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A Bovine Papillomavirus Constitutive Enhancer Is Negatively Regulated by the E2 Repressor through Competitive Binding for a Cellular Factor

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The bovine papillomavirus type 1 long control region (LCR) contains DNA sequence elements involved in the regulation of viral transcription and replication. Differences in the levels of transcription have previously been noted between bovine papillomavirus type 1-infected rodent cell lines and bovine cells. To investigate these differences, fragments of the LCR were cloned into an enhancer-deleted chloramphenicol acetyltransferase expression vector and assayed for enhancer activity. A strong constitutive enhancer was found in the 5′ portion of the LCR that was most active in primary bovine fibroblasts and had little activity in other cell types. Deletion mapping localized most of the activity to a 113-bp fragment from nucleotides (nt) 7162 to 7275, a region of the viral sequence that also contains the P_{7185} promoter and an E2-binding site at nt 7203. The enhancer activity of this element could be positively modulated by the full-length E2 transactivator or negatively modulated by the E2 repressor. Site-directed mutagenesis defined two *cis* elements, CE1 and CE2, which were both necessary for enhancer activity. The CE1 element was required for P_{7185} activity, whereas the CE2 element was dispensable for P_{7185} activity. The CE1 and CE2 elements both overlap the E2-binding site at nt 7203. In vitro DNA-binding studies revealed (i) a specific gel retardation complex associated with cellular factor binding at the CE1 element, (ii) a correlation between enhancer activity and the binding of factors to the CE1 element, and (iii) competitive binding between the E2 repressor and the cellular factor at the CE1 element.

Bovine papillomavirus type 1 (BPV-1) is a small DNA tumor virus that produces fibropapillomas in cattle and transforms some rodent cell lines in vitro (29, 32, 48). Papillomaviruses are of medical significance because of the association of specific human papillomaviruses with some types of human carcinomas (7, 15, 21, 59). Papillomaviruses show marked tissue specificity in their natural hosts and in in vitro culture systems. Late gene expression and vegetative replication are viral functions which are limited to terminally differentiated epithelium (5), and as yet there is no in vitro culture system for the complete productive cycle of the virus. BPV-1 has served as the prototype for studies with the papillomaviruses because of the availability of a quantitative focus-forming assay in susceptible cell lines (16, 38). In cells transformed by BPV-1, the viral genome is stably maintained as a multicopy plasmid within the nucleus (35). Eight overlapping early open reading frames (E1 to E8) are transcribed from a single DNA strand (2, 18, 27), utilize a common polyadenylation site, and encode a variety of protein products (4). A noncoding region (referred to as the long control region [LCR] or upstream regulatory region) contains several transcriptional promoters as well as *cis* elements postulated as necessary for viral DNA replication (called plasmid maintenance sequences) (40, 41). Several promoters have been mapped by analysis of the major 5′ ends of RNA species to nucleotides (nt) 7940, 89, 890, 2443, and 3080 (1, 5, 12, 56). In addition, the LCR contains a promoter referred to as P_{7185} (or P1) (5, 55), which maps to nt 7185, and a late promoter (P_L) with RNAs mapping heterogeneously between nt 7214 and 7256 (5). Promoters at nt 7940, 89, and 2443 are transactivated by the full-length viral E2 gene product via E2 DNA-binding motifs (3, 44, 45) located within the LCR (26, 28, 52, 53). In addition to the full-length E2 transactivator, truncated E2 products are produced both from P_{3080} and via a spliced E8/E2 product from P_{890} (12); these truncated E2 products encode the C-terminal DNA-binding and dimerization domain and can inhibit transactivation by the full-length E2 protein (33).

Although BPV-1 shows broader tissue and species specificity than do other papillomaviruses, there are quantitative as well as qualitative differences in transcription between infected mouse lines and bovine wart tissue or infected bovine cell lines (5, 8). For instance, late gene expression occurs only in differentiated bovine epithelial cells. To explore these differences in transcriptional activity, the BPV-1 LCR was assayed for tissue- or species-specific enhancer elements. An enhancer in the BPV-1 LCR was found that is strongly active in bovine cells and less active in cells of other species. The enhancer was found to contain an E2-binding site, and it was negatively regulated by the E2 repressor and transactivated by the E2 transactivator. Constitutive enhancer activity correlated with the binding of a specific cellular factor(s) at a site which overlapped the E2-binding site. The binding of cellular factors to the enhancer could be competed for by the E2 DNA-binding domain in vitro.

