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Mutational Analysis of the 3′ Open Reading Frames and the Splice Junction at Nucleotide 3225 of Bovine Papillomavirus Type 1

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Functional analysis of the 3′ open reading frames (ORFs) of bovine papillomavirus type 1 (BPV-1) has been complicated by the organization of that part of the genome. A region between nucleotides (nt) 3173 and 3551 contains three overlapping ORFs (E2, E3, and E4), as well as a 3′ splice junction at nt 3225 which is used by many of the BPV-1 transcripts. To more clearly assign functions to specific ORFs in this region, single-base substitution mutations were generated which introduced translational termination codons into each of the three ORFs; a fourth mutation substituted an A with a C at nt 3223, altering the 3′ splice junction consensus sequence from AG to CG. The E3- and the E4-specific mutants were wild type in their abilities to transform susceptible mouse C127 cells, to replicate as stable plasmids, and to *trans*-activate the E2 conditional enhancer. The E2-specific termination mutant was defective for plasmid replication, transformation, and *trans*-activation and could not be complemented for efficient transformation of a flat cell line which expressed the full-length E2 gene product. The splice junction mutant was defective for transformation of C127 cells and of a flat cell line expressing the full-length E2 gene product. These data extend previous analyses of the 3′ ORFs and suggest that a spliced E2 product is involved in cellular transformation. The splice junction mutant could replicate as a stable plasmid, indicating that there is no absolute requirement in plasmid replication for a viral gene product expressed solely from an mRNA using the 3′ splice junction at nt 3225.

The papillomaviruses are circular double-stranded DNA viruses which can cause benign tumors in a variety of higher vertebrates, including humans, and which have been associated with carcinomas in some species. The host range of these viruses is very specific, and their study has been hampered by the lack of an in vitro system for their propagation. Bovine papillomavirus type 1 (BPV-1) has provided a useful model for the study of the latent nonproductive phase of viral infection in transformed rodent tissue culture cells. The BPV-1 system has permitted the analysis of viral functions involved in cellular transformation, in viral transcriptional regulation, and in viral DNA replication (for a review, see reference 11).

Sequence analysis has revealed that all of the open reading frames (ORFs) in the BPV-1 genome larger than 400 base pairs are located on one of the two DNA strands (3), and transcription studies have revealed that only one strand is transcribed in transformed cells and in productively infected warts (1, 2, 7, 10). Viral transcription in transformed cells is nonetheless complex. At least five putative promoters have been identified by mapping the 5′ ends of viral RNAs in transformed cells (2, 27). There are multiple species of RNA with complicated patterns of splicing (27, 28). The genetic mapping of BPV-1 functions involved in cellular transformation and DNA plasmid replication has been difficult and complicated by the presence of genes whose products are involved in the regulation of transcription from some of the viral promoters. For instance, the E2 ORF has been shown to encode a factor which can *trans*-activate conditional enhancers located in the viral long control region (LCR) (25, 26). A factor further complicating the assignment of specific functions to specific ORFs is that there are regions of the genome where ORFs overlap.

In this study, we have focused our analysis on a segment of the BPV-1 genome between nt 3173 and 3551, in which the E2, E3, and E4 ORFs overlap in each of the three potential translation frames. Just downstream from this region is the E5 ORF, which partially overlaps the 3′ end of ORF E2. E5 encodes one of the two transforming proteins of BPV-1 (9, 17, 22, 23, 29).

There are a variety of ways the ORFs within this overlap region can be expressed. There are two transcriptional promoters, P_{2443} and P_{3080} located immediately upstream of this region, and a splice acceptor (SA) at nt 3225 which can be used to splice the 5′ ORFs expressed from the LCR promoters to the 3′ ORFs in this region (2, 27, 28). Several functions have been mapped to this overlap region. The product of the full E2 ORF is a *trans*-activator of enhancer elements located in the LCR (26). The 3′ portion of the E2 ORF encodes a transcriptional repressor which can inhibit viral transformation and repress E2-specific *trans*-activation (13). Although the product for the BPV-1 E4 ORF has not yet been demonstrated, the E4 ORF of human papillomavirus type 1a encodes a late protein, as its product is present in high levels in warts (5). An additional function involved in cellular transformation has been suggested by the studies of Rabson et al. and mapped to this overlap region (17). Some mutants with translation termination linkers (TTLs) inserted which affected the E2 and E4 ORFs could not be complemented for efficient transformation of YC-C59-3881 cells which express the full E2 gene product (17).

To further evaluate the viral functions encoded by this portion of the BPV-1 genome, we have constructed a series of ORF-specific mutants by substitution mutagenesis, introducing termination codons into each of the E2, E3, or E4 ORFs that should eliminate synthesis of the putative protein products. These ORF-specific mutants were then assessed for their ability to transform susceptible C127 cells, to

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replicate as stable plasmids, and to *trans*-activate the viral E2-responsive enhancer. An additional mutant was generated with a base substitution at nt 3223, altering the 3′ splice junction consensus sequence from AG to CG and, thereby, affecting the SA at nt 3225. This substitution creates a silent mutation in the overlapping E2 ORF and a leucine-to-phenylalanine substitution in the E4 ORF.

