JOURNAL OF VIROLOGY, Nov. 1984, p. 377–388
0022-538X/84/110377-12$02.00/0

Vol. 52, No. 2

Localization and Analysis of Bovine Papillomavirus Type 1 Transforming Functions

NAVA SARVER,† MICHAEL S. RABSON, YU-CHUNG YANG, JANET C. BYRNE, AND PETER M. HOWLEY*
Laboratory of Pathology, National Cancer Institute, Bethesda, Maryland 20205

Received 11 June 1984/Accepted 3 August 1984

Bovine papillomavirus type 1 (BPV-1) or cloned BPV-1 DNA can transform susceptible rodent cells, and the viral DNA remains as a stable extrachromosomal plasmid in the transformed cells. The transforming region of the BPV-1 genome has previously been localized to a specific fragment comprising 69% of the genome, which also contains the elements sufficient for extrachromosomal plasmid maintenance. To define more precisely the viral DNA sequences which are involved in cellular transformation, we have tested the ability of defined deletion mutants of BPV-1 DNA to morphologically transform mouse C127 cells. Cells containing the mutated DNAs have been examined for anchorage independence and tumorigenicity in nude mice. Several distinct regions of the BPV-1 genome were found to influence expression of the viral transformation functions. A transcriptional regulatory region located in the noncoding region 5′ to the early open reading frames is essential for transcriptional activity and transformation. A transcriptional enhancer element, located 3′ to the polyadenylation site for the viral RNAs expressed in transformed cells, has previously been shown to be essential for transformation (Lusky et al., Mol. Cell. Biol., 3:1108–1122, 1983). Deletion mutants affecting the E2 open reading frame, particularly the NH_2 half, are significantly impaired in their ability to transform, suggesting that the E2 gene product is an important transforming protein of BPV-1. Mutants lacking the E6 and E7 open reading frames are still able to induce transformation but at a lowered efficiency, and the transformants have altered characteristics. Mutations localized within the E1 open reading frame do not significantly affect the transforming functions but result in the integration of the viral genome in the transformed cells, implicating the E1 gene product in stable plasmid replication and maintenance.

Bovine papillomavirus type 1 (BPV-1) or its molecularly cloned DNA transforms mouse cells in vitro (10, 25) and persists in the transformed cell as an extrachromosomal multicopy plasmid without rearrangement (24). A subgenomic fragment (BPV_{69T}), delineated by the unique *Hin*dIII and *Bam*HI restriction sites, is sufficient to induce transformation in vitro (20, 25), and chimeric molecules in which the viral component consists of BPV_{69T} can be maintained as free plasmids in transformed cells (9, 24, 38). BPV_{69T} therefore contains the genetic elements required for cellular transformation as well as for autonomous plasmid replication.

None of the papillomaviruses has yet been successfully propagated as virus in tissue culture. Due to the absence of a permissive in vitro culture system, direct genetic studies of the papillomaviruses have not been possible. However, with the determination of the complete nucleotide sequence of BPV-1 DNA (5), in conjunction with known transcriptional data (2, 11, 18), the genomic organization of the viral genome has been established.

The viral DNA contains 7,945 base pairs, with nucleotide number 1 assigned to the first nucleotide in the recognition sequence of the single *Hpa*I site and numbering proceeding in the transcriptional-sense direction (5, 8). All of the detectable polyadenylated RNA species are transcribed from a single strand (2, 11), and all of the open translational reading frames (ORFs) greater than 400 bases are located on a single strand (5) (Fig. 1). A noncoding region containing two TATA boxes which might serve as functional components of transcriptional promoters is located 5′ to the eight significant ORFs located within BPV_{69T}. These eight ORFs are located in a segment of the genome which is actively transcribed in transformed cells (18), and most likely these ORFs serve as coding exons for the viral proteins required for transformation and for extrachromosomal plasmid maintenance. A polyadenylation recognition site (AATAAA) is located at base 4179 in a region where the 3′ ends of the viral RNAs present in the BPV-1-transformed cell map.

To further define and characterize the genomic elements required for cellular transformation, we have constructed a series of mutants with defined deletions within BPV_{69T} and have tested their ability to morphologically transform mouse C127 cells by focus assay. The biological function of certain BPV-1 mutant DNAs, which were negative by focus assay, was further analyzed by cotransformation, using drug resistance to G418 conferred by the aminoglycoside phosphotransferase gene from Tn5 (7, 41), thus permitting the isolation of cell lines harboring these mutated DNAs. Finally, cells containing the mutated BPV-1 DNAs were characterized for anchorage independence and tumorigenicity in nude mice.

At least four distinct regions of the BPV-1 genome which affect the expression of the viral transforming functions can be defined. A transcriptional regulatory region located in the noncoding segment 5′ to the ORFs in BPV_{69T} is essential for transcriptional activity and transformation. A transcriptional enhancer located 3′ to the polyadenylation site for the viral RNAs which are expressed in transformed cells is required for transformation (26). Mutants affected in the E2 ORF, particularly the NH_2 half, are significantly impaired in their ability to transform, indicating a requirement of the E2 gene product for morphological transformation. Mutants deleted of the E6 and E7 ORFs located between the *Hpa*I site (base 1) and the *Sma*I site (base 945) are still able to induce transformation, albeit at decreased efficiencies, and the

* Corresponding author.
† Present address: Biotechnology Research Center/Revlon, Springfield, VA 22151.

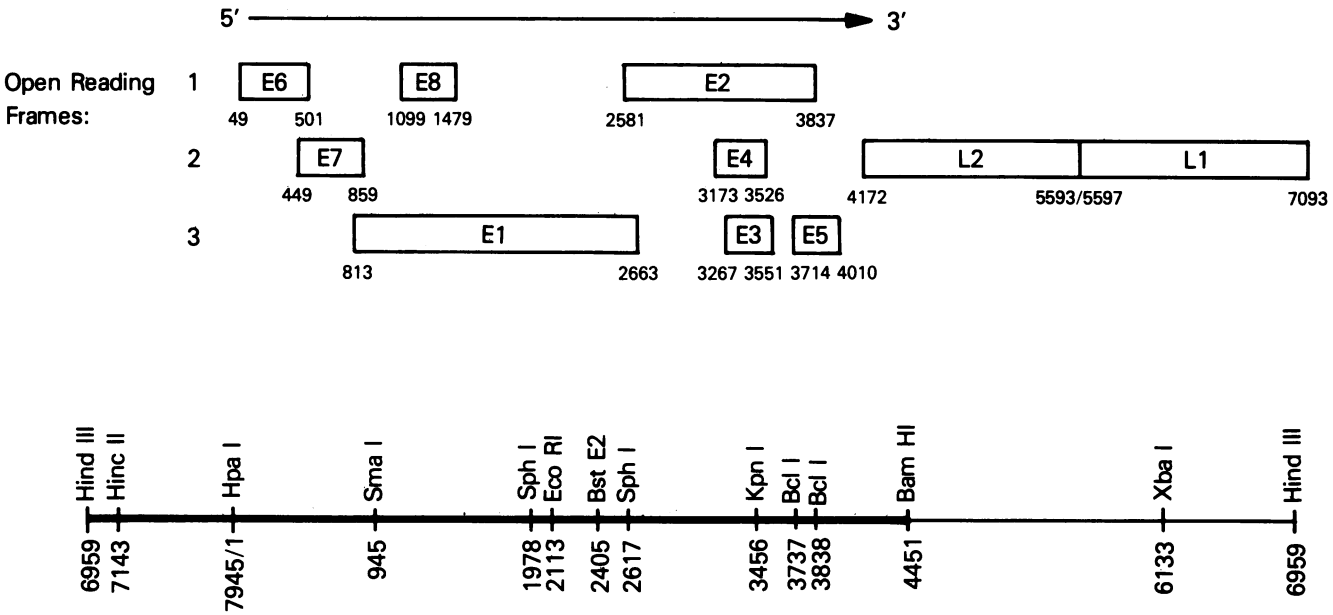

FIG. 1. Genomic organization of BPV-1 DNA. The full-length molecule (7,945 base pairs) of BPV-1 opened at the unique *Hin*dIII site (base 6959) is marked off with restriction endonuclease sites and bases at the bottom of this figure. The 69% *Hin*dIII-to-*Bam*HI segment of the genome previously shown to be sufficient for transformation (20, 25) and autonomous plasmid replication (24) is indicated by the heavy bar. The region transcribed in transformed cells and the direction of transcription are indicated by the arrow at the top of the figure (18). The open bars represent the ORFs and therefore the potential coding exons in each of the three translation frames predicted from the sequence (5). ORFs within the transforming region have been designated E1 to E8; the two ORFs, L1 and L2, within the 31% region not essential for transformation, are partitioned by a single stop codon. The numbers beneath the ORFs designate the first and last bases of the ORF. The information used to generate the ORFs is from the original sequence (5), with the base changes indicated by Danos et al. (8), and with an additional G added at base 3445 (U. Pettersson, and A. Stenlund, unpublished data).

transformants have altered characteristics. It is noteworthy that although mutations localized within the E1 ORF do not significantly affect transforming functions, they do result in the integration of the viral DNA in the transformed cells, thus implicating the E1 gene product in stable plasmid maintenance.

MATERIALS AND METHODS

Cells and transformation. Mouse C127 cells (10) were maintained in Dulbecco modified Eagle medium (DMEM; Meloy Laboratories, Springfield, Va.) complete with penicillin (10 U/ml), streptomycin (100 μg/ml), and heat-inactivated fetal calf serum (10%).

