JOURNAL OF VIROLOGY, Nov. 1986, p. 626–634
0022-538X/86/110626-09$02.00/0

Vol. 60, No. 2

Bovine Papillomavirus Type 1 3′ Early Region Transformation and Plasmid Maintenance Functions

MICHAEL S. RABSON,† CAROLE YEE, YU-CHUNG YANG,‡ AND PETER M. HOWLEY*
Laboratory of Tumor Virus Biology, National Cancer Institute, Bethesda, Maryland 20892

Received 1 April 1986/Accepted 6 August 1986

We examined bovine papillomavirus type 1 (BPV-1) DNAs mutated in the E2 open reading frame (ORF) to determine their ability (i) to transform C127 cells and (ii) to remain extrachromosomal in transfected cells. Results obtained with deletion mutants and insertion mutants containing a linker with translational termination codons in all possible reading frames indicated that an E2 ORF gene product(s) is necessary for efficient transformation, as well as viral plasmid replication and maintenance in the context of the full BPV-1 genome. Complementation assays in which mutant BPV-1 DNAs were transfected into cell lines expressing some viral functions from integrated BPV-1 cDNAs demonstrated that the E2 ORF product, when provided in *trans*, could allow BPV-1 E2 mutants to remain extrachromosomal. The E2 function could also augment transformation of some, but not all, BPV-1 E2 mutants, allowing identification of another region of BPV-1 involved in cellular transformation. It is likely that the role of the BPV-1 E2 product(s) in transformation and plasmid maintenance is indirect. A BPV-1 mutant altered in the E5 ORF is transformation defective and unable to replicate as a stable plasmid in C127 cells.

Bovine papillomavirus type 1 (BPV-1) is the prototype of a subgroup of papillomaviruses which can cause benign fibropapillomas in their natural hosts, induce fibroblastic tumors in heterologous species such as hamsters, and readily transform susceptible rodent cells in vitro (13). Although these viruses have not yet been successfully propagated in cell culture, the molecular biology and genetics of the papillomaviruses have been studied extensively with cloned DNA. Like intact virus, cloned BPV-1 DNA, or a subgenomic BPV-1 fragment comprising 69% of the viral genome (BPV_{69T}), can transform mouse C127 or NIH 3T3 cells in vitro (16). In transformed cells, fibroblastic tumors, and naturally occurring fibropapillomas, the viral DNA persists stably as an extrachromosomal multicopy plasmid (15).

The BPV-1 genome is a 7,945-base-pair (bp), circular, double-stranded molecule which has been sequenced (4). All of the open reading frames (ORFs) longer than 400 bp are located on the same strand (see Fig. 1). Eight ORFs, designated E1 through E8, have been identified within BPV_{69T}, which contains all of the viral genes required for transformation and plasmid replication and maintenance. Transcriptional analyses of the viral polyadenylated RNAs in BPV-1-transformed cells have shown that these RNAs map to BPV_{69T} (11). Both unspliced and spliced RNA species have been identified by electron microscopy and cDNA analysis (28, 29). Several different viral transcriptional promoters apparently function in transformed cells, evidenced by the fact that at least three sets of viral RNAs can be distinguished by their 5′ ends (28, 29). All share a common 3′ terminus at the polyadenylation site at BPV-1 base 4203 (29). Two transcriptional enhancer regions have been identified in BPV_{69T}. A conditional enhancer which can be activated in *trans* by the E2 ORF gene product is located in the 1,000-bp long control region (LCR) upstream of the eight early ORFs (26). This region was previously designated the noncoding region; however, in light of findings that this region contains coding exons for certain species of early and late viral RNAs (C. C. Baker and P. M. Howley, manuscript in preparation; M. R. Botchan, personal communication), we feel that this region is more appropriately termed the LCR. Another sequence which can activate a heterologous promoter in a position- and orientation-independent manner maps at the distal end of the BPV_{69T} region (3, 17). However, this distal enhancer has been shown not to be *cis* essential for plasmid maintenance or transformation by BPV-1 DNA in vitro (12).

Mutagenesis studies of full-length viral DNA and experiments with subgenomic DNA fragments expressed from surrogate promoters have mapped regions of the viral genome involved in cellular transformation (6, 19, 21, 23–25, 29). In addition to regions which have essential transcriptional control sequences, several loci which apparently encode proteins involved in transformation have been identified. A domain critical for transformation maps to the E5 ORF downstream from the methionine codon at base 3879; disruption of this part of the E5 ORF by introduction of an in-frame termination codon (30) or by frameshift mutations (25) has a marked inhibitory effect on transformation efficiency. A second viral function which is necessary for expression of the fully transformed phenotype maps to the region containing the E6 and E7 ORFs (23). Expression of the E6 ORF from a strong surrogate promoter is sufficient for morphologic transformation of C127 cells (24, 29). The BPV-1 E6 protein has recently been detected in transformed cells (2). A third region which is essential for transformation when the full-length BPV-1 region, rather than subgenomic fragments, is examined maps to the E2 ORF (6, 19, 23). Since the E2 gene has been shown to encode a product which can function to *trans* activate transcriptional regulatory sequences in the LCR (27), it is not clear whether the role of the E2 product in transformation is direct or indirect.

Genetic analyses of BPV-1 DNA have also defined regions of the genome involved in plasmid replication and maintenance. Two independent sequences, designated plasmid

* Corresponding author.
† Present address: Yale Law School, New Haven, CT 06520.
‡ Present address: Genetics Institute, Cambridge, MA 02140.

maintenance sequences (PMS-1 and PMS-2) have been described as *cis*-essential elements which are capable of supporting extrachromosomal plasmid maintenance when diffusible viral replication factors are provided in *trans* (18). The E1 gene product is essential for plasmid replication and maintenance. Mutations in this region invariably result in integration of viral DNA into the host chromosome (19, 23), and this function can be complemented in *trans* (18). Further, the E7 ORF product has been ascribed a role in regulating plasmid copy number based on mutagenesis of the E7 ORF and complementation studies with mutant DNAs (19). The role of the E2 ORF product in plasmid replication and maintenance has been disputed. In our studies (23), as well as those of DiMaio et al. (6), mutagenesis of the E2 ORF has resulted in loss of plasmid maintenance. However, Lusky and Botchan (19) have described mutants deleted of part or all of the E2 ORF which are able to replicate as stable extrachromosomal plasmids.

A limitation of the genetic analyses which we performed previously (23) was that our mutants with large deletions affected the integrity of more than one ORF because of the overlapping genetic organization of BPV-1. This study presents a further genetic analysis of BPV-1 with mutated DNAs with targeted alterations which affect specific ORFs or groups of ORFs. Experiments are described which examine the ability of these mutated DNAs to transform C127 cells and to stay extrachromosomal in such cells. Furthermore, we performed a series of complementation experiments in which various mutated BPV-1 DNAs and cDNAs were tested for their ability to provide functions in *trans* to defective counterparts in the same cell.

In this report we provide evidence that, in the full viral genomic background, expression of the E2 ORF is essential for stable plasmid maintenance and is important for efficient focus formation. We demonstrated that a product of the E2 ORF can act in *trans* to allow extrachromosomal plasmid maintenance by showing that, in cells expressing the E2 gene product(s) from an integrated cDNA, E2 ORF mutants are able to replicate as stable plasmids. Furthermore, we found that the product of the full E2 ORF can act in *trans* to augment transformation by certain BPV-1 E2 mutants but not others. These results indicated that the BPV-1 3′ ORFs encode in part a gene product which, in addition to the putative full E2 ORF product and the E5 protein, is involved in transformation.