MATERIALS AND METHODS

Cells. Mouse C127 cells (6), single cell subclones of C127 cells, and YC-C59-3881 cells were maintained in Dulbecco modified Eagle medium with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). The YC-C59-3881 cells have been previously described, are nontransformed, and contain a BPV-1 cDNA with an intact E2 ORF and a mutated E5 ORF which is expressed from simian virus 40 early promoter (17).

DNA plasmid transfections. Transfections of C127 and YC-C59-3881 cells were carried out by calcium phosphate coprecipitation as previously described (19) with the following modifications. Dishes (10 cm) of cells at approximately 80% confluence received 1 to 3.5 μg of plasmid DNA and 18 μg of herring sperm carrier DNA. This was followed at 2 h by treatment with 10% glycerol in phosphate-buffered saline for 1 min. The cells were then incubated in complete medium containing 4 mM sodium butyrate for approximately 12 h. When cells were selected with G418, medium containing 500 μg of the drug per ml was added 72 h posttransfection. Cells were fixed with 10% Formalin at approximately 14 days posttransfection and stained with methylene blue.

Substitution mutagenesis. Initially, the 1.3-kilobase *Eco*RI (2113)-to-*Kpn*I (3456) fragment from the wild-type BPV-1 plasmid 142-6 was cloned into M13 mp18 and mutagenized by the procedure used by Kunkel (12) with the following modifications. Briefly, a uracil-containing DNA template was grown in *Escherichia coli* BW313. Oligonucleotide-primed DNA synthesis was allowed to proceed at 37°C for 1 h and overnight at 14°C with 5 U of Klenow fragment of *Pol*I and 2 U of ligase. No uracil glycosylase or alkali treatment was used. The following antisense oligonucleotides were used as mismatch primers: 5′ CGATCTCGAAAATCAG (SA, nt 3230 to 3215); 5′ CCCAACCTAGGCCTG (E4, nt 3364 to 3350); 5′ TGCGAGCTAGGACCG (E3, nt 3386 to 3372); and 5′ GTGCCCTACACCGGG (E2 nt 3455 to 3441). The repaired DNA was transfected into JM107 *E. coli*, and bacteriophage was screened by selective hybridization with 5′-labeled oligonucleotides near the calculated T_m. The sequences of the base substitutions in positive phage were confirmed by the dideoxynucleotide method (18). The *Eco*RI-to-*Kpn*I fragment was then religated back into the full BPV-1 background and reprobed to confirm insertion of the substitution mutation. In addition, when possible, the DNAs were tested for newly created restriction sites (Fig. 1). Finally, C59-SA^{3225} was constructed by replacing the *Bst*EII-*Bst*XI fragment of C59 (29) with the analogous fragment from p1039-1.

CAT assays. Chloramphenicol acetyl transferase (CAT) assays were performed as described previously (26) with the following modifications. C127 cells were transfected with 1.0 μg of the CAT reporter plasmid (pSV2CAT, $pA_{10}CAT$, or p407-1), 1.0 μg of the plasmid being assayed for *trans*-activator function, and 18 μg of carrier DNA as described above. Fifty percent of the cell extract, prepared from 10-cm