DNA transformation was performed by the calcium phosphate coprecipitation method (13), followed by a 15% (vol/vol) glycerol (12) or a 25% (vol/vol) dimethyl sulfoxide (42) enhancement as described previously (36, 37).

Construction of deletion mutants. pdBPV-1 (142-6) is a plasmid containing the complete BPV-1 genome cloned at the *Bam*HI site of pML2d (36). Defined deletions were introduced into p142-6 DNA by cleavage with restriction endonucleases, followed by recircularization of the mutated plasmids. Briefly p142-6 DNA was cleaved with one or more enzymes, and the resultant fragments were separated by agarose gel electrophoresis. The DNA was recovered from the gel by electroelution into a dialysis bag, followed by extraction with phenol and precipitation with ethanol. When it was necessary to generate blunt-ended termini for subsequent ligation, T4 DNA polymerase was used. In some instances, synthetic linkers (Collaborative Research, Inc., Waltham, Mass.) were inserted at the site of the deletion. Transformation of *Escherichia coli* K-12 HB101 (21), screening of bacterial transformants (19), and propagation of recombinant plasmids (6, 44), as well as the molecular manipulations described above, were performed by conventional methodologies as detailed by Maniatis et al. (30, 31).

Analysis of cellular DNA. Total cellular DNA was extracted from transformed cells by a modification of the method of Gross-Bellard et al. (14) as described previously (24). Fractionation of digested DNAs was by agarose gel electrophoresis in Tris-acetate buffer (5 mM sodium acetate, 1 mM EDTA, 40 mM Tris [pH 8.0]) at 2.5 V/cm for 16 h. Partial depurination, denaturation in situ (43), transfer onto nitrocellulose filters (22, 40), preparation of radiolabeled DNAs (35), and blot hybridization were performed as described previously (24, 37).

Anchorage independence assays. The ability of the various cell lines containing mutated BPV-1 DNA to form colonies in soft agar was examined. For such assays, 60-mm^2 dishes were prepared with a bottom layer of 0.5% agar (Special Agar-Noble; Difco Laboratories, Detroit, Mich.) in DMEM. After the bottom layer had solidified, dishes were seeded with cells suspended in 0.3% agar in DMEM. Duplicate dishes were seeded with either 5×10^3 or 5×10^4 cells. Plates were fed twice weekly by overlaying them with 0.3% agar in DMEM. At 2 weeks, plates were scored for colony number, size, and shape (29).

Tumorigenicity studies. The ability of cells to induce tumors was evaluated in athymic nude mice. NIH BALB/c female nudes of ca. 4 weeks of age were injected with 10^6 cells each. The mice were observed for up to 10 weeks, and the time of appearance and size of the tumors, if any, were noted.

RESULTS

Generation of BPV DNA mutants and rationale. The approach used in studying BPV transformation was to generate a series of defined deletion mutants of the viral genome and initially assay for their ability to induce foci of mouse C127 cells. The starting DNA used for these studies was pdBPV-1 (142-6), which consists of the complete BPV-1 genome cloned at its unique *Bam*HI site in a derivative of pML2 which was deleted of additional pBR322 sequences between the *Hin*dIII site (base 29) and the *Bam*HI site (base 375). pML2 is a derivative of pBR322 deleted of sequences inhibitory to BPV-1-mediated DNA transformation (27). We have previously shown that this hybrid DNA molecule transforms mouse cells with an efficiency equivalent to that of the BPV-1 DNA molecule separated from the pBR322 moiety and that it replicates efficiently in mouse cells as a stable plasmid (36). In some instances, as noted in the figures, the deletions were made in BPV-1 DNA which had been cloned in pBR322. In such cases the mutant DNAs were unlinked from the pBR322 sequences by cleavage with the appropriate restriction endonuclease before the addition of the DNA to the cells.

Transforming activity of BPV deletion mutants. A series of the BPV-1 deletion mutants studied are listed in Fig. 2 along with the results of the transformation analyses performed by a focus assay with C127 cells. In these experiments, pdBPV-1 (142-6) had a transformation efficiency of ca. 200 foci per μg of DNA per 10^6 cells, which is similar to the efficiency we have previously observed (36).

One group of mutants was deleted of sequences located in the noncoding region mapping 5′ to the early ORFs. Mutant p214-1, deleted of 184 bases between the unique *Hin*dIII at the base 6959 site and the *Hin*cII site at base 7143, had a transformation efficiency similar to the full-length genomic DNA. Since this deletion mutant was cloned into pBR322 at the *Hin*dIII site, the DNA was first digested with *Hin*dIII to unlink it from the pBR322 sequences which are *cis* inhibitory to BPV DNA transformation (25, 36). Two deletion mutants which extend to the *Cla*I site at base 7477 (p267-34 and p268-17) are markedly impaired in their ability to induce foci in this transformation assay, indicating that sequences located between the *Hin*cII site at base 7143 and the *Cla*I site at base 7477 are important for focus formation. A deletion of virtually the entire noncoding region down to the *Hpa*I site (base 7945/1), as in mutant p141-1, abolishes the transforming activity in this assay.

Mutants deleted of sequences at the 3′ end of BPV_{69T},

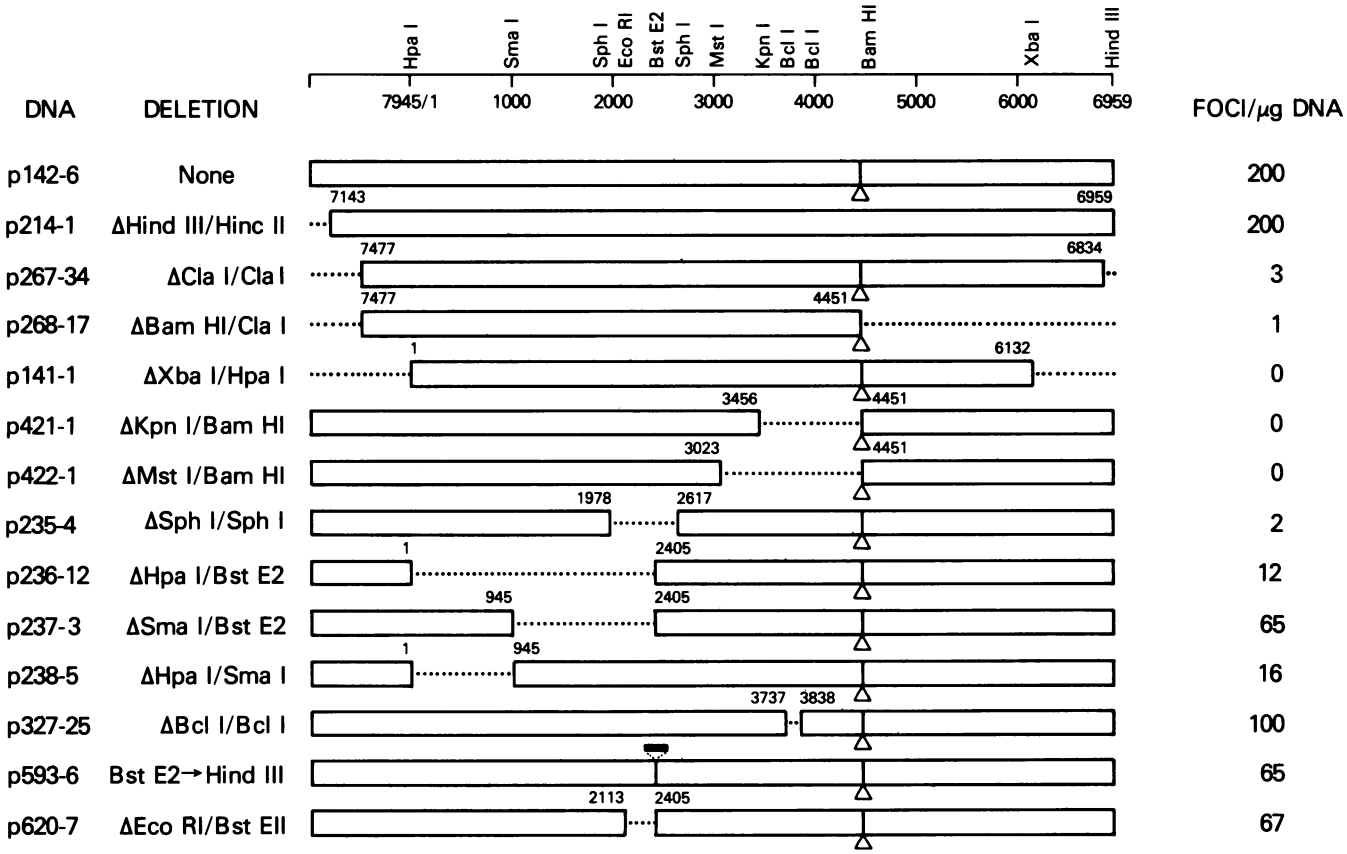

FIG. 2. Structure and transforming activity of BPV-1 deletion and insertion mutants. The recombinant plasmids and the restriction endonuclease sites used in generating the deletion mutant or linker insertion mutant are listed on the left. The transformation activity of each DNA was determined on mouse C127 cells, and each value represents the average of three separate experiments. For each plasmid except p214-1 and p141-1 the infectivity reflects that of the BPV-1 DNA sequences linked to the pML2d sequences at the *Bam*HI site, as indicated by the open triangle. The BPV-1 DNA segment in the p214-1 plasmid was cloned at the *Hin*dIII site located at the site of the deletion and was therefore digested with the *Hin*dIII enzyme before transfection. The BPV-1 DNA segment in p141-1 is cloned in the unique *Bam*HI site of PBR322 from which it was separated by a *Bam*HI digest before transfection. The retained sequences (▭) are covalently linked at the sites indicated and in the figures are shown separated by the deleted sequence (.....). In p593-6, the *Hin*dIII linker (▬) has been inserted into the *Bst*E2 site.