MATERIALS AND METHODS

Construction of enhancer-CAT plasmids. Bacterial transformations, plasmid preparations, and nucleic acid manipulations were performed by standard methods (42). BAL 31 deletions through the LCR had been previously constructed and cloned into the enhancer-deleted simian virus 40 (SV40) chloramphenicol acetyltransferase (CAT) expression vector $pA_{10}CAT$ (52). BAL 31 deletion plasmids p985, p984, p983,

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p982, p981, and p980 were cleaved with *Bam*HI and *Mlu*I, and the CAT-containing fragment was exchanged with a similar *Bam*HI-*Mlu*I fragment derived from the BAL 31 deletion plasmid p1005, to generate p1534, p1535, p1537, p1539, p1541, and p1543, respectively. This generated LCR enhancer plasmids with 5′ BAL 31 LCR deletion endpoints and a common 3′ end at BPV-1 nt 7386 (see Fig. 2). To obtain the same series of plasmids with the enhancer fragment in the antisense configuration, p1535, p1537, p1539, p1541, and p1543 were cleaved with *Sal*I and religated, yielding p1536, p1538, p1540, p1542, and p1544, respectively. 3′ deletions of the LCR enhancer fragment of p1534 were made by restriction of p1534 with *Sal*I, isolation of the LCR fragment, cleavage with either *Nar*I (to generate p1283 and p1284) or *Hpa*II (to generate p1295 and p1296), rendering the ends blunt with T4 DNA polymerase, and ligation into $pA_{10}CAT$ with *Sal*I linkers. In experiments assaying BPV-1 LCR fragments as enhancers, $pA_{10}CAT$ was used as the negative control, and pSV_2CAT (an SV40 early promoter-enhancer CAT expression vector) was the positive control (22). pC59 was used to express the E2 transactivator (58), and p1565 (previously designated pCW1-28/Δ1341-3078 [34]) was used to express the E2 repressor. Plasmid p1151, an Okayama-Berg expression vector without a cDNA insert (33), was used to equalize the total amount of cDNA transfected per plate.

Site-directed mutagenesis of the constitutive enhancer. Eight complementary overlapping synthetic oligonucleotides from BPV-1 nt 7143 (*Hin*cII site) to 7275 (*Nar*I site) with additional sequences for a 5′ *Bgl*II site and a 3′ *Sal*I site were kinase treated, annealed, ligated, and cloned into $pA_{10}CAT_{BS}$ (antisense orientation, with a *Sal*I-*Bgl*II cloning site) (52). Mutations were introduced into specific sites by wobbling bases during the oligonucleotide synthesis. All recovered plasmids were confirmed by sequence analysis of the entire BPV-1 insert.

Construction of BPV-1 P_{7185} CAT constructs. pUC-18 was cleaved with *Nde*I, filled in with Klenow DNA polymerase, and religated with a *Bgl*II linker. The resulting plasmid was cut with *Bgl*II and *Hin*dIII, and the large fragment was ligated to the *Bam*HI-*Hin*dIII CAT-containing fragment of pSV_2CAT (22), resulting in a promoter/enhancer-deleted CAT plasmid similar to pSV_0CAT (22). This plasmid was cleaved with *Asp*718, and the ends were filled in with Klenow DNA polymerase and religated with a *Bgl*II linker; the resulting plasmid was cleaved with *Bgl*II and ligated to the *Bam*HI-*Bcl*I (237-bp) fragment of SV40. The resulting plasmid, p1550, is a promoter/enhancer-deleted CAT plasmid with an SV40 polyadenylation signal upstream of the promoter cloning site. The *Bgl*II-*Sal*I enhancer fragments (spanning BPV-1 nt 7143 to 7275) from the oligonucleotide-reconstructed CAT expression plasmids were cloned in the sense orientation into the polylinker of p1550; the mutations with the associated plasmid numbers are shown in Fig. 4.

Construction of P_{89} and P_{7945} CAT expression plasmids. p1066 is a CAT expression plasmid that utilizes BPV-1 promoters P_{89} and P_{7940}; it contains the BPV-1 LCR from nt 6958 to 93 fused to the CAT gene (52). p1066 was cleaved with *Bam*HI and *Mlu*I, and the CAT-containing fragment was ligated to the non-CAT-containing *Bam*HI-*Mlu*I fragment of p985, p983, p982, and p981, to generate p1268, p1269, p1270, and p1271, respectively. This procedure generates a series of LCR-CAT expression vectors similar to p1066 except with 5′ LCR BAL 31 deletions (see Fig. 3).