MATERIALS AND METHODS

Cell lines. Mouse C127 cells (7) and C127 cells containing BPV-1 DNAs were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cell lines containing BPV-1 C59 and mutated C59 cDNAs have been previously described (30). C59 cDNA contains the complete BPV-1 E2, E3, E4, and E5 ORFs expressed from the simian virus 40 early promoter (29).

Transfections. We performed transfections of C127 cells by using a modification of the calcium phosphate coprecipitation which has been described previously (22). Briefly, each 60-mm (diameter) dish of cells received 10 μg of a precipitate containing 1 μg of BPV-1 DNA and 9 μg of salmon sperm carrier DNA. When cells were to be selected for drug resistance to G418, 1 μg of a plasmid in which the Tn*5* neomycin resistance gene is expressed from the mouse metallothionine promoter [pMMTneo(302-3)] (14) was added, and the amount of carrier DNA was reduced accordingly. Four hours after transfection, the cells were treated with 15% glycerol for 2 min and then washed. The cells were incubated in complete medium with 5 mM sodium butyrate for 24 h (9), and each 60-mm plate was split into two 100-mm dishes. For selection of drug-resistant cells, G418 (400 μg/ml) was added to the dishes 72 h after transfection, and the cells were maintained continuously under selection (5). For focus assays, the plates were stained after 2 weeks as previously described (22).

Cellular DNA extraction. Total cellular DNA was extracted from C127 cells by a method described previously (15). Restriction endonuclease digestion, gel electrophoresis, and Southern blot analysis of the DNAs were performed by standard methods (20).

Construction of BPV-1 mutant DNAs. The BPV-1 DNAs used in this study are diagrammed in Fig. 1. Except where otherwise noted, the starting plasmid for all of the BPV-1 mutant DNAs was p142-6, which contains the entire BPV-1 genome cloned at the *Bam*HI site of pML2d (22).

The BPV-1 mutant DNAs p593-6, p620-7, p327-25, and p448-5 have been described in detail previously (23). The plasmids p593-6 and p620-7 are mutated only in the E1 ORF; p593-6 was generated by insertion of a *Hin*dIII linker at the unique *Bst*EII site (base 2405) in BPV-1 DNA, whereas p620-7 is deleted of sequences between the *Eco*RI site (base 2113) and the *Bst*EII site (base 2405). The mutants p327-25 and p448-5 each contain deletions extending upstream from the *Bcl*I site at base 3838; p327-25 has a 101-bp deletion between the *Bcl*I sites at 3737 and 3838 which affects the 3′ end of the E2 ORF and the E5 ORF 5′ to the first methionine codon; p448-5 is deleted of the 383 bases between the *Kpn*I site (base 3455) and the *Bcl*I site (base 3838).

Two additional BPV-1 deletion mutants were constructed for this set of experiments. We produced the mutant p631-1, which is affected only in the E2 ORF, by cleaving p142-6 DNA with *Nco*I and religating, thereby removing a 211-bp *Nco*I fragment. We made p715-5 by deleting the sequences between the *Pst*I sites at 2775 and 4172; an *Xho*I linker is present at the site of the deletion. This mutant lacks all of the E3, E4, and E5 ORFs and most of the E2 ORF.

To target mutations which would lead to premature translational termination of the products of specific ORFs, we developed a different approach. An oligonucleotide linker element containing an *Hpa*I cleavage site was synthesized and inserted into the full-length clone of BPV-1 DNA (p142-6) at a variety of different restriction enzyme sites. This 12-bp element, a translational termination linker (TTL), as designed to contain a translation stop codon in each of the three reading frames when inserted, regardless of orientation. The sequence of the TTL is:

5′TTAGTTAACTAG 3′
AATCAATTGATC

The TTL was inserted in the E1 ORF at the *Pst*I site at base 1299 (p743-23) and in the E5 ORF by introduction of the TTL into the *Bst*XI site at base 3881 (p830-1). The p830-1 mutant is similar to the p744-1 plasmid, which has previously been described by us, except that p830-1 contains a single TTL and p744-1 contains multiple linkers. We generated two BPV-1 DNAs mutated in the E2 ORF by moving restriction fragments containing the TTL from the background of the BPV-1 C59 cDNA, which contains the E2, E3, E4, and E5 ORFs intact (29), into the p142-6 background. The cDNAs, C59-2878 and C59-3235, containing the TTLs have been described previously (30); each was cleaved with *Bst*E2 and