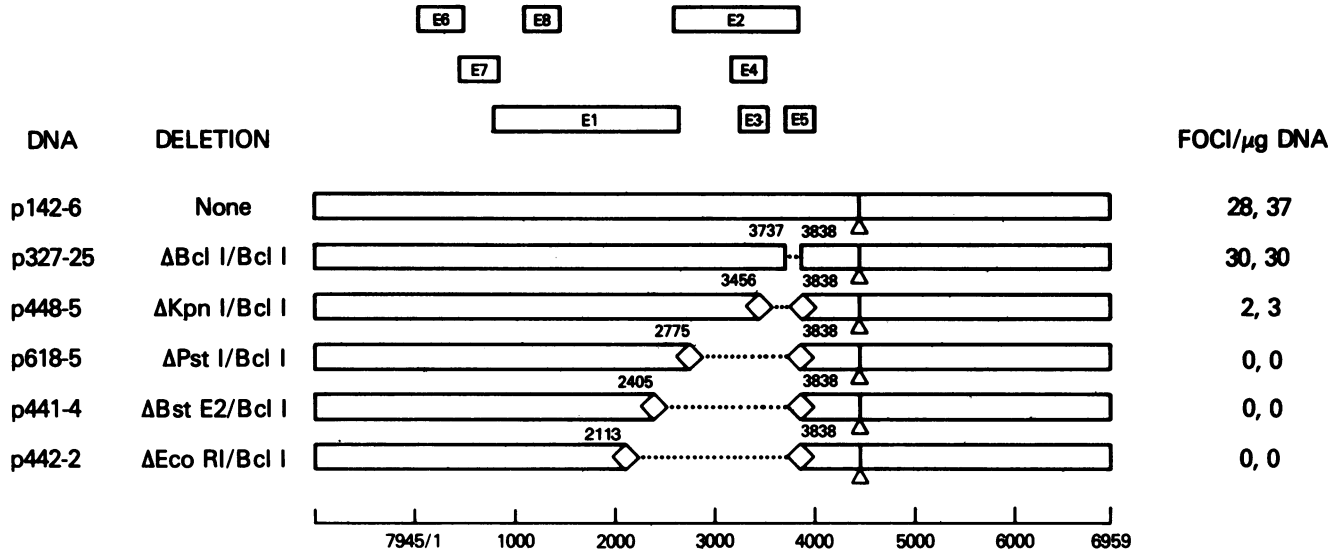

FIG. 3. Structure and transforming activity of BPV-1 DNA deletion mutants. The recombinant plasmid and the deletion are indicated on the left. The transformation efficiency was determined on mouse C127 cells, and the numbers represent the number of foci on duplicate plates assayed at 21 days after transfection. For each plasmid, the transformation activity was determined with the BPV-1 DNA sequences linked to the pML2d sequences at the *Bam*HI site, as indicated by the open triangle (△). The retained sequences (▭) are covalently linked at the sites indicated and in the figure are separated by the deleted sequences (.....). The *Xho*I linker is indicated (◇). The ORFs for the transforming segment are indicated at the top of the figure.

such as p421-1 and p422-1, have no transforming activity in the focus assay. This is most likely a consequence of deleting important transcriptional control sequences located in this region. In addition to affecting the E2, E3, E4, and E5 ORFs located at the 3′ of this transcriptional unit, these mutants eliminate the polyadenylation and 3′ processing sequences for the viral RNAs expressed in transformed cells (5, 18), as well as a transcription enhancer essential for the expression of these RNAs (26).

To identify regions of the BPV-1 genome which contain ORFs coding for the putative transforming proteins, a series of mutants were constructed in which the deletions primarily affected the ORFs located within the BPV-1 transforming segment. Two mutants were generated which are affected only in the E1 ORF. One mutant (p620-7) contained a deletion of the sequences between the *Eco*RI site at base 2113 and the *Bst*E2 site at base 2405. The deletion was generated by cleaving the parental DNA with *Eco*RI and *Bst*E2 restriction endonucleases, then repairing the ends with T4 DNA polymerase, and adding an *Xho*I linker. The second mutant (p593-6) is a mutant in which a *Hin*dIII linker was added at the *Bst*E2 site after the site was made blunt ended by treatment with T4 DNA polymerase. Each of the mutants is capable of transforming mouse C127 cells at ca. 30 to 35% the efficiency of the full-length genomic DNA (Fig. 2).

Three mutants with deletions between the *Hpa*I site at base 1 and the *Bst*EII site at base 2405 are still capable of transforming mouse cells, albeit at a frequency lower than that of the complete BPV-1 genome (Fig. 2). Two mutants, deleted of bases 1-2405 (p236-12) and deleted of bases 1-945 (p238-5) transform at an efficiency ca. 6 to 8% that of the full genome. A third mutant, p237-3, deleted from the *Sma*I site (base 945) to the *Bst*EII site (base 2405), transforms at an efficiency one third of that of the full genome. Thus, deletion mutants that lack the E6, E7, and E8 ORFs and most of the E1 ORF can still induce focus formation, although at a lower efficiency.

Another mutant, 235-4, deleted of sequences between the two *Sph*I sites and in which the deletion extends 3′ downstream to base 2617 is significantly impaired in its ability to induce foci. This mutant contains a 639-base-pair deletion which affects the carboxy terminus of the E1 ORF and removes the first 13 amino acids of the E2 ORF. The deletion in E2 includes the AUG codon at base 2608 which could possibly serve as an initiation methionine codon for the putative E2 ORF product. This result implicates the product of the E2 ORF as important for BPV-1 transformation.

Transforming activity of BPV-1 E2 deletion mutants. To further examine the role of the putative E2 gene product in BPV-1 cellular transformation, a series of mutants were made within the E2 ORF. The deletions in these mutants extend for various distances upstream from a fixed 3′ end delineated by the *Bcl*I site at base 3838. These mutants and their transforming activities are listed in Fig. 3. The p327-25 mutant with a deletion of only 101 bases extending to the *Bcl*I site at base 3737 affects the carboxy terminus of the E2 ORF as well as the E5 ORF. This mutant had a transforming activity similar to that of the full-length BPV-1 DNA. The mutant, p448-5, containing a larger deletion that extends an additional 281 bases upstream to the *Kpn*I site, affects the carboxy termini of the E2, E3, and E4 ORFs. This mutant is capable of inducing transformed foci at a significantly lower efficiency than p142-6, but the transformants do not have the refractile, spindled appearance characteristic of BPV-1 transformation (Fig. 4). The p618-5 mutant with a deletion from the *Pst*I site at 2775 to the *Bcl*I site at base 3838, which effectively eliminates all of the E2, E3, E4, and E5 ORFs, was negative for transformation in the focus assay used. Mutants with deletions extending further 5′ into the E1 ORF to the *Bst*E2 site (p441-5) or the *Eco*RI site (p442-4) were each negative for transformation in this experiment, although in other experiments they induced foci at a level ca. 100 fold-lower than that of the full BPV-1 genome. Thus, mutants with deletions affecting the E2 ORF, especially the amino terminus, were significantly impaired in their ability

to induce transformed foci in the focus assay employed.

To assess more accurately the consequences of introducing the mutant DNAs with deletions affecting the E2 ORF into cells, each of the DNAs (Fig. 3) were introduced into mouse C127 cells by cotransformation with pdMMTneo(302-3), and cotransformants were selected by virtue of their resistance to the drug G418. The pdMMTneo(302-3) plasmid contains the Neo^r gene of Tn*5* contained within a mammalian cell transcriptional cassette consisting of the mouse metallothionein promoter, the simian virus 40 (SV-40) small t-antigen splice donor and splice acceptor sequences, and the SV-40 early polyadenylation and 3′ processing sequences (23). The efficiencies of morphological transformation in DMEM and of drug resistance to the aminoglycoside G418 in selective media are listed in Table 1. Individual colonies were selected on the basis of drug resistance and analyzed for several properties of malignant transformation (Table 2).