Gel retardation assays. Nuclear extracts were prepared by the method of Dignan et al. (14). A 1-ng sample of end-labeled synthetic complementary oligonucleotides plus or minus corresponding unlabeled competing oligonucleotides from BPV-1 nt 7189 to 7225 was mixed with 2 to 3 μl of nuclear extract, reticulocyte lysate, or both and adjusted to a total of 10 μl with Digman buffer D. Poly(dI-dC) (500 ng) was added to inhibit nonspecific complex formation. The reaction was incubated on ice for 40 min and loaded on a 5% 29:1 polyacrylamide-bisacrylamide gel in 0.5× TBE salts and electrophoresed at 150 V at room temperature until the bromophenol blue marker lane reached the bottom (43). In vitro-transcribed and -translated $E2_{290-410}$, encoding the C-terminal 121-amino-acid DNA-binding domain (43), was generously provided by Alison McBride; 0.5 to 2 μl of translated or untranslated control lysate was used per binding reaction.

Transient expression assays. All cell lines were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). C127 (a mouse mammary tumor line), CV-1 (African green monkey kidney), and primary human fibroblasts were obtained from the American Type Culture Collection (Rockville, Md.). Bovine embryo fibroblasts (BEFs) were derived from a fetal skin after trypsinization and passaged in complete medium (Dulbecco modified Eagle medium supplemented with 10% serum selects against epithelial cells); cells were frozen at passage 3 and used in transfection assays by passage 6. For CAT transient expression assays, 5 μg of the CAT-expressing plasmid, 1 μg of an Okayama-Berg cDNA expression vector (where applicable), 0.25 μg of the β-galactosidase expression vector pCH110 (Pharmacia; used as an internal control of transfection efficiency), and high-molecular-weight salmon sperm DNA to a total of 10 μg were coprecipitated with calcium phosphate and applied to low-density cells in 6-cm-diameter plates (24). The cells were incubated for 3 to 5 h before a 1-min 15% glycerol shock. After incubation for 48 h in medium containing 2.5 mM sodium butyrate, cell extracts were prepared and assayed for CAT (22) and β-galactosidase activity. CAT assay results are the averages of duplicate plates and are expressed as the percent chloramphenicol acetylated, normalized to β-galactosidase activity. Each assay was repeated several times, with a single representative assay shown in each figure.

RESULTS

BPV-1 contains a constitutive enhancer within the 5′ LCR. A constitutive enhancer activity for the BPV-1 LCR was detected in BEFs by using a plasmid (p407) containing the complete LCR cloned in $pA_{10}CAT$ (an SV40 enhancer-deleted CAT expression vector). To further this analysis, subfragments of the BPV-1 LCR were cloned as enhancers into $pA_{10}CAT$. These plasmids were transfected into early-passage BEFs, and cell extracts were assayed for CAT activity at 48 h. Figure 1 shows strong enhancer activity with p1535, which contains the 5′ half of the LCR; this fragment had about three times the enhancer activity of the full-length LCR in plasmid p407. Although some enhancer activity could be detected in the 3′ half of the LCR in this experiment (Fig. 1, p979), we focused our attention on defining the enhancer activity within the 5′ LCR fragment of p1535. Deletion mutants of the 5′ LCR were constructed, cloned into $pA_{10}CAT$, and assayed for enhancer activity in sense and antisense orientations in primary BEFs (Fig. 2). A series of BAL 31 deletions of the 5′ LCR was made with nt 7386 as the common 3′ endpoint. Although enhancer activity