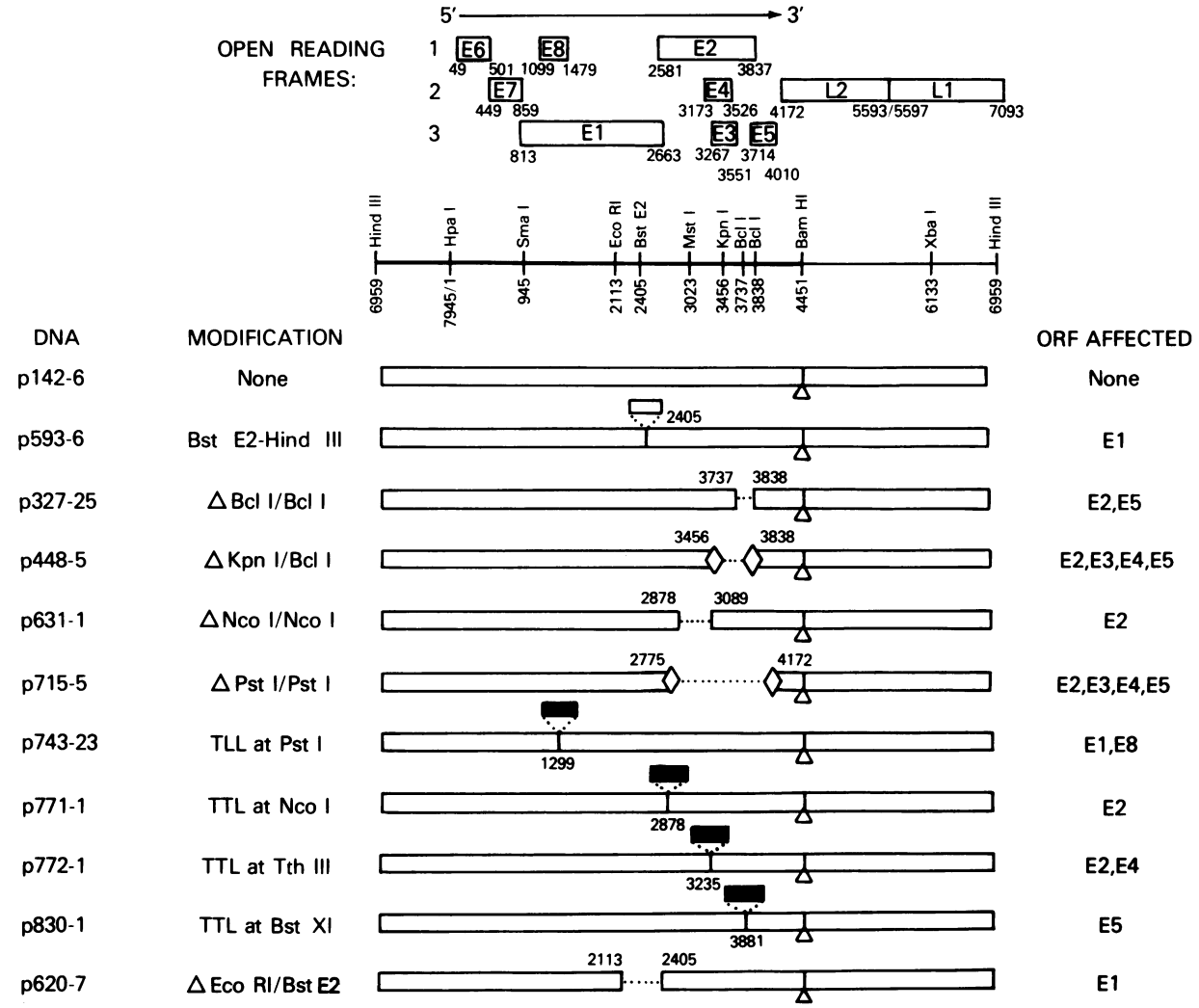

FIG. 1. Genomic organization of BPV-1 DNA and structure of BPV-1 mutants. The full-length molecule (7,945 bp) of BPV-1 opened at the unique *Hin*dIII site (base 6959) is marked off with restriction endonuclease sites and bases at the bottom of the figure. The BPV-1 subgenomic fragment which contains all of the sequences necessary for autonomous plasmid replication (15) and transformation (16) is indicated by a solid bar. The direction of transcription and the region transcribed in transformed cells are indicated by the arrow at the top of the figure (11). The open bars represent large ORFs and, therefore, potential coding sequences in each of the three translation frames predicted from the sequence (4). ORFs within the transforming region are designated E1 to E8. Those expressed only in productively infected fibropapillomas (1, 8) are designated L1 and L2. The numbers beneath the ORFs designate the first and last bases of an ORF. The recombinant plasmids are listed on the right, and the restriction endonuclease sites used in generating the various mutants are listed on the left. In each case, the plasmid consisted of BPV-1 DNA sequences linked to the pML2d vector sequences at the *Bam*HI site (△). Symbols: ▭, BPV-1 sequences covalently linked at the sites indicated; ◇, *Xho*I linker elements; ....., deleted sequences; ▬, linkers in TTL insertion mutants.

*Kpn*I, and the resultant 1,050-bp BPV-1 DNA fragment was transferred into p142-6, which had been deleted of the analogous fragment. Ligation of these DNA fragments generated the plasmids p771-1 and p772-1, which contain the TTL at bases 2878 (*Nco*I) and 3235 (*Tth*III), respectively, in the full-length BPV-1 genome.

RESULTS

These experiments were initiated as part of an analysis of the genetic elements involved in BPV-1 transformation and plasmid maintenance. Our previous analysis of E2 ORF deletion mutants, indicated that the product of this ORF had a role in both of these functions. We found that, compared with the full-length genome, BPV-1 DNAs deleted of sequences in the E2 ORF had diminished transforming ability (23). Analysis of the viral DNA in cells selected for transformation by these E2 mutants revealed that the DNA was integrated into the host chromosome (23). These results differed from those of Lusky and Botchan (19), who reported that the E2 ORF was important for transformation but that the E2 ORF product was not absolutely required for plasmid replication and maintenance. We initiated the experiments described in this report to examine in more detail the role of the E2 gene product in transformation and plasmid maintenance.

Transformation by E2 ORF mutants. Our initial experiments investigated the role of the E2 ORF in transformation. For these experiments, deletion and linker insertion mutants were generated in the E2 ORF.

Two deletion mutants, p715-5 and p631-1, were constructed. p715-5 had the viral sequences between the *Pst*I

TABLE 1. Transformation capacity of cloned mutated BPV-1 DNAs

Plasmid	ORF(s) affected	No. of foci/0.5 μg of DNA per 10^6 cells	
		Cleaved[a]	Uncleaved
p142-6	None	>500, >500	104, 93
p593-6	E1	276, 260	65, 71
p743-23	E1, E8	200	35, 38
p631-1	E2	1, 1	4, 6
p771-1	E2	26, 34	3, 4
p772-1	E2, E4	0, 0	0, 0
p448-5	E2, E3, E4, E5[b]	0, 1	ND[c]
p327-25	E2, E5[c]	ND	101, 91
p830-1	E5[d]	0, 0	6, 6
p715-5	E2, E3, E4, E5[e]	ND	0, 0

[a] *Bam*HI cleaved to release BPV-1 DNA sequences from pML2d sequences (23). Dual numbers refer to duplicate plates.

[b] The portion of the E5 ORF deleted in each of these mutants is located upstream of the first AUG methionine codon at bases 3879 to 3881.

[c] ND, Not determined.

[d] This mutant affects the E5 ORF 3' to the methionine ATG at bases 3879 to 3881.

[e] This mutant deletes the entire E5 ORF.

sites at bases 2775 and 4172 deleted; this mutant lacks all of the E3, E4, and E5 ORFs and all but the most 5' 194 bp of the E2 ORF. The BPV-1 distal enhancer and early-region polyadenylation signals are present. The mutant p715-5 is similar to the Bal 15 mutant previously described by Lusky and Botchan, which was reported to be capable of plasmid replication and maintenance (18). The p631-1 mutant was deleted of a 211-bp *Nco*I fragment which alters the reading frame of E2 but does not affect E3, E4, or E5.

Although the mutation in p631-1 is localized to the E2 ORF, the deletion of 211 bp could theoretically affect the expression of other viral genes, possibly by affecting mRNA stability. To alter the translational capacity of specific viral ORFs without grossly altering the viral genome, we decided to use linker insertion mutagenesis. A 12-bp linker element was designed which contained a translational termination codon in each of the possible reading frames in either orientation. The presence of this linker could be confirmed by virtue of the presence of an *Hpa*I restriction site contained within it.

Two mutant DNAs (p771-1 and p772-1) containing the TTL in the E2 ORF were generated. The mutant p771-1 contained the TTL inserted at base 2878, and p772-1 contained the TTL inserted at base 3235. Introduction of the TTL at base 3235 in p772-1 also introduces a stop codon into the E4 ORF. An additional TTL mutant (p830-1) which contained the TTL at the *Bst*XI site (base 3881) in the E5 ORF was made. The TTL was also inserted at base 1299 in the E1 ORF to make the mutant p743-23. The presence of a single linker at each of these sites was confirmed by DNA sequencing (data not shown).

Each of these BPV-1 mutants, either linked to the pML2d vector DNA or cleaved from the vector with *Bam*HI, was assayed for the ability to form foci on mouse C127 cells (Table 1). The E5 mutant p830-1 transformed at approximately 1% of the efficiency of p142-6. The mutants p715-5 and p772-1 induced no foci, whereas p631-1, p771-1, and p448-5 each transformed poorly, with an efficiency of approximately 1 to 10% of that of p142-6 DNA. As expected, the E1 mutants p593-6 and p743-23 transformed at approximately equal efficiencies, slightly less than the p142-6 control. These experiments confirmed our original findings and independent results from other laboratories that the integrity of the E2 ORF is required in the BPV-1 wild-type DNA context for efficient focus formation (6, 19).