Cells containing the p327-25 mutant have a morphology similar to that of mouse C127 cells transformed by the complete BPV-1 genome. In addition, the cells are fully anchorage independent as assayed in agar and are tumorigenic in nude mice. Thus, the 101-base deletion affecting the carboxy terminus of E2 ORF has no effect on the transformation capability of BPV-1 in the assays presented. Cells containing the p448-5 mutant DNA with the *Kpn*I-to-*Bcl*I deletion also exhibit several characteristics of transformation. They grow to high density and are not contact inhibited; however, they do not have the characteristic spindled cell appearance of C127 cells transformed by the full BPV genome or by the smaller *Bcl*I-to-*Bcl*I deletion mutant p327-25 (Fig. 4). Thus, the p448-5 mutant with a more extensive

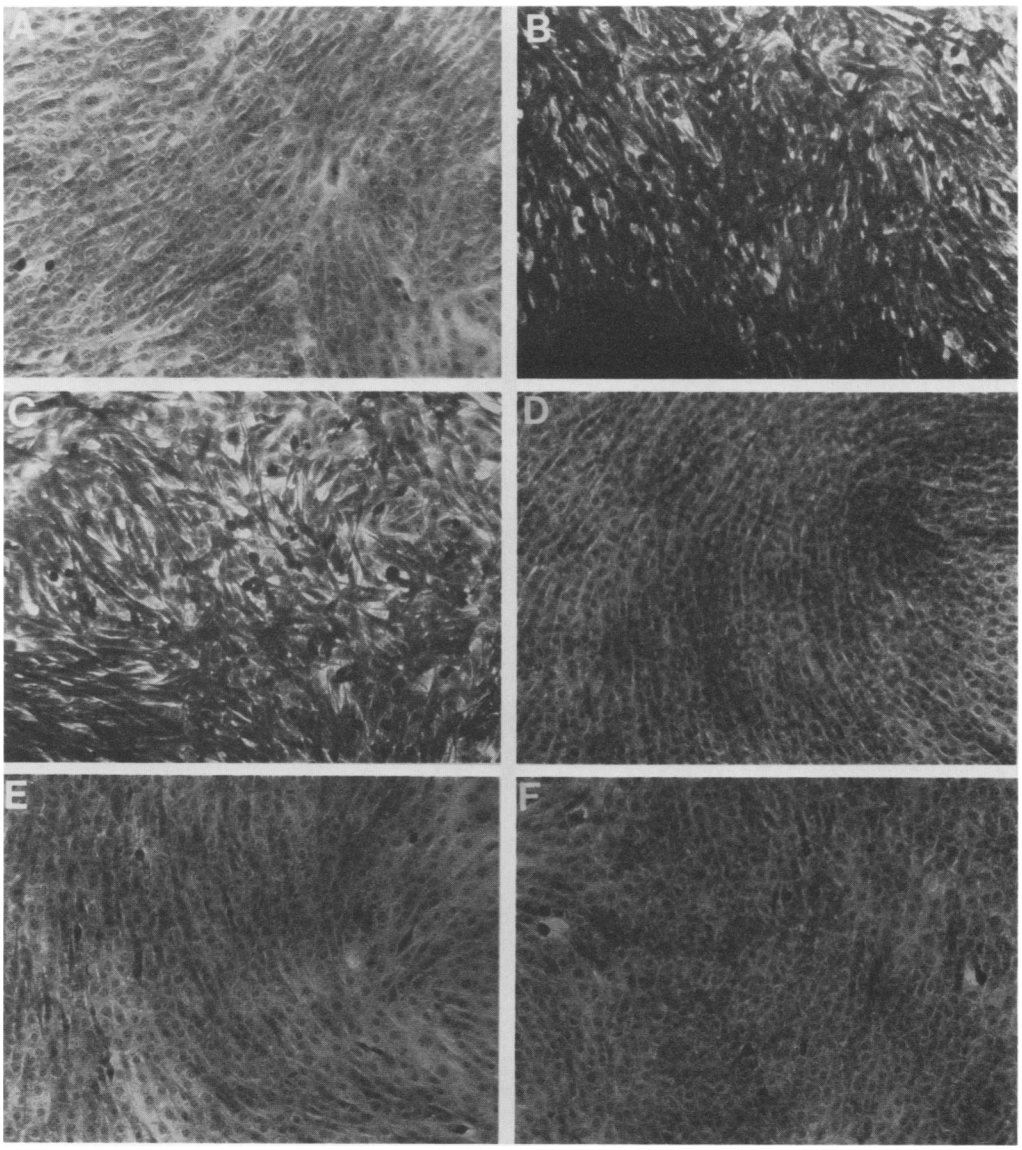

FIG. 4. Morphology of G418-resistant C127 cells transfected with (A) pdMMTneo (302-3) alone which exhibits a flat nontransformed phenotype, (B) pdMMTneo (302-3) and p327-25 which exhibit a transformed phenotype characteristic of BPV-1, (C) pdMMTneo (302-3) and p448-5 which exhibit a transformed phenotype, (D) pdMMTneo (302-3) and p618-5, (E) pdMMTneo (302-3) and p441-4, and (F) pdMMTneo (302-3) and p442-2. Cultures were maintained in DMEM plus G418 (400 μg/ml), fixed with 70% isopropyl alcohol, and stained with 1% methylene blue (magnification, ×52).

TABLE 1. Cotransformation of mouse cells by BPV-1 E2 ORF mutant DNAs and MMTneo(302-3)[a]

Plasmids	No. of foci in DMEM	No. of colonies in DMEM + G418
pdMMTneo(302-3) + p327-7	38, 49	95, 98
pdMMTneo(302-3) + p448-5	18, 19	135, 150
pdMMTneo(302-3) + p618-5	0, 0	32, 35
pdMMTneo(302-3) + p441-4	0, 2	73, 75
pdMMTneo(302-3) + p442-4	0, 0	90, 95
pdMMTneo(302-3) alone	0, 0	15, 16
p142-6 alone	42, 49	0, 0
pBR322	0, 0	0, 0

[a] Mouse C127 cells (5×10^5 cells per plate) were transfected with 1.25 μg of each of the plasmid DNAs listed above with salmon sperm DNA (20 μg/ml) as the carrier, using the calcium phosphate precipitation technique (13). The cells were exposed to 15% glycerol for 2 min at 4 h after exposure to the DNA. At 72 h after exposure to DNA, the cells were split at a ratio of 1:6 and maintained in DMEM or under selection in medium containing 400 μg of the amino glycoside G418 (GIBCO Laboratories, Grand Island, N.Y.) per ml as previously described (23). At 21 days, the cultures were evaluated in duplicate for focus formation or colony formation.

deletion in the E2 ORF expresses only a partially transformed phenotype. The larger deletion mutants which eliminate essentially all of the E2 ORF (p618-5, p441-4, and p442-4) do not have any apparent morphological effect on mouse C127 cells into which the DNA has been introduced by cotransformation. The cells are morphologically indistinguishable from nontransformed C127 cells, are contact inhibited, and are anchorage dependent. However, certain clones were tumorigenic, although at a reduced frequency when compared to wild-type BPV-1 transformants, and the tumors were also slower to develop.

Phenotypic analysis of transforming BPV-1 deletion mutants. Transformed lines generated from the focus assay mutants with deletions mapping outside the E2 ORF were further studied with respect to their properties of transformation. Each of the mutants (Fig. 2) which were positive by focus assay and which contained the E2 ORF region intact were further analyzed for anchorage independence in agar and for tumorigenicity in nude mice (Table 3). The lines transformed with p593-6 or p620-7 which are affected only in the E1 ORF have properties indistinguishable from the BPV-1 wild-type transformant ID13. The NS237-1 cell lines which contained the mutant DNA deleted of sequences between the *Sma*I site and the *Bst*E2 site have properties of anchorage independence in agar and tumorigenicity in nude mice similar to that of ID13 cells. The deletion in this mutant removes most of the E1 ORF and all of the E8 ORF, indicating that the putative gene products encoded by the E1 and E8 ORFs are not essential for focus formation or for tumorigenicity. In contrast, cells transformed by p236-12 or p238-5, which are deleted of sequences from the *Hpa*I site to the *Bst*E2 site or to the *Sma*I site, respectively, are significantly impaired in their properties of anchorage independence and tumorigenicity. The cells do not form colonies in soft agar, and although some of the lines were still tumorigenic, the tumors appeared later and were smaller. Thus, although the E2 ORF apparently encodes the major transforming protein, a function maps between the *Hpa*I site and the *Sma*I site which is also required for the expression of the full transformed phenotype.

State of the viral DNA in the transformants. The viral DNA has been previously shown to exist as a stable extrachromosomal plasmid in BPV-1 viral transformants (24), as well as in mouse C127 cells transformed by p142-6 (36). The state of the viral DNA was therefore examined in the cells transformed by the BPV-1 deletion mutants. Of the deletion mutants (Fig. 2) which could induce transformation, only p214-1 existed extrachromosomally in the transformed cells (Fig. 5). Transformants induced by mutants with deletions and alterations located upstream from the E2 ORF all contained integrated BPV-1 DNA sequences. As illustrated in the case of p236-12-induced transformants (Fig. 6A), *Sal*I-

TABLE 2. Properties of G418-resistant cells cotransformed with BPV-1 E2 ORF mutants

Cell lines	Bases deleted	Morphology	Anchorage independence[a]		Tumorigenicity	
			Colonies per 10^4 cells	Colony size (mm)	No.	Time of appearance (wk)
NS327-25 GT-A	3737–3838	Fully transformed	8×10^2	>1	4/4	3–4
NS327-25 GT-B	3737–3838	Fully transformed	4×10^2	>1	4/4	3–4
NS448-5 GT-A	3456–3838	Transformed[b]	20	<1	2/3	8
NS448-5 GT-B	3456–3838	Transformed	60	<1	2/4	8
NS618-5 GT-A	2775–3838	Flat	0		1/4	5
NS618-5 GT-B	2775–3838	Flat	0		0/4	
NS618-5 GT-C	2775–3838	Flat	0		0/4	
NS441-4 GT-A	2405–3838	Flat	4	<1	1/4	8
NS441-4 GT-B	2405–3838	Flat	ND[c]		3/4	8
NS442-4 GT-A	2113–3838	Flat	0		0/4	
NS442-4 GT-B	2113–3838	Flat	40	<1	2/4	8
ID13	None	Fully transformed	10^3	>1	4/4	3
C127	No BPV DNA	Flat	0		0/4	
NS302-3U-A	No BPV DNA	Flat	0		0/4	

[a] Anchorage independence was assayed at 2 weeks after suspension of single cells in 0.30% agar suspension as described in the text.
[b] Transformed cells lack the spindled, refractile appearance characteristic of BPV-1-transformed C127 cells.
[c] ND, Not done.