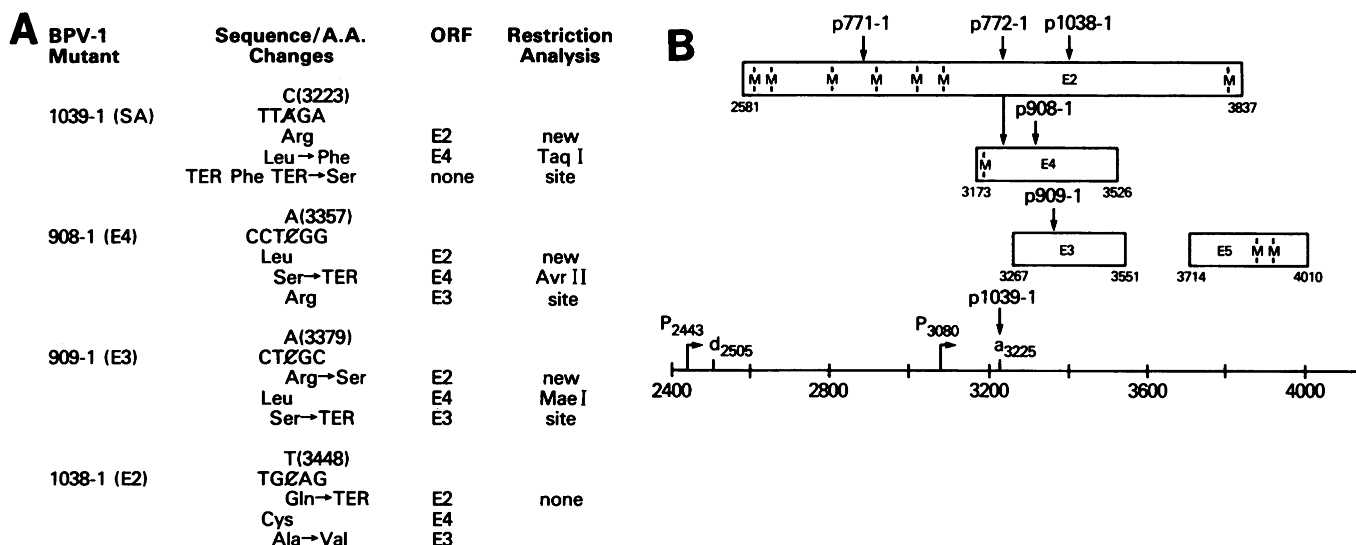

FIG. 1. Mutant structure and genomic organization of the BPV-1 E2 overlap region. (A) The recombinant plasmids are shown in the first column on the left. The second column indicates the nucleotide substitution effected in each particular mutant, the specific sequence number mutated in parentheses, and the resulting amino acid (A.A.) changes shown below. The crossed-out nucleotide represents the wild-type nucleotide removed, and its substitution is indicated above. The resulting A.A. changes encoded for by the mutant are indicated by arrows. The ORF in which the A.A. substitution takes place is indicated in the third column. The fourth column indicates the presence of any newly created restriction sites. All mutations were transposed into the full-length BPV-1 p142-6 plasmid background. (B) The genomic organization of the region. The open bars represent ORFs E2, E3, E4, and E5. The numbers below the ends of each ORF and M within the open bars indicate the presence of methionine codons of that ORF. The positions of the four mutants created by oligonucleotide-directed site-specific mutagenesis are indicated by arrows. Two additional mutants p771-1 and p772-1 are also shown at sequences 2878 and 3235, respectively. These mutants are described previously (17) and are linker insertion mutants containing a termination codon in all translational frames. Over the sequence marker at the bottom of the figure, the promoters P_{2443} and P_{3080} (2) are indicated, as well as the splice donor site d_{2505} and the major SA site a_{3225}.

dishes 72 h after removal of sodium butyrate, was assayed for CAT activity in an overnight incubation. Acetylated and nonacetylated forms of [^{14}C]chloramphenicol were separated by thin-layer chromatography and quantitated by scintillation counting.

Replication analysis. C127 cells were transfected with 0.5 μg of one of three plasmids expressing a *neo*-selectable marker, 1.0 μg of the BPV-1 DNA, and 18 μg of carrier herring sperm DNA. The plasmids pMMTneo(302-3) and pSV2neo have been described previously (14, 24). The plasmid p1042-1 (LCR-SVneo) was constructed by Carl Baker by replacing the CAT sequences in p407-1 (*Hin*dIII-to-*Bam*HI fragment) with the analogous drug resistance gene fragment from pSV2neo. Transfected cells were placed in G418 for selection at 72 h after sodium butyrate removal (8). Pooled resistant cells were selected at 3 weeks as described previously (17).

RESULTS

Construction of BPV-1 mutants. Four specific base substitution mutations were generated by oligonucleotide-directed site-specific mutagenesis. The mutations were initially made in the *Eco*RI-to-*Kpn*I fragment of BPV-1 and exchanged into the full-length wild-type BPV-1 plasmid as described in Materials and Methods. Three of these mutations introduced a termination codon specifically into the E2, E3, and E4 ORFs to generate the mutants p1038-1, p909-1, and p908-1, respectively (Fig. 1). An additional mutant, p1039-1, was generated which alters the AG of the SA at nt 3225 to CG (Fig. 1). The specific mutations generated, their locations in the BPV-1 genome, and their effect on each of the translation frames are indicated in Fig. 1. In addition, the location of the previously described TTL insertion mutants, p771-1 and p772-1, are indicated in Fig. 1 (17). Because of the overlapping translation frames in this region of the viral genome, it was difficult to create base substitution mutants resulting in a termination codon in one translational frame that were also silent in the other two frames. Only one of the four mutants, the E4 ORF-specific termination mutant p908-1, was silent in the other two reading frames. The E3 ORF termination mutant, p909-1, created by changing a C at nt 3379 to an A, also causes a missense mutation in E2 ORF, substituting a serine for an arginine. The E2 ORF termination mutant, p1038-1, generated by changing a C at nt 3448 to a T, also substitutes a valine for an alanine in the E3 ORF. The A-to-C mutation in p1039-1 which alters the SA site at nt 3225 changes a termination codon upstream of the E3 ORF to a serine residue. We feel this is unlikely to be a significant alteration, however, since another termination signal remains intact 2 codons further upstream in the E3 translation frame. In addition, it results in a missense mutation in the E4 ORF, substituting a phenylalanine for leucine.