The E2 ORF product is necessary for stable plasmid maintenance. We determined the state of the viral DNAs of each of the E2 mutants by examining the total intracellular DNAs from cell lines transfected with the mutant BPV-1 DNAs. When possible, individual foci were selected and expanded, and the DNA was analyzed by Southern blot analysis. Additionally, each mutant DNA was cotransfected with pMMTneo(302-3) into C127 cells, and G418-resistant colonies were selected and expanded to obtain DNA for Southern blot analysis.

The results from these experiments are listed in Table 2. The data from Southern blot analysis of DNA from cells established from foci induced by p631-1 or from G418-resistant colonies established by cotransfection with this mutant DNA and pMMTneo(302-3) are shown in Fig. 2 (lanes c to f). Regardless of the selection, the viral DNA was integrated. These data are in agreement with the results of DiMaio et al. (6), who found that an identical deletion mutant integrated into the host chromosome regardless of whether cells were selected for focus formation or for expression of an unlinked second marker. The state of the viral DNA was also assessed in cell lines established from foci and G418-resistant cotransformants for the E2 mutant p771-1 and the E2-E4 mutant p772-1. The viral DNA of each of these mutants was found to be integrated into the host chromosome regardless of the selection. The integrated state of the viral DNA is shown in Fig. 2 for three lines containing p771-1 (lanes g, h, and i) and for two lines containing p772-1 (lanes j and k) selected for drug resistance.

A mutation in the E5 ORF affects stable plasmid maintenance. We also tested the ability of p830-1, which contains the TTL at base 3881 in the E5 ORF, to replicate as a plasmid in C127 cells. This DNA, which has very low transforming ability (Table 1), was cotransfected into C127 cells with pMMTneo(302-3). After selection in G418, the drug-resistant foci were pooled and intracellular DNA was prepared. Southern blot analysis of the total cell DNA revealed viral DNA migrating with high-molecular-weight cellular DNA and extrachromosomal monomeric circular DNA (Fig. 3A, lane f). Similar analysis of the E2 mutant p771-1 and of the E1 mutant p620-7, revealed only integrated

TABLE 2. State of mutant BPV-1 viral DNAs in mouse C127 cells[a]

Plasmid	ORF(s) affected	State of DNA in transformed cells	State of DNA in neo^r cells
p142-6	None	Extrachromosomal	Extrachromosomal
p593-6	E1	Integrated	Integrated
p743-23	E1, E8	Integrated	Integrated
p631-1	E2	Integrated	Integrated
p771-1	E2	Integrated	Integrated
p772-1	E2, E4	ND[b]	Integrated
p448-5	E2, E3, E4, E5	Integrated	Integrated
p327-25	E2, E5	Integrated	Integrated
p830-1	E5	ND	Unstable[c]

[a] In each case, the physical state of the recombinant plasmid consisting of the mutated BPV-1 DNA cloned in pML2d was determined. Mouse C127 cells were transfected with intact hybrid plasmid. Transformed foci were selected and expanded or neomycin-resistant colonies were selected and expanded for DNA analysis.

[b] ND, Not determined.

[c] The DNA was apparently integrated in three of four clonal lines examined. In the line 4 it was rearranged. In pooled resistant colonies examined 3 weeks after selection, extrachromosomal plasmid DNA could be detected (Fig. 3).

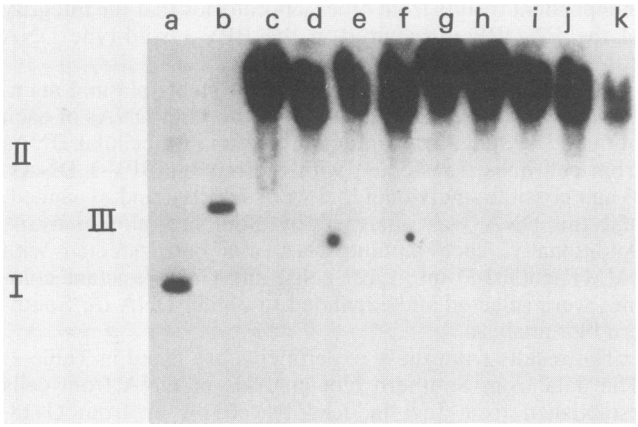

FIG. 2. Southern blot analysis of total cellular DNA from C127 cells transformed with BPV-1 ORF E2 mutants. Lanes a and b contained, respectively, 10 pg of uncleaved p142-6 marker DNA and 10 pg of *Xba*I-cut, linearized p142-6 DNA. Lanes c to k each contained 10 μg of *Xho*I-cleaved DNA from the following cell lines (lanes): c, MR 631-1 G-B; d, MR 631-1 G-C; e, MR 631-1 A; f, MR 631-1 C; g, MR 771-1 G-A; h, MR 771-1 G-B; i, MR 771-1 G-C; j, MR 772-1 G-A; k, MR 772-1 G-C. *Xho*I does not cut within p631-1, p771-1, or p772-1. Cell lines designated with the letter G represented in lanes c, d, g, h, i, j, and k were expanded from individual G418 resistant colonies. All other cell lines were established from individual BPV-1-transformed foci. A nick-translated ^{32}P-labeled full-length BPV-1 genome probe was hybridized to this filter. Note that the viral DNA was integrated into the high-molecular-weight DNA in all of these lines. The small quantity of faster-migrating DNA in lanes c, f, and j appears to be integrated DNA. The roman numerals indicate the positions of migration of form I (supercoiled circles), II (nicked circles), and III (linear molecules) DNAs.

DNA (Fig. 3A, lanes d and e). These data suggested that the E5 ORF product, which is important in transformation, is not absolutely essential for plasmid replication. To study this point further, we examined the state of p830-1 DNA in four independent cell lines expanded from individual G418-resistant foci. Total cellular DNA was examined, and the viral DNA migrated with the high-molecular-weight cellular DNA in three of the lines (Fig. 3B), indicating integration. In the fourth cell line (MR830-1B), some extrachromosomal DNA could be detected in addition to the integrated DNA, although the restriction endonuclease cleavage patterns indicated that the plasmid DNA had undergone rearrangements. Thus, it appears that the integrity of the E5 ORF is required for stable plasmid maintenance in the background of the full viral genome.

The E2 function for plasmid replication can be complemented in *trans*. The inability of the E2 mutants to be maintained as stable plasmids in mouse C127 cells suggested that a product of the E2 ORF was required for this function. We therefore assessed whether this plasmid replication and maintenance function could be provided in *trans* in cells constitutively expressing the E2 gene function. For these studies we cotransfected each BPV-1 mutant DNA with pMMTneo(302-3) into YC-C59, a transformed C127 cell line containing and expressing an integrated copy of the BPV-1 cDNA, C59. This cDNA contains the complete BPV-1 E2, E3, E4, and E5 ORFs expressed from the simian virus 40 early promoter (29). The YC-C59 cell line was established from a single transformed focus induced by C59 DNA and has been shown to express the E2 *trans*-activation function, which affects a BPV-1 LCR transcriptional regulatory element (26).