TABLE 3. Properties of BPV-1 deletion mutant transformed lines

Cell lines	Bases deleted	ORFs affected	Anchorage independence[a]		Tumorigenicity	
			Colonies per 10^4 cells	Colony size (mm)	No.	Time of appearance (wk)
NS236-12U-A	1–2405	E6, E7, E8, E1	0		3/4	5–6
NS236-12U-B	1–2405	E6, E7, E8, E1	0		4/4	5–6
NS237-1U-A	945–2405	E8, E1	4.4×10^2	>1	4/4	3
NS237-1U-B	945–2405	E8, E1	1×10^3	>1	4/4	3
NS237-1U-C	945–2405	E8, E1	1.6×10^2	>1	4/4	3
NS238-5U-A	1–945	E6, E7, E1	0		0/4	
NS238-5U-B	1–945	E6, E7, E1	0		N.D.[b]	
NS593-6U-A	None[c]	E1	10^3	≥1	4/4	3
NS593-6U-B	None	E1	10^3	≥1	N.D.	
NS620-7U-A	2113–2405	E1	80	≥1	4/4	4
NS620-7U-B	2113–2405	E1	80	≥1	N.D.	
ID13	None		10^3	>1	4/4	3
C127	No BPV-1DNA		0		0/4	

[a] Anchorage independence was assayed at 2 weeks after suspension of single cells in a 0.30% agar suspension as described in the text.
[b] ND, Not done.
[c] The mutant used to generate these transformants was an insertion mutant generated by inserting a decamer *Hin*dIII linker into the *Bst*E2 site of pdBPV (142-6) after the generation of blunt ends with T4 polymerase.

cleaved DNA (a no-cut enzyme for the viral plasmid) from four independent p236-12-induced cell lines migrated with high-molecular-weight DNA. Cleavage with either *Xba*I or *Hin*dIII (one-cut enzymes) generated a multitude of bands indicative of integration. The same was observed for p237-3 (Fig. 6B)-, p238-5 (not shown)-, p593-6 (Fig. 7A)-, and p620-7 (Fig. 7B)-induced transformants. Thus, it is apparent that sequences upstream from E2 are required for stable plasmid maintenance and autonomous plasmid replication.

In the case of NS236-12C, it is clear from the number of offsize bands that are present that only two to three copies of the genome are integrated. In the other lines there are many viral genomes integrated. Thus, unlike cells transformed by the full BPV-1 genome, which invariably contain multiple copies (20 to 400 copies per diploid cell) (24), only one or a few viral genomes are required for transformation when the DNA is integrated.

Two mutants which we studied contained lesions which are limited to the E1 ORF. The p593-6 mutant contains a 10-base *Hin*dIII linker inserted at the *Bst*EII site at base 2405. The p620-7 mutant is deleted of sequences between the *Eco*RI site and the *Bst*E2 site (bases 2113 to 2405). The DNA of each of these is integrated in the transformants induced by these DNAs, implicating the putative E1 ORF gene product as possibly being required in *trans* for autonomous plasmid maintenance. This hypothesis assumes that sequences required in *cis* either for transcriptional activity or for plasmid replication or maintenance are not located at the sites of these engineered mutations.

Functional complementation of the 5′ noncoding region. The deletion mutants p267-34, p268-17, and p141-1 each were effectively negative in the focus forming assay (Fig. 2), and each lacks a critical segment of the BPV-1 genome in the 5′ noncoding region. The analysis of the deletion mutants in which the noncoding segment was intact showed that the apparent coding sequences for focus formation mapped to the E2 ORF, whereas an additional function required for the full transformed phenotype is located between bases 1 and 945. We therefore examined whether the critical 5′ noncoding domain delineated in this study by the deletion from the *Hin*cII site at base 7143 and the *Hpa*I site at base 1 represents a transcriptional regulatory element. A 344-base-pair segment of SV-40 DNA delineated by the *Pvu*II site and the *Hin*dIII site containing the early transcriptional promoter, as well as the 72-base-pair repeated enhancer elements, was inserted in either orientation into the unique *Xba*I site of p141-1 (Fig. 8). The mutant p141-1 has no activity in the transformation focus assay (Fig. 1). When the SV-40 early promoter (EP) was inserted in the same transcriptional direction relative to the BPV-1, the resultant recombinant (p213-2) induced 23 colonies per μg of DNA per 10^6 cells or ca. 10% that of the control. Of interest is the result that the

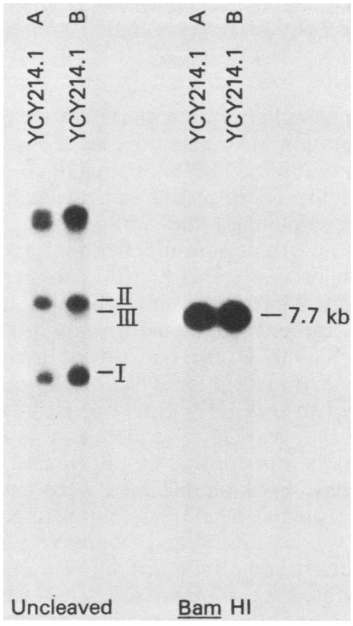

FIG. 5. Southern blot analysis of BPV-1 DNA in two independent mouse C127 cells transformed by p214-1. High-molecular-weight DNA (5 μg per lane) from transformed cell lines YCY214-1 A and B was subjected to agarose gel electrophoresis either uncleaved or after digestion with the restriction endonuclease *Bam*HI and transferred to nitrocellulose filters. The filters were hybridized with a ^{32}P-labeled nick-translated probe as previously described (24). The roman numerals indicate the migration of forms I (supercoiled circles), II (nicked circles), and III (linear 7.7-kb molecules).

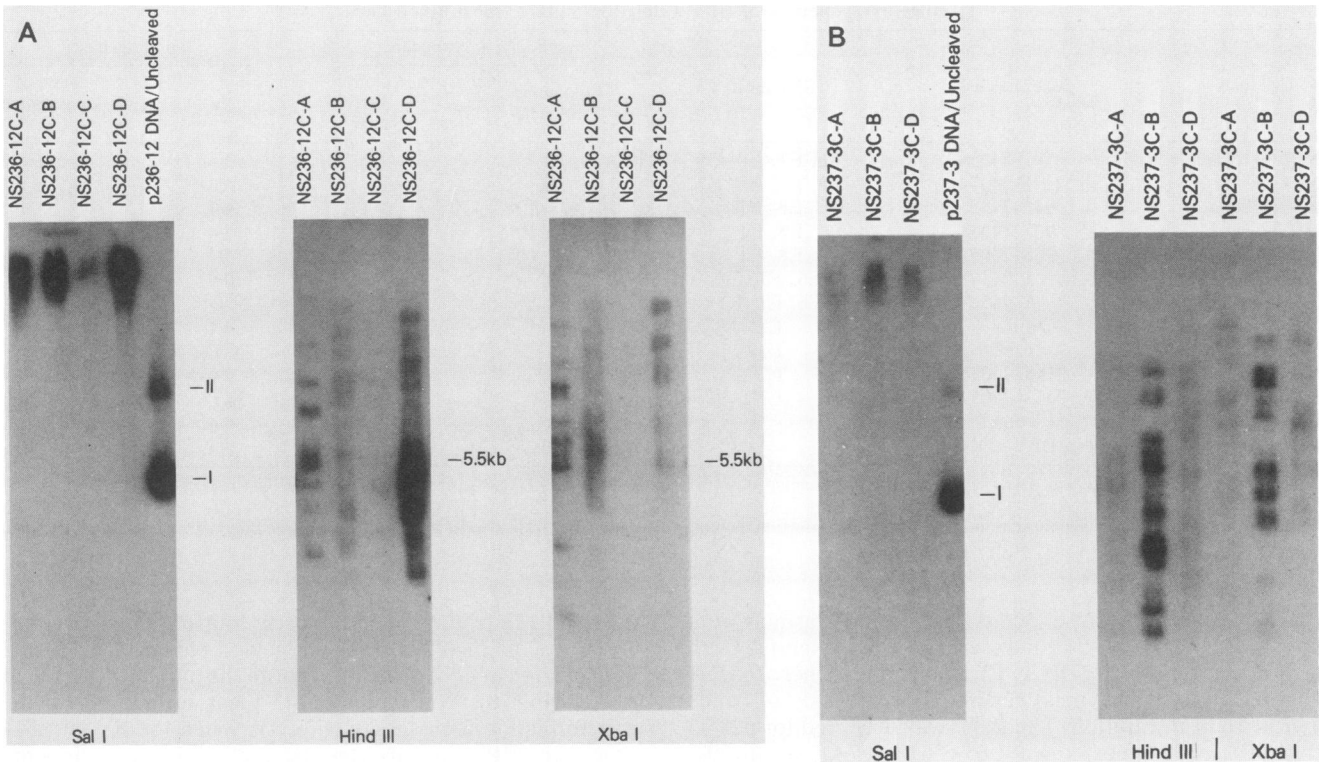

FIG. 6. Southern blot analysis of BPV-1 DNA in mouse cells transformed by either (A) p236-12 DNA or (B) 237-3 DNA. These individual transformants were cloned from cells transfected with plasmid DNAs cleaved with *Bam*HI, which freed the BPV-1 DNA sequences from the pML2d sequences. Total cellular DNA was extracted as previously described (24), and 5 μg was digested to completion with the indicated restriction endonuclease and fractionated in a 0.6% agarose gel in Tris-acetate buffer. The DNA was partially depurinated, denatured, and transferred to nitrocellulose filters as previously described (24). Marker lanes contain 400 pg of the bacterially generated plasmid cleaved as indicated. The positions of migration of the appropriate sized supercoiled (form I) and nicked circular (form II) DNAs are indicated as I and II. The immobilized DNAs were hybridized to a ^{32}P-labeled nick-translated BPV-1 DNA probe.