Transformation of C127 cells by E2 region mutants. Each of these four mutants was assayed for its ability to induce foci on murine C127 cells after DNA transfection (Table 1). In this experiment, each of the BPV-1 DNAs was left intact with the procaryotic pML2d plasmid sequences. The wild-type complete BPV-1 plasmid (p142-6) induced approximately 200 transformants per plate in this experiment. The E2-specific termination mutant (p1038-1) transformed at a level approximately 6% that of the wild type. The E4-specific termination mutant (p908-1) transformed at levels comparable with that of p142-6. The E3-specific mutant (p909-1) transformed with an efficiency slightly higher than the wild type, and the foci appeared, on average, to be slightly larger than those induced by the wild type. The SA mutant (p1039-1) produced no foci. Thus, the E2 mutant behaved as many other described E2 mutants, in that it was significantly impaired in its ability to transform mouse C127 cells when assayed in a wild-type genomic background (4, 9, 17). The data with the E3 and E4 ORF-specific mutants indicate that their gene products are not directly involved in transformation of rodent cells. Whether the slight increase in transformation observed with p909-1 is a consequence of the termination codon in E3 or the amino acid alteration in E2 is not known at this time. The SA mutant (p1039-1) did not induce any foci in this assay, indicating that the integrity of the SA at 3225 is critical for the expression of the transformed phenotype.

TABLE 1. Transformation of C127 cells by BPV-1 mutant plasmid DNAs

Plasmid	Mutation	No. of foci/μg of DNA[a]	Relative activity[b]
p142-6 (wild type)	None	170, 240, 195	100
p1038-1	E2 ORF	11, 12	5.7
p909-1[c]	E3 ORF	450, 280	180
p908-1	E4 ORF	180, 160	84
p1039-1	SA^{3225}	0, 0	0

[a] Numbers refer to independent plates.
[b] Relative transforming activity determined from the average number of foci per plate, with p142-6 defined as 100.
[c] For this mutant, the foci appeared, on the average, to be slightly larger than those of the wild type induced by p142-6.

To determine whether mutation of the SA in p1039-1 has an effect on the expression of the E5 gene, this mutation was introduced into the viral cDNA, C59. The C59 cDNA contains the BPV-1 3′ ORFs intact and can efficiently transform C127 cells (28, 29). Mutational analysis of this cDNA has previously established that the E5 ORF encodes the transforming protein and that mutations in the E2 ORF of this cDNA had no direct effect on transforming ability (29). The structure of the mRNA encoding the E5 gene has not yet been definitely identified, however. Two types of viral RNAs can be predicted for the C59 construct, one with no splice in the BPV-1 sequences and the other using the SA at nt 3225 and possibly the viral splice donor at nt 2505. As with studies of the SA mutation in the full BPV-1 background, the C59-SA recombinant was markedly decreased in its ability to induce transformation compared with the parental C59 clone. In independent plates with a subclone of C127 cells selected for high transformation susceptibility, C59 plasmid produced 83 and 94 foci per μg of DNA, whereas C59-SA^{3225} produced 3 and 6 foci per μg of DNA. As expected, the C59-SA recombinant retained full E2 *trans*-activation activity (data not shown). This result suggests that expression of the E5 transforming protein involves an RNA(s) which uses the SA at nt 3225. A candidate for the mRNA which may encode E5 is the species derived from P_{2443} using the splice junctions at nt 2505 and 3225 (28). This species of RNA is relatively abundant in transformed mouse cells and in the dermal fibroblasts of a bovine fibropapilloma (2). A cDNA representative of this mRNA species in an Okayama-Berg expression vector was originally reported to be transformation negative (28) but can transform a subclone of C127 cells particularly sensitive to BPV-1 transformation but at a very low efficiency (P. L. Hermonat and P. M. Howley, unpublished observation).

Transformation of E2 region transformation-defective mutants in YC-C59-3881 cells. Several different classes of E2

TABLE 2. Complementation of BPV-1 3′ ORF mutant DNAs in YC-C59-3881 cells

Plasmid	Mutation position (nt)	Mutation	No. of foci/μg of DNA		Relative activity[a]
			Expt A	Expt B	
p142-6 (wild type)		None	200	210	100
p771-1	2878	E2 ORF	56	53	27
p772-1	3235	E2, E4 ORF	0	0	0
p1038-1	3448	E2 ORF	6	16	5.4
p1039-1	3223	SA^{3225}	0	0	0

[a] Numbers based on comparison of average foci per plate, with p142-6 defined as 100.