After pMMTneo(302-3) cotransfection of the YC-C59 cell

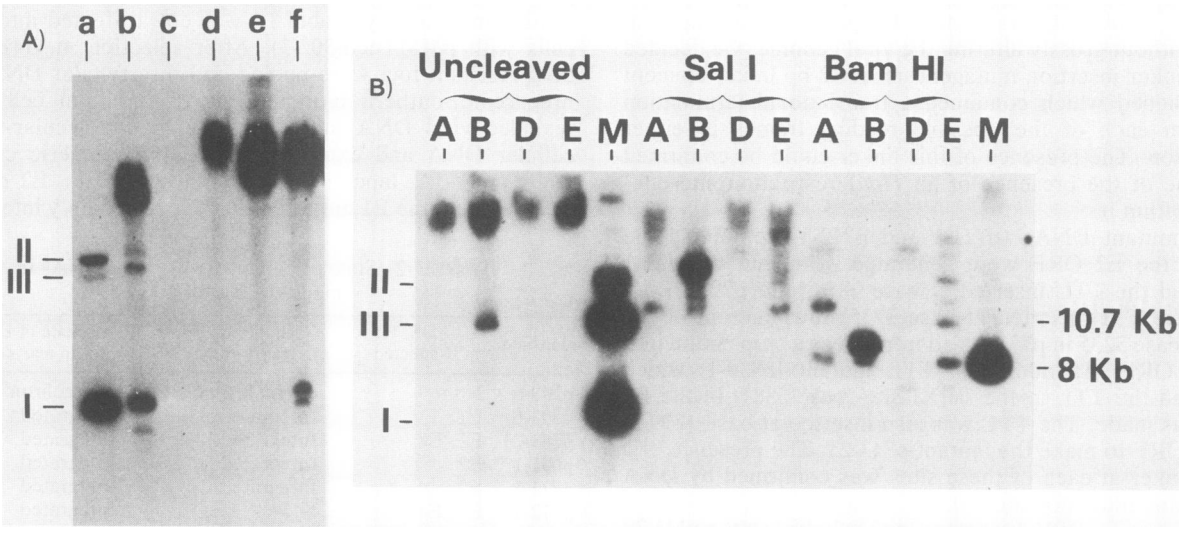

FIG. 3. (A) Southern blot analysis of total cellular DNA from pooled G418-selected C127 cells cotransfected with pMMTneo(302-3) and the indicated mutated BPV-1 cloned DNAs. DNA was prepared from pooled G418-selected colonies. Lane a contained 50 pg of the marker pBPV-1(142-6) (forms I, II, and III). Lanes b to f contained 10 μg of sheared cellular DNA from the pooled cotransfected colonies (lanes): b, cotransfected with wild-type pBPV-1(142-6); c, transfected with pMMTneo(302-3) alone; d, cotransfected with the BPV-1 E1 mutant p620-1; e, cotransfected with BPV-1 E2 mutant p771-1; f, cotransfected with BPV-1 E5 mutant p830-1. The blot was hybridized to nick-translated ^{32}P-labeled BPV-1 DNA (2×10^8 cpm/μg) and washed under stringent conditions. (B) Southern blot analysis of total cellular DNA from selected foci of C127 cells cotransfected with p830-1 and pMMTneo(302-3). DNAs were prepared from four individual G418-resistant cell lines (A to D) established from individual foci. Each lane contained 10 μg of DNA from each of the cell lines treated with no enzyme, *Sal*I (a one-cut enzyme for p830-1), or *Bam*HI (a two-cut enzyme for p830-1). The marker lane (M) to the left contained 400 pg of p830-1 DNA divided among forms I, II, and III of the plasmid. The marker lane (M) to the far right contained 200 pg of p830-1 cleaved with *Bam*HI, releasing the 8.0-kilobase (Kb) BPV-1 DNA insert. The blot was hybridized and washed as described above.

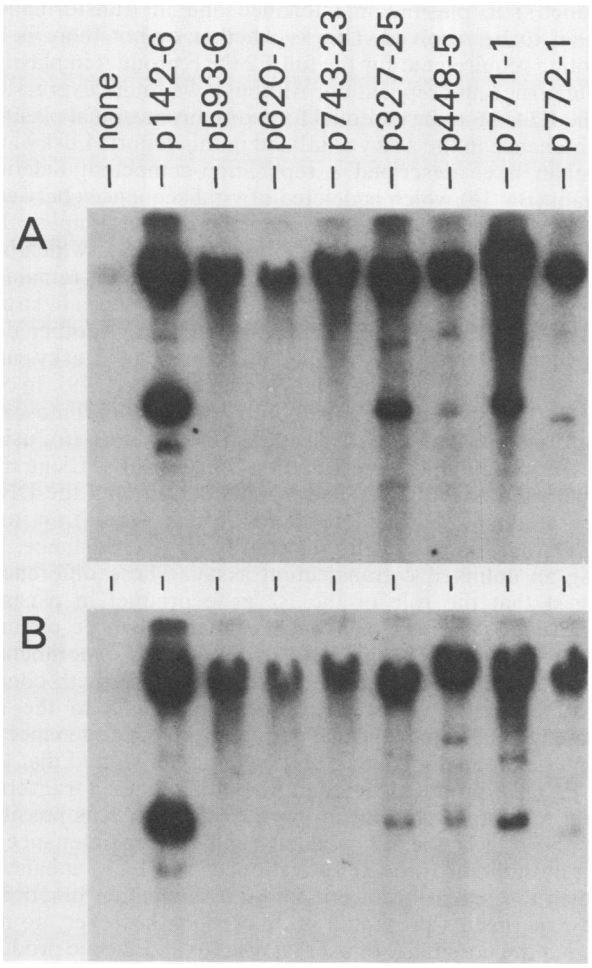

FIG. 4. Southern blot analysis of cellular DNAs from line YC-C59 cells transfected with BPV-1 DNAs. E1 and E2 mutants of BPV-1 were cotransfected with pMMTneo(302-3) on YC-C59 cells which contain integrated C59 DNA containing the BPV-1 E2, E3, E4, and E5 ORFs intact. Intracellular DNA was prepared from pooled G418-selected colonies. For A, we gently sheared the DNA by passing it through a 26-gauge needle 20 times, and for B we cleaved the DNA with *Apa*I, a no-cut enzyme for the recombinant DNA plasmids used. DNA was subjected to electrophoresis through 0.85% agarose, transferred to nitrocellulose membranes, and hybridized to ^{32}P-labeled, full-length BPV-1 DNA under stringent conditions. Cellular DNA (10 μg) was loaded in each lane. p142-6 is the wild-type BPV-1 recombinant in pML2d. p593-6, p620-7, and p743-23 contain mutations in the E1 ORF. p327-25, p448-5, p771-1, and p772-1 contain mutations in the E2 ORF.

line with various mutant BPV-1 DNAs and G418 selection, multiple-drug-resistant colonies were pooled, total cellular DNA was prepared, and the state of the input mutant viral DNA was examined. The results of this experiment are presented in Fig. 4A, in which the DNAs were analyzed after mild shearing of the total cellular DNA, and in Fig. 4B, in which the cellular DNA was cleaved with *Apa*I, which is a no-cut enzyme for the recombinant DNA plasmids used. The first lanes contained DNA from nontransfected YC-C59 cells and demonstrated the integrated BPV-1 cDNA sequences. The second lanes contained the positive controls, in which wild-type p142-6 DNA was transfected; multiple extrachromosomal DNA copies were apparent. ORF E1 mutants (p593-6, p620-7, and p743-23) served as negative controls; the DNAs were found integrated. Four E2 ORF mutants were tested in this experiment. The E2 deletion mutants p327-25 and p448-5 were each found to remain as extrachromosomal plasmids in the YC-C59 cells. The TTL mutants p771-1 and p772-1, which integrated into C127 cells (Fig. 2), were found to replicate as stable plasmids in YC-C59 cells. Thus, the YC-C59 cell line provided a factor which acted in *trans* to permit the E2 mutants, but not the E1 mutants, to replicate as stable plasmids. It should be noted, however, that the wild-type BPV-1 DNA (p142-6) was present at a higher copy number in these cells than were any of the E2 ORF mutant DNAs (Fig. 4). This suggested that, although the YC-C59 cells provided a factor which acted in *trans* to permit the E2 mutants to replicate as stable plasmids, the cells may not provide a fully permissive environment for plasmid replication and maintenance for the E2 mutants. Indeed, two of the E2 mutants (p448-5 and p772-1) were present at very low copy number in these cells, suggesting that other viral factors encoded in part by the E2 or E4 ORF may be involved in plasmid replication and maintenance. Further experiments are in progress to resolve this question.