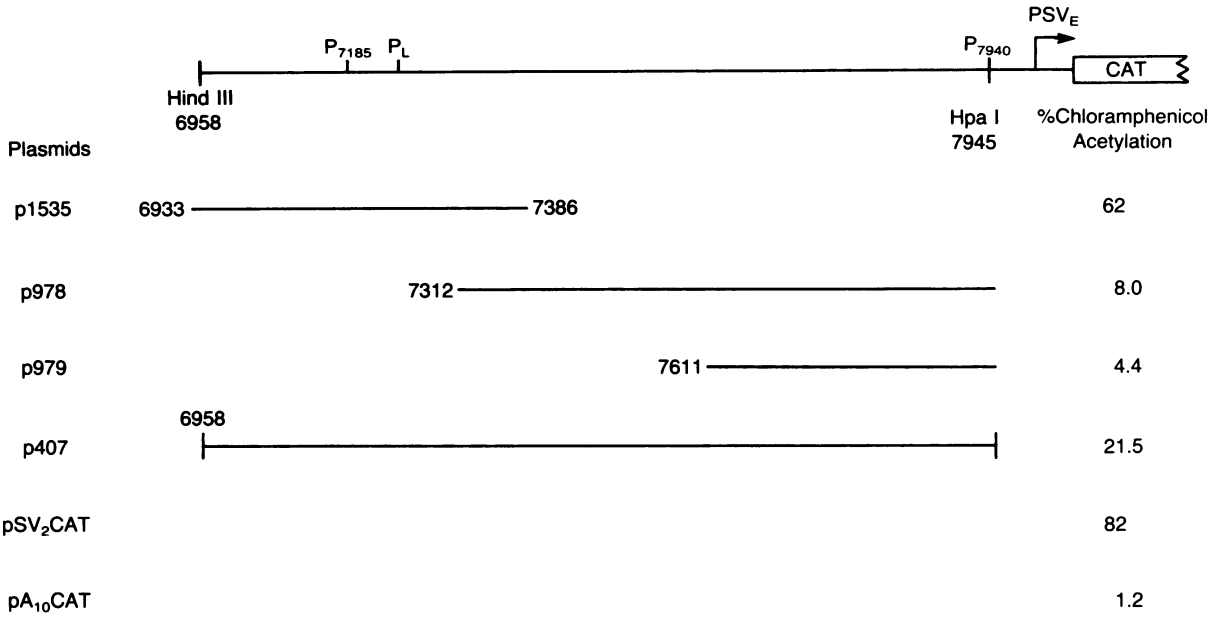

FIG. 1. Enhancer assay of BPV-1 LCR fragments. The CAT expression vector p407 (the BPV-1 LCR from *Hin*dIII 6958 to *Hpa*I 7945 cloned as an enhancer in pA$_{10}$CAT) is shown at the top. Subfragments of the BPV-1 LCR were cloned in the sense orientation into the CAT expression vector pA$_{10}$CAT and transfected into early-passage BEFs as detailed in Materials and Methods. LCR deletion endpoints are shown next to horizontal lines indicating the approximate position of the assayed fragment within the entire LCR. Data are reported as percent chloramphenicol acetylation normalized to β-galactosidase activity.

dropped with progressive deletion, the greatest drop was seen between nt 7162 and 7228, mapping a 5′ boundary to this enhancer activity at nt 7162 (Fig. 2). Deletions from the 3′ end of p1534 were constructed at nt 7275 and 7210; activity was maintained up to nt 7275 but was lost with the deletion to 7210. Thus, a strong constitutive enhancer was mapped within the 5′ LCR to between nt 7162 and 7275 (Fig. 2). This fragment was shown to have enhancer activity in