recombinant molecule p213-8 with the SV-40 EP insert in the opposite orientation was able to transform cells at levels comparable to that of p213-2, inducing 20 colonies per μg of DNA per 10^6 cells. These results indicate that the SV-40 EP segment can complement the 5′ noncoding sequences of BPV_{69T} but in an orientation-independent manner. Clearly, at least in the case of p213-8, this cannot simply be by providing an active promoter since the promoter element in the SV-40 EP segment will be incorrectly oriented. It seems likely that the SV-40 72-base-pair repeat enhancer elements are activating a transcriptional promoter element located within the *Hpa*I-to-*Bam*HI segment of BPV_{69T}.

The state of the viral DNA sequences was next examined in the mouse cells transformed by p213-2 and p213-8 recombinant molecules. Four clonal lines were established from transformants induced by p213-2 and p213-8, and the total cellular DNA was extracted and examined for BPV-1 DNA sequences as described above. Each line contained multiple copies of integrated recombinant DNA molecules. No extrachromosomal supercoiled or nicked circular viral DNA sequences could be detected in uncleaved DNA analyzed from any of the eight cell lines examined, two of which are presented in Fig. 9. The plasmid markers are indicated in the lanes labeled p213-2 DNA. The viral DNAs in each line are apparently integrated in tandem intact, in that cleavage of the cellular DNA with the single-cut restriction enzyme *Sal*I resulted in a prominent band of 10.8 kilobases (kb) corresponding to the size of the linearized plasmid. Additional bands possibly representing host-viral DNA junction fragments were also present. Further evidence that the BPV–SV-40 EP segment was intact in each of the transformed lines was provided by the fact that *Bam*HI or *Bam*HI plus *Cla*I cleavages of the input DNA resulted in fragments which comigrated with those from the input plasmid. These data argue that the 5′ noncoding sequences in BPV_{69T} contain sequences essential for plasmid replication and maintenance.

DISCUSSION

Two principal features, transformation of the host cell and autonomous plasmid replication of the viral genome as a multicopy plasmid, characterize the interaction of BPV DNA with susceptible mouse cells. Initial studies with BPV-1 cloned DNA localized these functions to a specific subgenomic fragment (BPV_{69T}) comprising 69% of the viral genome extending from the *Hin*dIII site (base 6959) to the *Bam*HI site (base 4451) (20, 25). The absence of a productive tissue culture system for BPV-1 has prevented a standard genetic analysis of this virus in that it has hindered the generation of conditional mutants of BPV-1. The approach we used to study the properties of this virus and the interaction of BPV-1 with mouse cells has been to generate a series of defined BPV-1 deletion mutants. The studies presented here delineate four discrete regions of the genome required for the expression of the full BPV-1 transformed phenotype. A transcriptional control element which maps in the 5′ noncod-

ing region can be functionally reconstituted by a segment of SV-40 DNA which has sequences for promotion and enhancement of RNA transcription. A transcriptional enhancer element of BPV-1 has previously been localized to a 60-base-pair fragment at the 3′ end of this transforming unit (26). A region mapping in the E2 ORF is essential for efficient focus formation, anchorage independence, and tumorigenicity, implicating the gene product of the E2 ORF as the major transforming protein. In addition to the region mapping in the E2 ORF, sequences located between the *Hpa*I site (base 1) and the *Sma*I site (base 945) encompassing the E6 and E7 ORFs must be present for full expression of the transformed phenotype.

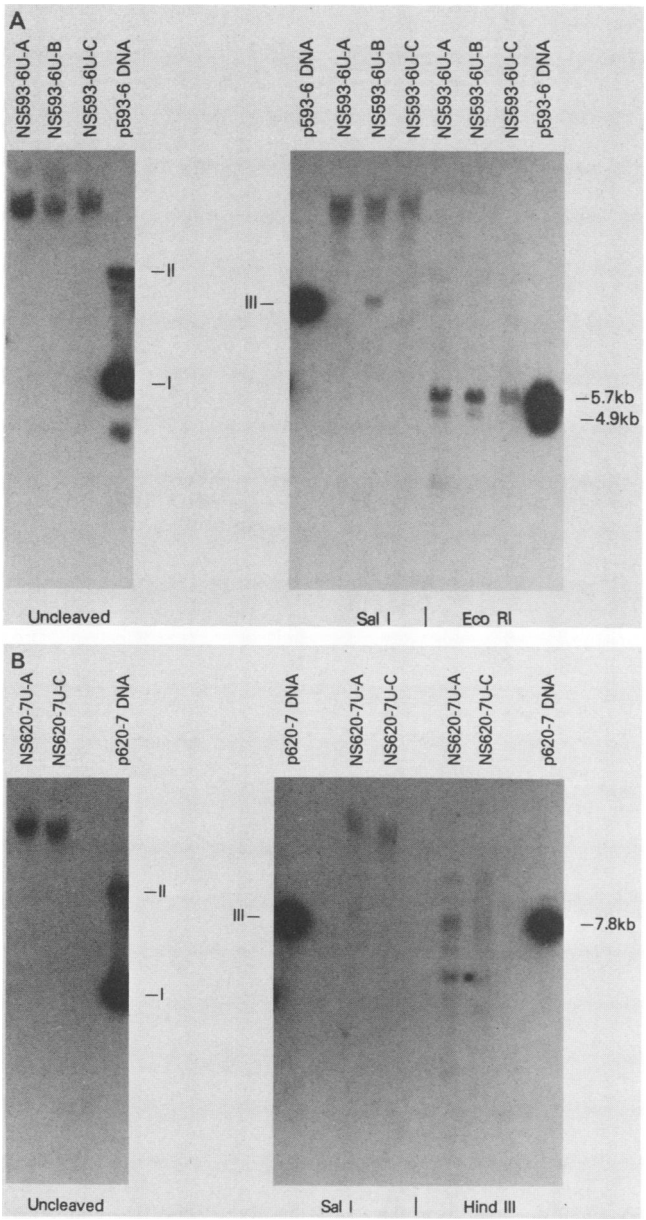

FIG. 7. Southern blot analysis of BPV-1 DNA in mouse cells transformed by either (A) p593-6 DNA or (B) p620-7 DNA. Individual transformants were cloned from cells transfected with intact p593-6 DNA or intact p620-7 DNA. Total cellular DNA was prepared as previously described (24) and for each lane 5 μg was analyzed uncleaved or digested with the indicated restriction endonuclease. The DNAs were subjected to electrophoresis through 0.6% agarose in Tris-acetate buffer and transferred to nitrocellulose membranes after partial depurination. Marker lanes contain 400 pg of bacterially generated plasmid. The position of migration of supercoiled (I), nicked circular (II), and linear (III) forms of the input plasmids are marked. The probe utilized was nick-translated ^{32}P-labeled BPV-1 DNA.

Others have reported that a 2.2-kb deletion in BPV_{69T} between the *Hae*III sites at 7587 and 1805 did not abolish transforming activity in a focus assay (34). One region essential for transformation in the 5′ noncoding region was shown to be a transcriptional control element in that it could be functionally complemented by a retrovirus long terminal repeat with promotional and enhancement activity for RNA transcription (34). Similarly we map a transcriptional control element to this 5′ noncoding region. It can be functionally complemented by the SV-40 EP which also has transcriptional promotion and enhancement activities (3, 17, 33). The SV-40 EP fragment functions equally well in either orientation, indicating that at least in one orientation the complementation must occur through the activation of a functional promoter element within the *Hpa*I-to-*Bam*HI fragment.

The viral transcripts present within BPV-1-transformed cells are not abundant, map to the segment between *Hpa*I and *Bam*HI site, and have a 3′ coterminus at 0.53 map units (18). At least five discrete transcripts have been detected, the RNA species are generated by differential splicing, and the 5′ ends of the majority of the RNA species map at base 89 (1, 18; Y. C. Yang and P. M. Howley, unpublished data). Based on the presence of a TATAAA sequence at nucleotide 58 and on the position of the 5′ ends of the BPV-1 RNA species in transformed cells, it seems likely that this TATA box forms part of the BPV-1 transcriptional promoter functioning in transformed cells. Studies have established that in addition to the TATA box, which accurately positions the initiation of transcription, sequences upstream for this element are required for efficient transcription in vivo (4, 15, 16, 32). Thus, the deletions upstream from this TATA box which extend 5′ from the *Hpa*I site would be expected to impair this promoter function.