As mentioned above, the C59 cDNA which was used to generate the YC-C59 cell line contains the BPV-1 E2, E3, E4, and E5 ORFs intact. Therefore, in addition to expressing the E2 transcriptional *trans*-activation function, this line could also potentially express viral gene products from the E3, E4, and E5 ORFs. To test whether the factor expressed in YC-C59 cells did indeed arise from the E2 ORF, we determined whether BPV-1 mutant DNAs could replicate as stable plasmids in cell lines transformed with mutated C59 DNAs which were altered in the E2 ORF by insertion of the TTL. The mutant C59 cDNAs, C59-2878 and C59-3235, have been described previously (30). C59-2878 contains the TTL at the *Nco*I site at base 2878, and C59-3235 contains the TTL inserted at the *Tth*III site at base 3235. Each of these mutant cDNAs can transform C127 cells because of expression of the E5 ORF, but they do not express the E2 *trans*-activation function (30). Cell lines transformed with each of these mutated cDNAs were cotransfected with the full-length BPV-1 mutant DNAs and pMMTneo(302-3). After selection in G418, intracellular DNA was isolated from pooled drug-resistant foci and analyzed by Southern blot analysis. Several of the BPV-1 mutant DNAs tested in YC-C59 cells were also tested in the two cell lines described above. Only wild-type p142-6 DNA remained extrachromosomal in the latter two lines. The E1 mutant p620-7 integrated, as did each of the E2 mutants (p771-1 and p772-1) tested (data not shown). Thus, it is the E2 ORF that encodes a diffusible factor which is capable of acting in *trans* to complement E2 mutant DNAs and which allows them to remain extrachromosomal in C127 cells.

An E2 ORF product can act in *trans* to increase transformation efficiency. Having shown that an E2 ORF product can act in *trans* to allow E2 mutant DNAs to remain as extrachromosomal multicopy plasmids, we asked whether the E2 ORF product might also act to augment the transformation capacity of the E2 mutants in *trans*. To address this question, we transfected full-length BPV-1 mutant DNAs into either C127 cells or the YC-C59-3881 cell line containing an integrated copy of a mutated C59 cDNA (C59-3881). We used this approach rather than cotransfection to avoid potential problems introduced by recombination between heterologous DNAs during the transfection procedure. The C59-3881 cDNA contains a TTL in the E5 ORF at the *Bst*XI site at base 3881. The cDNA is cloned behind the simian virus 40 early promoter and expresses the E2 *trans*-

TABLE 3. Complementation of BPV-1 mutant DNAs in C127 cells expressing integrated BPV-1 C59-3881 cDNA functions[a]

Plasmid	No. of foci/0.5 μg per 10^6 cells	
	C127	YC-C59-3881
p142-6	168, 132	165, 159
p631-1	3	162, 152
p771-1	7, 17	135, 160
p772-1	0, 0	0, 0
p448-5	0, 0	0, 0
p830-1	0, 0	0, 2

[a] Transformation ability is expressed as the number of transformed foci per 60-mm (diameter) dish receiving 0.5 μg of plasmid DNA. We cleaved the plasmid DNAs with *Bam*HI to release BPV-1 DNA from procaryotic pML2d sequences. Foci were counted 21 days after transfection after staining as shown in Fig. 5. Dual numbers refer to duplicate plates.

activation function but is transformation negative. The YC-C59-3881 cell line containing this cDNA was selected by cotransfection with pMMTneo(302-3) and is not morphologically transformed. The presence of the intact BPV-1 DNA segment was confirmed by Southern blot analysis.

The results from these experiments are shown in Table 3. As expected, neither the wild-type (p142-6) nor the E5 (p830-1) mutant DNA showed any difference in transformation efficiency on the two cell lines. By contrast, two of the E2 mutants, p631-1 and p771-1, transformed C59-3881 cells much more efficiently than C127 cells, indicating that the E2 mutation in each of those mutants could be complemented in *trans*. It is noteworthy that these mutants are altered only in the E2 ORF. These mutants approached wild-type levels in their transforming ability in C59-3881 cells; the foci were larger and visible at earlier times after transfection (Fig. 5). To our surprise, the p448-1 and p772-1 mutants were not complemented in transformation by the E2 gene product expressed in the YC-C59-3881 cell line. Plates demonstrating enhanced transformation of the p771-1 plasmid in YC-C59-3881 and C127 cells are shown in Fig. 5.

These data suggested that, in addition to the full E2 ORF product and the E5 transforming gene, there is a domain of an additional BPV-1 function involved in transformation, which maps to the 3′ ORFs. From the data presented in this report, it is unclear to which ORF this function maps. The E2-specific mutants mapping upstream of the splice acceptor at base 3224 are not mutated in this function. The E2 mutant p772-1, which contains the TTL at base 3235, is mutated in the E4 ORF, as well as in the E2 ORF downstream from the splice acceptor at base 3225, suggesting that this additional function maps to one of these coding domains.

DISCUSSION

The experiments described here extend our previous genetic analyses, which identified regions of the BPV-1 genome involved in transformation and plasmid replication and maintenance. With the study of BPV-1 DNA deletion and TTL mutants, we demonstrated that mutations in the E2 ORF reduce the transforming capacity of the DNA and destroy the ability of the viral DNA to remain extrachromosomal. Whereas the mode of action of the E2 product(s) in transformation and plasmid maintenance needs to be more fully defined, we showed with complementation experiments that the E2 product(s) can act in *trans* to provide each of these functions to certain E2 mutants.