mutants have been described on the basis of their transformation characteristics (17). One class consists of mutants mapping in the amino-terminal domain of E2 which are defective in transformation of C127 cells but which can efficiently transform a flat nontransformed cell line YC-C59-3881 expressing E2 from an integrated C59-3881 cDNA. A second class of E2 mutants is exemplified by the mutant, p772-1, which contains a TTL, inserted at nt 3235. This class of mutant is defective for transformation of C127 cells as well as of the YC-C59-3881 cell line. Since this mutant (p772-1) contains a linker which introduces a nonsense codon in both the E2 and E4 ORFs, the defect could not be assigned to a specific ORF. Since the E4 ORF-specific mutant p908-1 has wild-type transforming activity, it seemed likely that the function required for transformation of the YC-C59-3881 line must be encoded by the 3′ portion of the E2 ORF. To test this, we assayed the E2 and SA mutants, p1038-1 and p1039-1, for their abilities to transform YC-C59-3881 cells. In this experiment, two mutants, p771-1 and p772-1, which have been previously described and which contain a TTL inserted at nt 2878 and 3235, respectively, were also assayed (Table 2). As previously shown, the 5′ E2-specific mutant p771-1 was capable of transforming YC-C59-3881 cells, whereas p772-1 with the TTL inserted at nt 3235 downstream of the 3′ splice junction at nt 3225 was unable to transform. The E2-specific mutant (p1038-1) transformed the YC-C59-3881 cell line at the same low efficiency as C127 cells, indicating a lack of complementation by the BPV-1 functions constitutively expressed in the cell line, thereby confirming that this additional function required for transformation maps to the 3′ portion of the E2 ORF. The SA mutant (p1039-1) produced no foci in YC-C59-3881 cells, suggesting that access to the 3′ portion of the E2 ORF is through the splice junction at nt 3225. We have not yet identified which upstream coding sequences are spliced to the E2 ORF through the 3′ splice junction at nt 3225 to provide such a function. By primer extension analysis of viral RNA in C127 cells containing p1039-1, we have confirmed that the base substitution at nt 3223 does result in the loss of use of the 3′ splice junction at nt 3225 (data not shown).

Plasmid maintenance by E2 overlap region mutants. To determine if any of the base substitution mutants described in this study were defective in plasmid maintenance, each of the mutant DNAs was transfected with the plasmid, pMMTneo(302-3), encoding a selectable drug resistance marker into C127 cells. Total DNA was extracted from pooled G418-resistant colonies and analyzed by Southern blot hybridization 4 weeks after transfection. The ability of a mutant DNA to replicate as a stable extrachromosomal plasmid was assayed by evaluating the cellular DNA from the pooled drug-resistant colonies for supercoiled plasmid DNA. In this analysis, the E3- and E4-specific mutants (p909-1 and p908-1) were indistinguishable from the wild-type p142-6 in their abilities to replicate as stable plasmids (Fig. 2). Thus, the E3 and E4 ORFs appear to be nonessential for plasmid maintenance. The negative control for this experiment was p745-1, which has a mutation at nt 945 in the 5′ portion of the E1 ORF, and was previously shown to be defective for stable plasmid replication (16). The E2-specific mutant plasmid, p1038-1, exhibited no form I DNA, in agreement with previous studies indicating that a gene product encoded by the full E2 ORF is required for stable plasmid maintenance (4, 17). Although no form I DNA was noted in this exposure of the lane containing DNA from cells transfected with the SA mutant, P1039-1, a small amount of form I DNA was evident after long exposure (Fig. 2).

During these experiments, it was noted that cotransfection of p1039-1 with pMMTneo resulted in fewer G418-resistant colonies than with other BPV-1 mutants. To investigate this observation further, increasing amounts of p1039-1 DNA, the E2 ORF mutant DNA (p1038-1), and wild-type p142-6 DNA were transfected with pMMTneo(302-3) into C127 cells and selected with G418 (Table 3). Transfection of an increasing amount of either p142-6 DNA or p1038-1 DNA resulted in a slight increase in the number of G418-resistant colonies. In contrast, increasing the amount of p1039-1 DNA resulted in a decrease of G418-resistant colonies. It was possible that the 3′ splice junction mutant somehow down-regulated expression of the TN5 neomycin resistance gene from the MMT promoter or directly expressed a factor which was cytotoxic. We were, therefore, concerned that assessment of the ability of p1039-1 to replicate as a stable plasmid in G418-resistant cells selected with pMMTneo(302-3) could be flawed. Therefore, the analysis was repeated using plasmids in which the drug resistance gene was under the control of other promoters. In one set of experiments, we used a

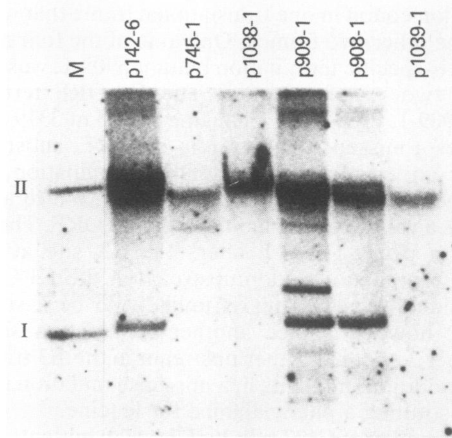

FIG. 2. Southern blot analysis of total cellular DNA from pooled G418-selected C127 cells cotransfected with pMMTneo(302-3) and the indicated BPV-1 mutant DNA. DNA was digested with *Apa*I, a no-cut enzyme for the BPV-1 plasmids. Lane M contains 250 pg each of form I and form II p142-6 plasmid marker DNA with 10 μg of herring sperm DNA. All other lanes contain 10 μg of the indicated total cellular DNA from pooled G418-selected cells. The blot was hybridized to nick-translated P-labeled BPV-1 DNA and washed under stringent conditions. In this experiment, the p909-1-transfected cell line contains aberrant forms of plasmid DNA. A repeat experiment showed no aberrant forms for p909-1. p745-1 is an E1 mutant, previously described, defective for plasmid maintenance (16).