The mechanisms allowing for the functional complementation by the SV-40 EP segment could be mediated through the SV-40 early promoter only in one orientation. In the orientation in which the promoter is directed away from the BPV-1 segment, the functional complementation is most likely through the activation of a promoter element in the *Hpa*I-to-*Bam*HI segment. This could be the residual BPV-1 promoter segment with the TATA box at base 58 or could be through the activation of a cryptic promoter element located downstream in the BPV-1 segment. In this regard, this same SV-40 EP segment, as well as the mouse sarcoma virus enhancer element, can enhance transformation by the *Eco*RI-to-*Bam*HI segment of BPV-1 (bases 2113 to 4451) in a position- and orientation-independent manner (N. Sarver and P. M. Howley, unpublished data). Similarly, the Harvey sarcoma virus long terminal repeat which contains transcriptional enhancement sequences can function in an orientation-independent manner to increase the frequency of transformation of the *Eco*RI-to-*Bam*HI segment of BPV-1 DNA (34). Whether the apparent cryptic promoter which can be activated in this DNA segment is normally functional in BPV-1 transformed cells is not yet known.

The BPV-1 DNA segment containing the coding sequences for a gene product sufficient for inducing focus formation in mouse fibroblasts has previously been localized to the 2.3-kb *Eco*RI-to-*Bam*HI segment (34). By defined

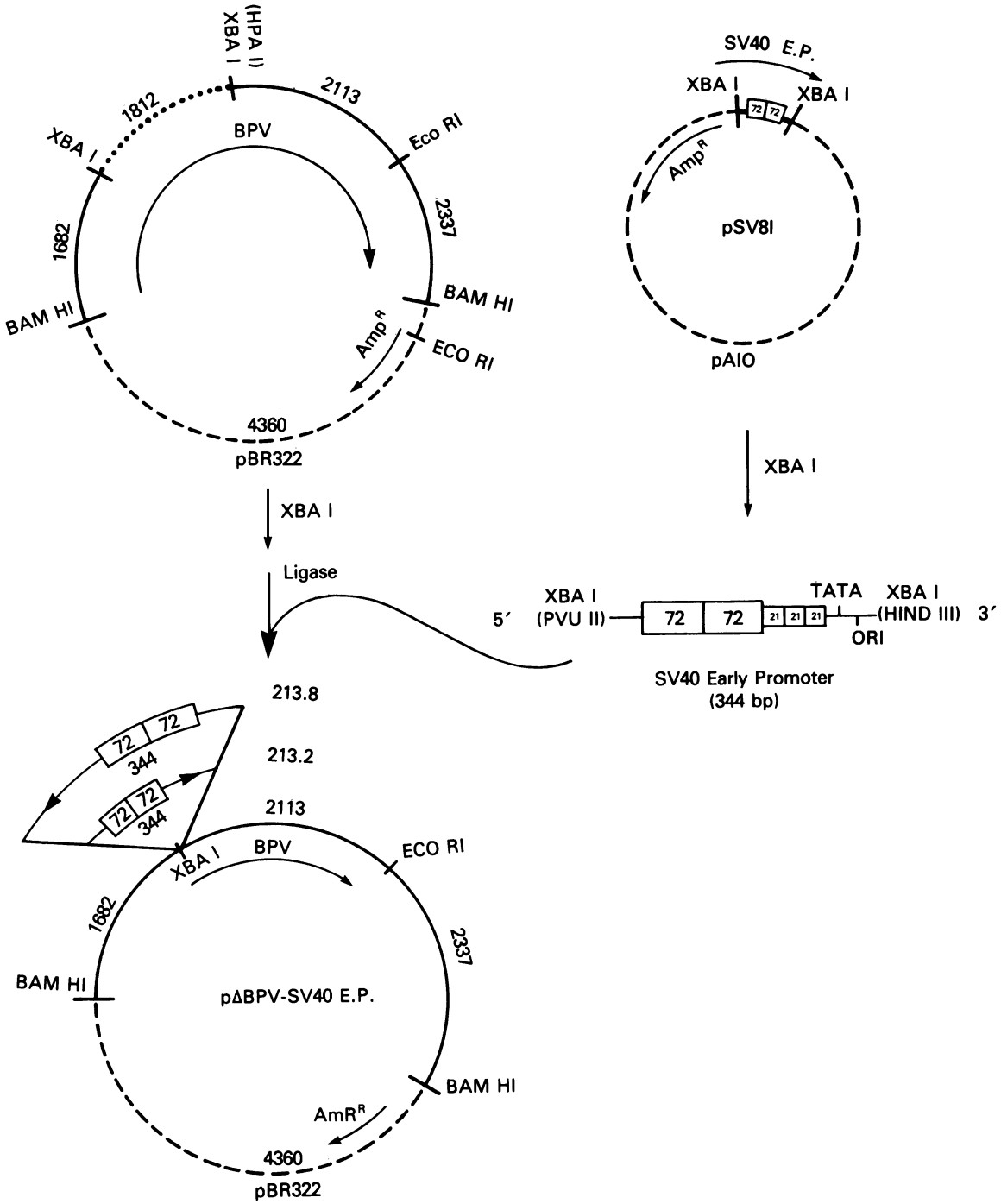

FIG. 8. Construction of pΔBPV–SV-40 EP recombinant DNAs. The SV-40 EP segment from the *Pvu*II site to the *Hin*dIII site modified with *Xba*I linkers (obtained from L. Laimons and G. Khoury) was cloned at the site of deletion in p141-1 (pBPV-1 deleted of the *Xba*I-to-*Hpa*I fragment with a reconstituted *Xba*I site). In the case of p213-2 the promoter direction of the SV-40 element is the same as the transcriptional direction of BPV-1, and in the case of p213-8, it is opposite. Symbols: —, BPV-1 DNA; – – – –, pBR322; ....., deleted BPV-1 DNA segment. bp, Base pairs.

deletion mutagenesis, we localize this function to the E2 ORF which could by itself encode a 48-kilodalton protein. The E2 ORF is apparently transcribed intact in transformed cells, in that a polydenylated RNA species with a body whose 5′ end maps to the NH_2 terminus of the E2 ORF has been described (18). The NH_2 half of the protein product encoded by the E2 ORF is well conserved among different papillomaviruses including human papilloma virus type 1a and human papillomavirus type 6 (8, 39). There is also structural and amino acid homology within the 86 amino acids at the COOH-terminal of the E2 ORFs of the BPV-1, human papillomavirus types 1a and 6b, and cottontail rabbit

papilloma virus genomes, and limited amino acid homology with this region can be found with the human c-*mos* gene (O. Danos and M. Yaniv, *in* G. F. Vande Woude, A. J. Levine, W. C. Topp, and J. D. Watson, ed., *Cancer Cell*, in press). What role the putative E2 gene product may have in benign warts is unknown, although its function may be in the induction of dermal fibroblastic proliferation seen in bovine fibropapillomas induced by BPV-1 and possibly in the induction of the epidermal cell proliferation characteristic of all papillomavirus-induced benign lesions.

Expression of the E2 ORF by itself is not sufficient for the manifestation of the full BPV-1 transformed phenotype. Recombinants expressing the E2 ORF under the control of the Harvey-mouse sarcoma virus long terminal repeat (34) or of the SV-40 EP segment (N. Sarver, M. S. Rabson, and P. M. Howley, data not shown) can induce transformed foci; however, the resultant transformed mouse cells are less tumorigenic in nude mice in that tumors are smaller and appear later, and the cells are less anchorage independent when assayed in soft agar (M. S. Rabson, N. Sarver, and P. M. Howley, data not shown). In the presence of an intact E2 ORF, deletion mutation analysis maps a function essential for the fully transformed phenotype between the *Hpa*I and *Sma*I sites. Two significant open translation frames, E6 and E7, map in this region. Recent analyses have revealed that transcripts exist in BPV-1-transformed cells which could direct the synthesis of a 15.5-kilodalton E6 product, a 20-kilodalton E6/E4 product, and a 21-kilodalton E6/E7 gene product (Y.-C. Yang, H. Okayama, and P. M. Howley, unpublished data), any of which could be candidates for providing this function. Formal complementation analyses to test these possibilities are in progress.

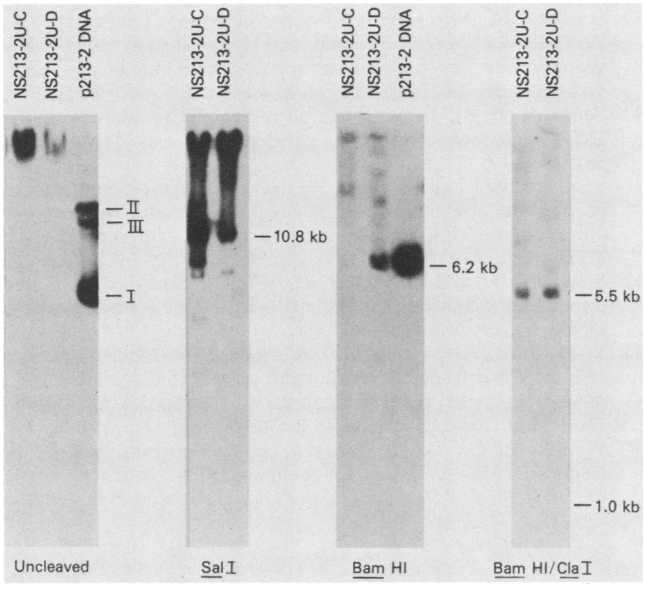

FIG. 9. Southern blot analysis of BPV-1 DNA in mouse cells transformed by p213-2 DNA. Two individual transformed lines, NS213-2U-C and NS213-2U-D, were cloned from cells transfected with intact p213-2 DNA. Total cellular DNA was extracted as previously described (24), and for each lane 5 μg was treated as indicated and fractionated in a 0.6% agarose gel in Tris-acetate buffer. The DNA was partially depurinated, denatured, and transferred to nitrocellulose membranes as previously described (24). Marker lanes contain 400 pg of bacterially generated plasmid DNA and the positions of migration of supercoiled (I), nicked circular (II), and linear (III) forms of p213-2 DNA are indicated. The probe utilized was nick-translated, ^{32}P-labeled BPV-1 DNA.