Several questions concerning the role of the E2 gene product(s) in plasmid maintenance and in transformation remain to be resolved. One is whether or not there is an absolute requirement for the full E2 ORF product in plasmid maintenance and replication. Although we found expression of the E2 ORF to be essential for extrachromosomal plasmid maintenance in the assays outlined in this report, Lusky and Botchan have described a replication-competent deletion mutant (Bal 15) which is deleted of viral sequences between bases 2694 and 4172 and includes almost the complete E2 ORF (18). None of our E2 mutants, even p715-5, which has a deletion somewhat analogous to that in Bal 15, remained extrachromosomal in C127 cells. These differences in structure may be critical in determining function. Another discrepancy between our results and those of Lusky and Botchan pertains to *Nco*I deletion mutant p631-1 (19). In our assay, this mutant integrated into the host chromosome regardless of the mode of selection. DiMaio et al. (6), using an identical mutant, have found the same results. Using this mutant, however, Lusky and Botchan (19) found the DNA integrated in cells selected for focus formation and extrachromosomal in cells selected for G418 resistance, by using an unlinked cotransfection assay. These differences suggest that the role of the E2 gene product in plasmid replication is indirect and that its function can be circumvented in some cell lines and under certain experimental conditions. It is possible that, under certain growth conditions, a cellular factor functionally analogous to the E2 product could be induced and permit plasmid maintenance of E2 mutants. Spalholz et al. (27) have demonstrated that the full E2 ORF product can act in *trans* to activate a transcriptional regulatory element in the BPV-1 LCR. It is possible that the role of the E2 product in plasmid maintenance is entirely indirect through activation of the LCR enhancer, leading to expression of critical viral replication functions. The experiments presented in this report, however, do not rule out the possibility of a direct role for an E2 gene product in plasmid maintenance. Another question which remains to be addressed is whether or not there is an additional 3′ ORF function involved, directly or indirectly, in stable high-copy-number BPV-1 plasmid maintenance. Our results indicate that expression of the full E2 ORF product in YC-C59 cells can complement E2 mutants, permitting plasmid replication. The reduced copy number of monomeric plasmids observed (Fig. 4) could be due to the absence of a viral gene product in these cells. Such a product could be necessary for establishment or maintenance of a high plasmid copy number. Potentially, products encoded in part by ORFs upstream of E2, joined through RNA splicing to E2 or E4, could play such a role.

It is also not clear whether the role of the *trans*-acting E2 gene product in transformation is direct or indirect. Although it was previously suggested that the E2 gene might encode a transforming protein (23), in view of the role of E2 in transcriptional transactivation (27) it now seems likely that the role of E2 in transformation is indirect. Most of the E2 mutants analyzed in this study have a similar phenotype; they are (i) significantly impaired in their ability to transform, (ii) replication incompetent, and (iii) unable to *trans* activate the LCR enhancer (23, 27, 30). The *Bcl*I deletion mutant (p327-25), however, has a different phenotype. Although this mutant is replication incompetent in C127 cells and is unable to *trans* activate the LCR enhancer (30), it transforms at an efficiency comparable to that of the wild-type BPV-1 plasmid, p142-6 (Table 1) (23). Therefore, it seems that the reduced transformation capabilities exhibited by most of our E2 mutants cannot be fully explained by

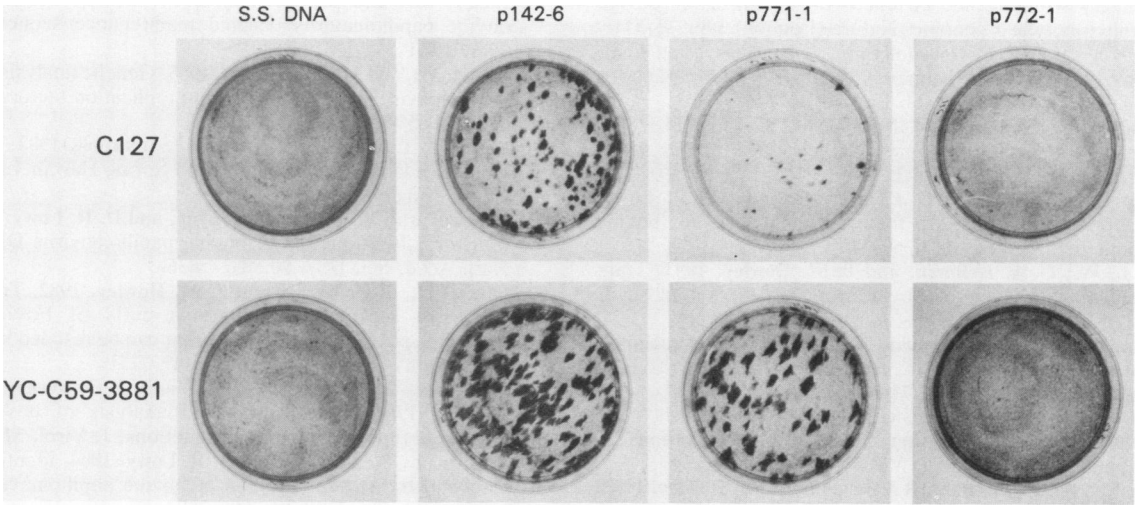

FIG. 5. Representative plates of C127 mouse cells and YC-C59-3881 cells 21 days after transfection with the indicated DNA. DNA transformation was performed by a modified calcium phosphate precipitation technique as described in Materials and Methods. Plates were stained with 1% methylene blue and washed extensively before counting. p142-6 is the full-length BPV-1; p771-1 and p772-1 contain a TTL in the E2 ORF (Fig. 1). Note the larger size of the foci in YC-C59-3881 cells transfected with p142-6 and p771-1 DNAs compared with those in C127 cells. See Table 3 for the full compilation of data from this experiment. S.S. DNA, salmon sperm DNA.

invoking only an indirect role for the E2 gene product(s) in viral transcriptional control.

It should be stressed, however, that a direct transforming function for the E2 gene has not yet been identified. When expressed from either the simian virus 40 early promoter or a Harvey sarcoma virus long terminal repeat, the primary transforming function of the 3′ ORFs (E2, E3, E4, and E5) maps to the E5 ORF, downstream from the first AUG at 3879, not to the E2 ORF (25, 30). We carried out these transformation experiments by assaying for focus formation on the immortalized cell lines C127 and NIH 3T3. It remains possible that a direct role for E2 in transformation could be demonstrated in alternative assays with nonimmortalized or epithelial cells.

Our study of E5 mutant p830-1 confirms previous reports that expression of the 3′ portion of the E5 ORF is essential for transformation by BPV-1 (25, 30). Although in short-term plasmid replication studies the BPV-1 ORF mutant can be found as a plasmid, we found, in agreement with Groff and Lancaster (10), that this mutant did not remain as a stable plasmid in clonal lines selected for G418 resistance. Recent studies from this laboratory have revealed the presence of the E5 transforming protein in BPV-1-transformed cells (26).

The mechanism by which the mutation in the E5 ORF at the *Bst*XI site at base 3881 influences plasmid stability and replication remains to be determined. It is possible that this region of the BPV-1 genome contains an element required in *cis* to assure plasmid stability. Alternatively, the E5 product itself (26) or another product generated from a spliced RNA containing the E5 ORF as a coding exon joined to an upstream ORF could be involved in plasmid maintenance. The mutant Bal 15 described by Lusky and Botchan (18) is replication competent and is deleted of the E2 and E5 ORFs. Thus, it is unlikely that a *cis* element critical for plasmid maintenance is located at this site or that the product of the E5 ORF has a direct role in plasmid maintenance.