TABLE 3. G418-resistant colony formation of C127 cells by dual plasmid transfection[a]

DNA	Amt of BPV-1 DNA (μg)	No. of colonies/plate
pMMTneo(302-3)	None	125
pMMTneo(302-3) + p142-6	0.1	360
pMMTneo(302-3) + p142-6	1.0	TNTC[b]
pMMTneo(302-3) + p142-6	3.0	TNTC
pMMTneo(302-3) + p1038-1	0.1	200
pMMTneo(302-3) + p1038-1	1.0	400
pMMTneo(302-3) + p1038-1	3.0	420
pMMTneo(302-3) + p1039-1	0.1	100
pMMTneo(302-3) + p1039-1	1.0	60
pMMTneo(302-3) + p1039-1	3.0	34

[a] Each plate received 0.5 μg of pMMTneo(302-3) plus the indicated amount of BPV-1 DNA.

[b] Too numerous to count (>500).

plasmid in which the *neo*^{r} gene might be activated by the BPV-1 E2 gene product. The mutant p1039-1 contains an intact E2 ORF and can express E2, as assayed by its ability to *trans*-activate an element in the viral LCR. We, therefore, chose a plasmid (p1042-1) in which the Tn*5* neomycin resistance gene is expressed from the simian virus 40 early promoter enhanced by the BPV-1 LCR. Selection with p1042-1 would presumably favor selection of those cells expressing E2. Wild-type p142-6 DNA, the E1 mutant p745-1, and the 3′ splice junction mutant p1039-1 were transfected into C127 cells along with the LCR-SVneo plasmid, and G418-resistant colonies were selected. Total cellular DNA was prepared from pooled G418-resistant colonies and analyzed for extrachromosomal DNA (Fig. 3). The restriction endonuclease *Dpn*I was used to cleave the DNA before Southern blot analysis. This enzyme will only cleave DNA methylated after replication in *dam*$^+$ bacteria and does not cleave DNA which has replicated in mammalian cells. The positive control p142-6 replicated in cells cotransfected with pLCR-SVneo(1042-1) but the E1 replication-defective mutant, p745-1, did not (Fig. 3). In contrast to the results obtained with coselection with pMMTneo(302-3), the SA mutant p1039-1 was present as a stable plasmid in cells selected with LCR-SVneo(1042-1). To confirm that the DNA present in these drug selected cells was p1039-1, the DNA was assayed by hybridization analysis after cleavage with *Taq*I (Fig. 3). The A-to-C substitution generates a new *Taq*I site which is in the middle of a 2.8-kilobase fragment (Fig. 1). Analysis (Fig. 3) confirms that the BPV-1 DNA present in these cells is indeed p1039-1. These experiments were also carried out with pSV2-neo as a selectable marker. In these experiments, the p1039-1 DNA also replicated as a stable plasmid (data not shown). These data, therefore, indicate that there is no absolute requirement in plasmid replication for a viral gene product which is solely expressed from an mRNA using the SA at nt 3225.

LCR enhancer *trans*-activation by E2 region mutants. Each of these E2 region mutants was assessed for its ability to *trans*-activate the LCR E2-responsive enhancer. *Trans*-activation was determined measuring CAT expression from a BPV-1 LCR-CAT plasmid, p407-1, in which the LCR serves to enhance the enhancer-deleted simian virus 40 early promoter (26). In these assays, C127 cells were used, since expression of the BPV-1 functions was from homologous viral promoters. Lysates were prepared 72 h after sodium butyrate removal, and the reactions were allowed to continue overnight (see Materials and Methods) (Fig. 4). In agreement with the previous genetic analysis of the BPV-1 LCR *trans*-activation function, only the E2-specific mutant, p1038-1, was defective in this *trans*-activation function, with levels approximately 8% that of the wild type. In contrast, the E3, E4, and SA mutants had *trans*-activation activities comparable with or slightly higher than that of the wild type.

DISCUSSION

Genetic dissection of the 3′ ORFs of the BPV-1 genome has been complicated by the fact that the E2, E3, and E4 ORFs overlap and by the transcriptional organization of the region. Our approach was to individually mutate each of the ORFs in this region as well as the SA so as to introduce silent or, hopefully, neutral changes in the other reading frames. Such mutants permit a more precise mapping of functions located in this region to specific ORFs.

