prevent translation of either ORF E7 or E6/E7 do not result in transformation defects, but simultaneous inactivation of both ORF E7 and ORF E6/E7 inhibits anchorage-independent growth, even in the presence of an intact E6 gene. One defective mutant, E7oc1/E67SA, is generated by the combination of ORF E7 and E6/E7 mutations, neither of which individually caused a defect. These results strongly suggest that translation of ORF E6 and the 3′ end of ORF E7 is required for complete transformation by the full-length BPV1 genome and that either the E7 protein or the E6/E7 protein can fulfill the latter requirement. Formal proof that ORF E6 and the 3′ end of ORF E7 encode separate proteins that are both necessary for efficient colony formation requires complementation of the transformation defect in cells expressing the two mutant genomes. The increased tumorigenicity and colony-forming ability of cells transformed by the E67SA mutant suggest that the E6/E7 gene product exerts negative as well as positive effects on C127 cell transformation.

TABLE 5. Summary of ORF E6 and E7 analysis

Activity	ORF required for transformation
Focus formation with:	
Full-length genome containing ORF E5 mutation	E6; E7 or E6/E7[a]
LTR construct	E6
Colony formation with:	
Full-length genome	E6; E7 or E6/E7
LTR construct	E6
Tumorigenicity with full-length genome	E6; E7 or E6/E7[b]

[a] Mutants unable to express both ORF E7 and ORF E6/E7 were less defective than ORF E6 mutants in this assay, suggesting that the requirement for ORF E6 is more stringent.

[b] A partial requirement for ORF E7 or ORF E6/E7 in tumorigenicity is inferred from the partial defect of cells transformed by E7oc2 and the lack of a defect of cells transformed by E67SA.

The 3′ end of ORF E7 is not required for induction of either foci or colony growth by the LTR construct. The increased expression of ORF E6 in this construct (2) may circumvent the need for E7 expression. Alternatively, if the major role of ORF E7 is to regulate expression of the E6 ORF from its endogenous regulatory signals, this regulatory circuit may be bypassed in the LTR construct, rendering transformation independent of ORF E7. However, expression of ORF E7 by the intact viral genome does not affect ORF E6 RNA species detectable by primer extension.

Unlike the other E6 mutations, E6fs1 did not significantly inhibit colony formation in agarose. This frameshift mutation is located 3′ to the first potential initiation codon in ORF E6, suggesting that translation from this codon is not required for colony formation. This ATG is only 2 bases from the major mRNA cap site at nucleotide 89 (1, 3). In other systems in which an ATG is less than 10 bases from the 5′ end of the mRNA, translation can initiate at both the first and second ATG codons (16). Since the E6fs1 mutation does inhibit the weak focus-forming activity of ORF E6, it is possible that the full-length E6 protein is required for focus formation but that the internally initiated one suffices for colony formation.

Although other workers have reported that some ORF E6 and E7 mutations cause substantial defects in the focus-forming activity of the full-length viral DNA, most of the previously reported mutations in this region affected more than one ORF (4, 5, 21, 29). We detected a decrease in focus formation only when the E6 mutants were assayed in the absence of an intact E5 ORF. It is possible that under certain assay conditions, more E6 protein or less E5 protein is expressed and that this results in more readily detectable E6 focus-forming activity. In agreement with the results of Rabson et al. (26), who found that mutations in ORF E7 in the full-length genome had no effect on the efficiency of focus formation or plasmid copy number, the mutations described here did not result in reduced viral DNA copy number. Some ORF E6 and E7 mutants, including *dl576*, have been reported to display the low-copy-number phenotype (4, 5, 21, 22), but in our hands this mutant appeared to

replicate normally. These different results are most likely due to assay differences, which may include the use of different substrains of C127 cells or different procedures for transfection and passaging of cells.

Our results emphasize several aspects of cell transformation by BPV1. (i) Multiple viral genes are required for full transformation of the immortalized C127 cell line. (ii) Different viral transforming genes have relatively specialized activities. In our assays, ORF E5 seemed to be the primary viral gene involved in focus formation, whereas ORF E6 and the 3′ end of ORF E7 were involved primarily in inducing anchorage independence. (iii) BPV1 is genetically redundant in that there appear to be alternative mechanisms for expressing the transforming activity of the 3′ end of ORF E7. (iv) The genetic context in which BPV1 transforming genes are expressed can markedly influence their activity. For example, in our assays the focus-forming activity of ORF E6 was apparent only in the absence of the E5 ORF, and the requirement for the 3′ end of ORF E7 for colony formation was not evident in the LTR constructs. (v) Undefined assay differences among different laboratories can affect the apparent properties of the same mutant (e.g., *dl*576). These complexities indicate that additional genetic and biochemical analysis of the BPV1 transforming proteins is required to further our understanding of cell transformation by this virus.

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