By complementation assays with mutant BPV-1 DNAs and cDNAs, we demonstrated the existence of yet another viral function which affects transformation. Whereas the nature of this viral function is still obscure, it is clear that its function cannot be activated or augmented in *trans* by a product from the E2 ORF. The newly identified viral function was mapped to the 3′ end of the early region and is mutated in the p772-1 and p448-5 plasmids. As such, it maps to a portion of the BPV-1 genome where the E2 and E4 ORFs overlap downstream from the splice acceptor site at base 3225 (29). Unpublished data indicate that mutations specific for the E4 ORF do not affect transformation (P. Hermonat and P. M. Howley, manuscript in preparation; K. Neary, B. Horwitz, and D. DiMaio, submitted for publication). It is possible that the function is a *cis*-essential regulatory element, which perhaps influences expression of the E5 ORF or affects viral mRNA stability. However, indirect evidence suggesting that this region is important as a protein coding sequence, rather than as a *cis*-essential regulatory region, comes from the structural analysis of cDNAs of viral mRNAs in BPV-1-transformed cells (29). Viral cDNAs have been characterized which contain this region (downstream sequences from the splice acceptor at base 3225) of the BPV-1 genome spliced to upstream sequences. Therefore, proteins composed of amino acids from upstream sequences linked to the product of the E4 ORF or the colinear E2 ORF sequences may play a role, either direct or indirect, in BPV-1 transformation.

ACKNOWLEDGMENTS

We are grateful to Richard Schlegel and Barbara Spalholz for critical comments on the manuscript. We thank Nan Freas for preparation of the manuscript.

This work was supported in part by grant 1597 from the Council for Tobacco Research, U.S.A., Inc., to M.S.R.

LITERATURE CITED

1. **Amtmann, E., and G. Sauer.** 1982. Bovine papillomavirus transcription: polyadenylated RNA species and assessment of the direction of transcription. J. Virol. **43**:59–66.
2. **Androphy, E., J. T. Schiller, and D. Lowy.** 1985. Identification of the protein encoding by the E6 transforming gene of bovine papillomavirus. Science **230**:442–445.
3. **Campo, M. S., D. A. Spandidos, J. Lang, and N. M. Wilkie.** 1983. Transcriptional control signals in the genome of bovine papillomavirus type 1. Nature (London) **303**:77–80.
4. **Chen, E. Y., P. M. Howley, A. Levinson, and P. Seeburg.** 1982.

The primary structure and genetic organization of the bovine papillomavirus type 1 genome. Nature (London) **299**:529–534.

5. **Colbere-Garapin, F., F. Horodniceanu, P. Kourilsky, and A. C. Garapin.** 1981. A new dominant hybrid selective marker for higher eukaryotic cells. J. Mol. Biol. **150**:1–14.
6. **DiMaio, D., J. Metherall, K. Neary, and D. Guralski.** 1986. Nonsense mutation in open reading frame E2 of bovine papillomavirus DNA. J. Virol. **57**:475–480.
7. **Dvoretzky, I., R. Shober, S. Chattopadhy, and D. R. Lowy.** 1980. A quantitative in vitro focus forming assay for bovine papillomavirus. Virology **103**:369–375.
8. **Engel, L. W., C. A. Heilman, and P. M. Howley.** 1983. Transcriptional organization of bovine papillomavirus type 1. J. Virol. **47**:516–528.
9. **Gorman, C. M., and B. Howard.** 1983. Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate. Nucleic Acids Res. **11**:7631–7648.
10. **Groff, D. E., and W. D. Lancaster.** 1986. Genetic analysis of the 3′ early region transformation and replication functions of bovine papillomavirus type 1. Virology **150**:221–230.
11. **Heilman, C. A., L. Engel, D. R. Lowy, and P. M. Howley.** 1982. Virus-specific transcription in bovine papillomavirus transformed mouse cells. Virology **119**:22–34.
12. **Howley, P. M., E. T. Schenborn, E. Lund, J. C. Byrne, and J. E. Dahlberg.** 1985. The bovine papillomavirus distal "enhancer" is not *cis* essential for transformation or for plasmid maintenance. Mol. Cell. Biol. **5**:3310–3315.
13. **Lancaster, W. D., and C. Olson,** 1982. Animal papillomaviruses. Microbiol. Rev. **46**:191–207.
14. **Law, M.-F., J. C. Byrne, and P. M. Howley.** 1983. A stable bovine papillomavirus hybrid plasmid that expresses a dominant selective trait. Mol. Cell. Biol. **3**:2110–2115.
15. **Law, M.-F., D. R. Lowy, I. Dvoretzky, and P. M. Howley.** 1981. Mouse cells transformed by bovine papillomavirus contain only extrachromosomal viral DNA sequences. Proc. Natl. Acad. Sci. USA **78**:2727–2731.
16. **Lowy, D. R., I. Dvoretzky, R. Shober, M.-F. Law, L. Engel, and P. Howley.** 1980. In vitro tumorigenic transformation by a defined subgenomic fragment of bovine papillomavirus DNA. Nature (London) **287**:72–74.
17. **Lusky, M., L. Berg, H. Weiher, and M. Botchan.** 1983. Bovine papilloma virus contains an activator of gene expression at the distal end of the transcription unit. Mol. Cell. Biol. **3**:1108–1122.
18. **Lusky, M., and M. Botchan.** 1984. Characterization of the bovine papillomavirus plasmid maintenance sequences. Cell **36**:391–401.
19. **Lusky, M., and M. R. Botchan.** 1985. Genetic analysis of bovine papillomavirus type 1 *trans*-acting replication factors. J. Virol. **53**:955–965.
20. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Nakabayashi, Y., S. Chattopadhyay, and D. R. Lowy.** 1983. The transformation function of bovine papillomavirus DNA. Proc. Natl. Acad. Sci. USA **80**:5832–5836.
22. **Sarver, N., J. C. Byrne, and P. M. Howley.** 1982. Transformation and replication in mouse cells of bovine papillomavirus/pML2 plasmid vector that can be rescued in bacteria. Proc. Natl. Acad. Sci. USA **79**:7147–7151.
23. **Sarver, N., M. S. Rabson, Y.-C. Yang, J. C. Byrne, and P. M. Howley.** 1984. Localization and analysis of bovine papillomavirus type 1 transforming functions. J. Virol. **52**:377–388.
24. **Schiller, J., W. C. Vass, and D. R. Lowy.** 1984. Identification of a second transforming region of bovine papillomavirus. Proc. Natl. Acad. Sci. USA **81**:7880–7884.
25. **Schiller, J. T., W. C. Vass, K. H. Vousden, and D. R. Lowy.** 1986. The E5 open reading frame of bovine papillomavirus type 1 encodes a transforming gene. J. Virol. **57**:1–6.
26. **Schlegel, R., M. Wade-Glass, M. S. Rabson, and Y.-C. Yang.** 1986. The E5 transforming gene of bovine papillomavirus directs synthesis of a small hydrophobic protein. Science **233**:464–466.
27. **Spalholz, B. A., Y.-C. Yang, and P. M. Howley.** 1985. Transactivation of a bovine papillomavirus transcriptional regulatory element by the E2 gene product. Cell **42**:183–191.
28. **Stenlund, A., J. Zabielski, H. Ahola, J. Moreno-Lopez, and U. Pettersson.** 1985. The messenger RNAs from the transforming region of bovine papillomavirus type 1. J. Mol. Biol. **182**:541–544.
29. **Yang, Y.-C., H. Okayama, and P. M. Howley.** 1985. Bovine papillomavirus contains multiple transforming genes. Proc. Natl. Acad. Sci. USA **82**:1030–1033.
30. **Yang, Y.-C., B. A. Spalholz, M. S. Rabson, and P. M. Howley.** 1985. Dissociation of transforming and transactivation functions for bovine papillomavirus type 1. Nature (London) **318**:575–577.