Our data are consistent with previous published data that the transcriptional *trans*-activator encoded by the full-length E2 ORF is important for both stable plasmid maintenance and cellular transformation. Its role, however, may be entirely indirect through the activation of the LCR promoters via the E2-responsive elements (25, 26). The E2-specific mutant, p1038-1, was defective in plasmid maintenance, transformation, and *trans*-activation functions (Table 1 and Fig. 4). Recently, Rabson et al. provided data which suggests that the 3′ portion of the E2 ORF or overlapping portion of the E4 ORF may encode an additional product

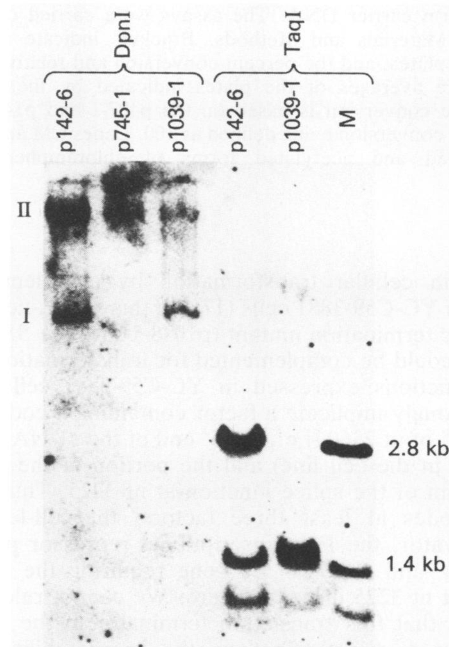

FIG. 3. Southern blot analysis of total cellular DNA from pooled G418-selected C127 cells cotransfected with LCR-SVneo(1042-1) and the indicated BPV-1 mutant DNA. DNA was digested with *Dpn*I and *Taq*I where indicated. *Taq*I can distinguish between p142-6 (wild type) and p1039-1 (SA) DNA by the presence of an additional *Taq*I site in p1039 at sequence 3223. Lane M contains 500 pg of p142-6 marker DNA with 10 μg of herring sperm DNA. All other lanes contain 10 μg of the indicated total cellular DNA from pooled G418-selected cells. The blot was hybridized as done in Fig. 1. p745-1 is an E1 mutant, previously described (16), defective for plasmid maintenance. Although p1039-1 was found to be defective for plasmid maintenance when using MMTneo as a selectable marker (Fig. 1), when the LCR-SVneo plasmid was used, this mutant was positive for plasmid maintenance. kb, Kilobase.

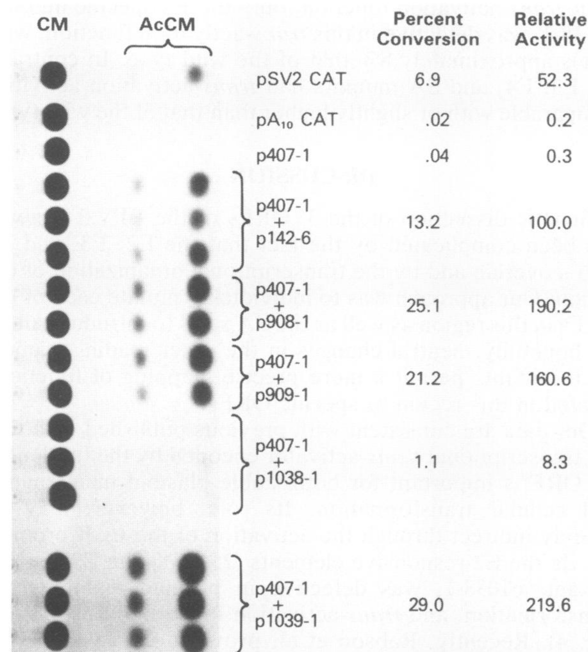

FIG. 4. *Trans*-activation of p407-1 (LCR-CAT) by cotransfection with various BPV-1 constructs. C127 cells were transfected with 1 μg each of the constructs indicated in the first column plus 10 μg of herring sperm carrier DNA. The assays were carried out as described in Materials and Methods. Brackets indicate redundant transfected plates, and the percent conversion and relative activity columns are averages of the plates indicated by the brackets. The relative conversion is based on the p407-1 and p142-6 average percent conversion being defined as 100. Lanes CM and AcCM, nonacetylated and acetylated forms of chloramphenicol, respectively.

involved in cellular transformation by complementation analysis in YC-C59-3881 cells (17). In this study, neither the E2-specific termination mutant (p1038-1) nor the SA mutant (p1039-1) could be complemented for transformation by the BPV-1 functions expressed in YC-C59-3881 cells. These results strongly implicate a factor containing a coding exon mapping 5′ of nt 2360 (i.e., the 5′ end of the cDNA segment expressed in the cell line) and the portion of the E2 ORF downstream of the splice junction at nt 3225. Thus the E2 ORF encodes at least three factors: the full-length E2 *trans*-activator, the E2 transcriptional repressor promoted from P_{3080}, and the new E2 gene requiring the 3′ splice junction at nt 3225 discussed here. We cannot rule out the possibility that this translation terminator in the E2 ORF might have a direct effect on the translatability of the downstream E5-transforming gene.

The gene product of the E4 ORF appears to be dispensable for both transformation and plasmid replication in tissue culture. These results are similar to those of Neary et al. who have also found that the putative product of the E4 ORF is nonessential for plasmid maintenance and transformation in vitro (15). The E4 gene product for BPV-1 has not yet been identified; however, the analogous E4 ORF for human papillomavirus type 1 has been shown to encode an abundant cytoplasmic protein that is expressed coordinately with the major capsid protein (5). Analysis of RNA from bovine fibropapillomas substantiates the fact that the E4 ORF of BPV-1 encodes a late or wart-specific protein, in that the 5′ ORF of the most abundant RNA species transcribed from the late promoter (P_L) would be the E4 ORF (2).