This study demonstrates that BPV-1-induced transformation can occur in the absence of extrachromosomal plasmid replication, unlinking transformation and autonomous plasmid maintenance. Two additional deletion mutants described by Lusky and Botchan (28) which leave the E1, E6, E7, and E8 ORFs intact are capable of autonomous plasmid maintenance but are not capable of inducing foci. A deletion mutant (p560-7) and a linker insertion mutant (p593-6), which are each affected in only the E1 ORF, are each capable of inducing transformed foci at a level one-third that of the intact full BPV-1 genome. The resultant transformants have a fully transformed phenotype, and the viral DNA is integrated. Other BPV-1 DNA deletion mutants which affect the E1 ORF, as well as the E6, E7, or E8 ORFs or a combination of these, also induce focus formation but are not capable of autonomous plasmid maintenance. The putative gene product of the E1 ORF is thus necessary for autonomous plasmid maintenance in transformed cells but is not required for transformation.

ACKNOWLEDGMENT

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

We are grateful to C. R. Schlegel for a critical reading of this manuscript and for helpful discussions in the course of these experiments.

LITERATURE CITED

1. **Ahola, H., A. Stenlund, J. Moreno-Lopez, and U. Pettersson.** 1983. Sequences of bovine papillomavirus type 1 DNA—functional and evolutionary implications. Nucleic Acids Res. **11**:2639–2650.
2. **Amtmann, E., and G. Sauer.** 1982. Bovine papilloma virus transcription: polyadenylated RNA species and assessment of the direction of transcription. J. Virol. **43**:59–66.
3. **Banerji, J., and W. Schaffner.** 1981. Expression of a β-globin gene is enhanced by remote SV40 DNA sequences. Cell **27**:299–308.
4. **Benoist, C., and P. Chambon.** 1981. *In vivo* sequence requirements of the SV40 early promoter region. Nature (London) **290**:304–310.
5. **Chen, E. Y., P. M. Howley, A. D. Levenson, and P. H. Seeburg.** 1982. The primary structure and genetic organization of the bovine papilloma-virus type 1 genome. Nature (London) **299**:529–534.
6. **Clewell, D. B.** 1972. Nature of the Col E_1 plasmid replication in *Escherichia coli* in the presence of chloramphenicol. J. Bacteriol. **110**:667–676.
7. **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.
8. **Danos, O., L. W. Engel, E. Y. Chen, M. Yaniv, and P. M. Howley.** 1983. Comparative analysis of the human type 1a and bovine type 1 papillomavirus genomes. J. Virol. **46**:557–566.
9. **DiMaio, D., R. Treisman, and T. Maniatis.** 1982. A bovine papilloma-virus vector which propagates as an episome in both mouse and bacterial cells. Proc. Natl. Acad. Sci. U.S.A. **79**:4030–4034.
10. **Dvoretzky, I., R. Shober, and D. Lowy.** 1980. Focus assay in mouse cells for bovine papillomavirus. Virology **103**:369–375.
11. **Engel, L. W., C. A. Heilman, and P. M. Howley.** 1983. Transcriptional organization of the bovine papillomavirus type 1. J. Virol. **47**:516–528.
12. **Frost, E., and J. Williams.** 1978. Mapping temperature-sensitive and host-range mutations of adenovirus type 5 by marker rescue. Virology **91**:39–50.

13. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–461.
14. **Gross-Bellard, M., P. Oubert, and P. Chambon.** 1973. Isolation of high molecular weight DNA from mammalian cells. Eur. J. Biochem. **36**:32–38.
15. **Grosschedl, R., and M. L. Birnstiel.** 1980. Spacer DNA sequences upstream of the T-A-T-A-A-A-T-A sequence are essential for promotion of H2A histone gene transcription *in vivo*. Proc. Natl. Acad. Sci. U.S.A. **77**:7102–7106.
16. **Grosveld, G. C., E. de Baer, C. K. Skewmaker, and R. A. Flavell.** 1982. DNA sequences necessary for transcription of the rabbit β-globin gene in vivo. Nature (London) **295**:120–126.
17. **Gruss, P., R. Dhar, and G. Khoury.** 1981. Simian virus 40 tandem repeated sequences as an element of the early promoter. Proc. Natl. Acad. Sci. U.S.A. **78**:943–947.
18. **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.
19. **Holmes, D. S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114**:193–197.
20. **Howley, P. M., M.-F. Law, C. A. Heilman, L. W. Engel, M. C. Alonso, W. D. Lancaster, M. A. Israel, and D. R. Lowy.** 1980. Molecular characterization of papillomavirus genomes, p. 233–247. *In* M. Essex, G. Todaro, and H. zur Hausen (ed.), Viruses in naturally occurring cancers. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Hutchinson, K. W., and H. O. Halvorson.** 1980. Cloning of randomly sheared DNA fragments from a 105 lysogen of *Bacillus subtilis*—identification of prophage-containing clones. Gene **8**:267–278.
22. **Ketner, G., and T. Kelly, Jr.** 1976. Integrated simian virus 40 sequences in transformed cell DNA: analysis using restriction endonucleases. Proc. Natl. Acad. Sci. U.S.A. **73**:1102–1106.
23. **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.
24. **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. U.S.A. **78**:2727–2731.
25. **Lowy, D. R., I. Dvoretzky, R. Shober, M.-F. Law, L. Engel, and P. M. Howley.** 1980. *In vitro* transformation by a defined subgenomic fragment of bovine papillomavirus DNA. Nature (London) **287**:72–74.
26. **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 transcriptional unit. Mol. Cell. Biol. **3**:1108–1122.
27. **Lusky, M., and M. Botchan.** 1981. Inhibitory effect of specific pBR322 DNA sequences upon SV40 replication in simian cells. Nature (London) **293**:79–81.
28. **Lusky, M., and M. R. Botchan.** 1984. Characterization of the bovine papillomavirus plasmid maintenance sequences. Cell **36**:391–401.
29. **MacPherson, I., and I. Montagnier.** 1964. Agar suspension culture for the selective assay of cells transformed by polyoma virus. Virology **23**:291–294.
30. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. *In* T. Maniatis and E. F. Fritsch (ed.), Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Maniatis, T., R. C. Hardison, E. Lacy, C. Lauer, D. Quon O'Connel, G. K. Sim, and A. Efstratiadis.** 1978. The isolation of structural genes from libraries of eukaryotic DNA. Cell **15**:687–701.
32. **McKnight, S. L., E. R. Gavis, R. Kingsbury, and R. Axel.** 1981. Analysis of transcriptional regulatory signals of the HSV thymidine kinase gene: identification of an upstream control region. Cell **25**:385–398.
33. **Moreau, P., R. Hen, B. Wasylyk, R. Everett, M. P. Gaub, and P. Chambon.** 1981. The SV40 72 base pair repeal has a striking effect on gene expression both in SV40 and other chimeric recombinants. Nucleic Acids Res. **9**:6047–6068.
34. **Nakabayashi, Y., S. K. Chattopadhyay, and D. R. Lowy.** 1983. The transforming functions of bovine papillomavirus DNA. Proc. Natl. Acad. Sci. U.S.A. **80**:5832–5836.
35. **Rigby, P. D., M. D. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase. J. Mol. Biol. **113**:237–251.
36. **Sarver, N., J. C. Byrne, and P. M. Howley.** 1982. Transformation and replication in mouse cells of a bovine papillomavirus-pML2 plasmid vector that can be rescued in bacteria. Proc. Natl. Acad. Sci. U.S.A. **79**:7147–7151.
37. **Sarver, N., P. Gruss, M.-F. Law, G. Khoury, and P. M. Howley.** 1981. Bovine papilloma virus deoxyribonucleic acid: a novel eucaryotic cloning vector. Mol. Cell. Biol. **1**:486–496.
38. **Sarver, N., S. Mitrani-Rosenbaum, M.-F. Law, J. C. Byrne, and P. M. Howley.** 1984. A bovine papillomavirus/pML2 hybrid vector—a dual host replicon, p. 81–88. *In* N. L. Sternberg and M. L. Pearson (ed.), Gene transfer and cancer. Raven Press, Publishers, New York.
39. **Schwarz, E., M. Durst, C. Demankowski, O. Lattermann, R. Zech, E. Wolfsperger, S. Suhai, and H. zur Hausen.** 1983. DNA sequence and genome organization of genital human papillomavirus type 6b. EMBO J. **2**:2341–2348.
40. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
41. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
42. **Stowe, N. D., and N. M. Wilkie.** 1976. An improved technique for obtaining enhanced infectivity with Herpes simplex type 1 DNA. J. Gen. Virol. **33**:447–458.
43. **Wahl, G. M., M. Stern, and G. R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. U.S.A. **76**:3683–3687.
44. **Wesnik, P. C., D. J. Finnegan, J. E. Donelson, and D. S. Hogness.** 1974. A system for mapping DNA sequences in the chromosomes of *Dropsophila melanogaster*. Cell **3**:315–325.