It has been suggested that the BPV-1 E3 ORF does not encode a protein, since it has no methionine initiation codon and it is not highly conserved among other papillomaviruses. Our data does not provide any evidence to counter this suggestion. The E3 mutant p909-1 did have a slightly higher level of transformation than that of the wild-type plasmid p142-6. This difference may not be a result of the E3 mutation but, rather, because of the missense mutation in E2 created in this mutant (Fig. 1) (i.e., serine replacing arginine). It is conceivable that this amino acid substitution in the 3′ domain of E2 produces a protein with altered biological properties. Certainly, the E3 mutant, p909-1, was not defective in transformation or plasmid replication, permitting us to conclude that the E3 ORF does not encode an essential protein for these processes. Whether it encodes a protein at all remains an open question.

The SA^{3225} mutant p1039-1 is interesting since it displays an altered phenotype, yet the mutation does not introduce an amino acid change in the E2 ORF. We conclude, therefore, that the effects manifested are due to the elimination of the 3′ splice junction at nt 3225. By primer extension analysis with a 5′-end-labeled oligomer complementary to the RNA spanning the splice from nt 2505 to 3225, we have confirmed that the SA^{3225} mutant is indeed defective for this splice (data not shown). It is possible that other cryptic SAs are used in this mutant. From analyses of cDNA libraries isolated in this laboratory, it is apparent that many BPV transcripts use the 3′ splice junction at nt 3225 (2, 28). One might expect this mutation to have pleiotropic effects by affecting genes created by the splicing of exons at this junction as well as by affecting exons located entirely upstream or downstream of this junction but expressed from RNAs using this splice junction. At this time, we have not defined the mechanism underlying the transformation defect of p1039-1. BPV-1 contains two independent transforming genes, E5 and E6 (20, 21, 29). Analysis of a cDNA library has revealed that an mRNA which could encode E6 and which has slight transforming activity uses the 3′ splice junction at nt 3225 (28). Therefore, it is possible that expression of the E6 protein is lower in this mutant. This, however, is not sufficient to explain the defect because p1039-1 cannot be complemented to wild-type transformation levels by the cDNA encoding E6 (data not shown). It seems more likely that this mutation affects the level of E5 expression. Although the RNA that serves as the message for E5 has not been determined, it has been suggested that a spliced RNA from P_{2443} using the 3′ splice junction at 3225 may be the E5 transcript (2). Our data with the C59-SA mutant which contains the E5 ORF intact but which is still impaired in its ability to transform would support this idea. Furthermore, we have analyzed the levels of E5 protein in C127 cells in which p1039-1 was introduced by selection with the LCR-SVneo construct p1042-1 and found only low levels of the protein (data not shown). These data suggest that the transformation defect of the SA mutant (p1039-1) may be due to altered expression of at least two genes. First, through elimination of the splice junction at nt 3225, it would prevent the synthesis of the spliced message that may normally encode E5. Secondly, it would prevent the synthesis of a spliced E2 product which might act directly or indirectly to regulate expression of the viral transforming factors.

It is also possible that the transformation defect is not due to the direct expression of one of the transforming genes but may be indirectly due to the expression of a nontransforming

gene involved in the regulation of gene expression. The ability to express the E2 *trans*-activator is intact in this plasmid (Fig. 4); however, it is possible that the SA^{3225} mutation could affect expression of other viral products which could indirectly affect transformation. We have been unable to complement p1039-1 for transformation with a series of spliced cDNAs which used the 3′ SA at nt 3225 (28) either singly or in combination (data not shown).

Although the p1039-1 mutant was defective for transformation, it was able to replicate as a stable plasmid in cells cotransfected with the LCR-SVneo plasmid (p1042-1) and with pSV2neo. Thus, unlike transformation, there is no absolute requirement for products of RNAs using the m3225 SA for plasmid replication functions. These data contrast with our results in which cells taking up p1039-1 DNA were selected by cotransfection with p302-3 expressing the neo^R gene from the mouse metallothionein promoter. The majority of the 1039-1 DNA was integrated in C127 cells selected with pMMTneo(302-3). A titration experiment demonstrated that increasing amounts of p1039-1 DNA led to fewer drug-resistant colonies. This experiment suggested that p1039-1 was somehow inhibitory to the mouse metallothionein promoter. One possibility is that the full E2 product may affect expression of the metallothionein promoter. p1039-1 expressed higher levels of the E2 *trans*-activator than the wild-type plasmid, p142-6 (Fig. 4). It is also a formal possibility that the p1039-1 and the pMMTneo(302-3) DNAs might compete for a factor required either for efficient transcription or for BPV-1 DNA replication. Experiments to further evaluate the effect of BPV-1-encoded factors on cellular promoters including the MMT promoter are in progress.

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