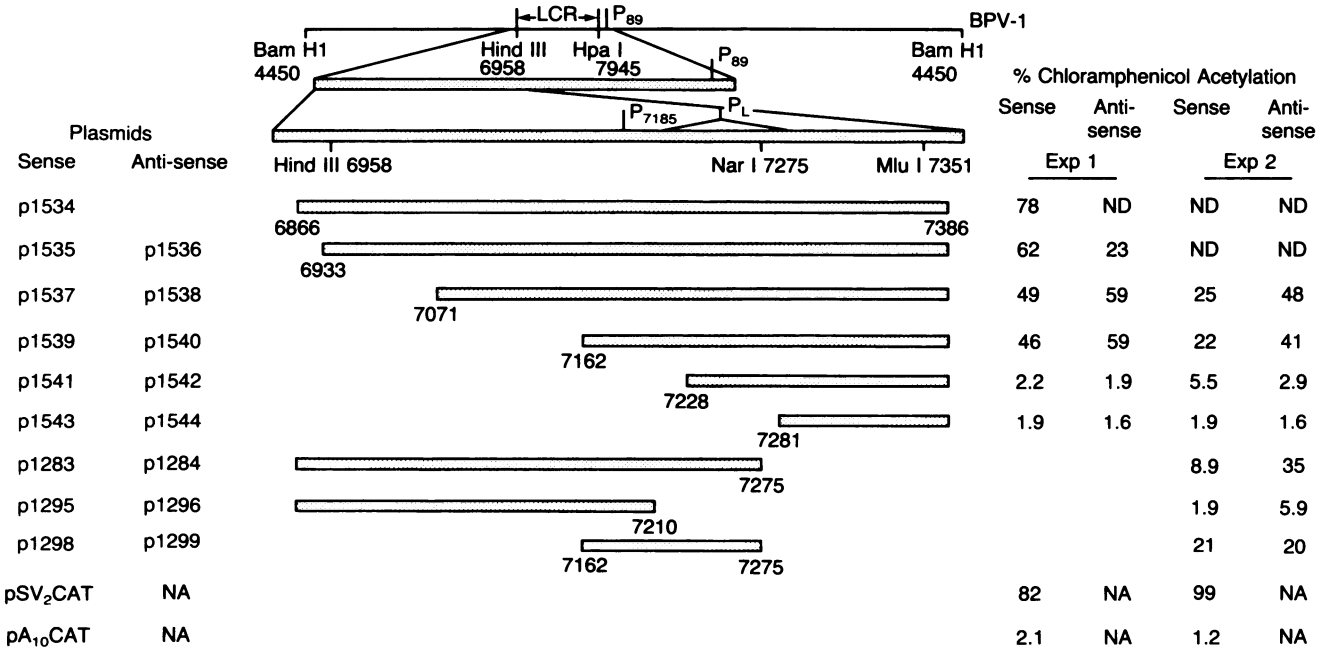

FIG. 2. Deletion mapping of constitutive enhancer activity within the LCR. The BPV-1 genome linearized at the *Bam*HI site is shown at the top along with locations of promoter P$_{89}$ and the LCR. Below is shown an expanded LCR, and below that is an expanded 5′ LCR. Deletions of the 5′ LCR were made as described in Materials and Methods. The approximate sizes and positions of the assayed fragments are indicated by a horizontal bar in the figure along with the actual deletion endpoints. Transient CAT assays were in early-passage BEFs; data are expressed as percent chloramphenicol acetylation normalized to β-galactosidase activity. ND, not done; NA, not applicable.

TABLE 1. Cell type specificity of the BPV-1 constitutive enhancer

Plasmids	% Chloramphenicol acetylation in given cell type[a]			
	BEF	Primary human HF fibroblasts	C127	CV-1
$pA_{10}CAT$	1.4 (1.0)	0.25 (1.0)	0.9 (1.0)	0.25 (1.0)
pSV_2CAT[b]	98	17	95	94
p1535	73 (52)	1.6 (6.4)	4.0 (4.4)	ND
p1537	61 (43)	ND	ND	0.76 (3.0)
p1539	15 (11)	ND	0.6 (0.66)	0.95 (3.8)
p1541	5.9 (4.2)	ND	0.83 (0.92)	0.48 (1.9)

[a] Data are normalized to β-galactosidase activity. Numbers in parentheses indicate enhancement relative to that of the enhancer-deleted control plasmid $pA_{10}CAT$. ND, Not determined.
[b] Contains the SV40 enhancer and early promoter as a positive control.

BEFs with plasmids p1298 and p1299, containing the fragment in the sense and antisense orientations, respectively (Fig. 2). Many of the plasmids tested in Fig. 2 showed different activities in sense versus antisense orientations, which was probably an effect of spacing between the enhancer and the promoter. For example, p1298 and p1299 (in which flanking sequences have been removed) and p1537 and p1538 (which have similar amounts of 5′- and 3′-flanking sequence on the minimal enhancer domain) showed no difference in activity in the sense versus antisense orientations.

The constitutive enhancer is most active in primary BEFs. Previous experiments using monkey CV-1 cells, mouse C127 cells, or several other nonbovine cell lines of fibroblast lineage had failed to reveal constitutive enhancer activity in the LCR comparable to that observed here with primary bovine cells (25, 26, 39, 50, 53). Therefore, the BPV-1 5′ LCR constitutive enhancer activity was compared in primary bovine and human fibroblasts, CV-1 cells, and C127 cells (Table 1). The enhancer was approximately 10 times more active in BEFs than in any of the other cell types tested. In preliminary experiments (not shown), this enhancer was also found to be quite active in primary bovine squamous epithelial cells. The minimal enhancer fragment in p1298 and p1299 is also inactive in CV-1 and C127 cells (data not shown). These experiments and those shown in Fig. 2 therefore defined and mapped an enhancer element in the BPV-1 genome between nt 7162 and 7275 that was principally active in bovine cells and was not dependent on viral factors in *trans*.

The constitutive enhancer does not activate the major early promoter P_{7940} or P_{89}. It had previously been demonstrated that promoters P_{89} and P_{7940} can be transactivated by the full-length E2 transactivator in CV-1 and C127 cells. We wished to determine whether the LCR constitutive enhancer activated LCR promoter P_{7940} or P_{89} in BEFs. The 5′ LCR-deleted fragments depicted in Fig. 2 were reconstructed back into the full BPV-1 LCR to assay the effect of the deletions on the expression of CAT activity from BPV-1 promoters in BEFs. There was only a slight difference in CAT activity between plasmids containing the constitutive enhancer and those deleted of the enhancer (p1268, p1269, and p1270 versus p1271) (Fig. 3). Thus, the strong constitutive enhancer mapping in the 5′ LCR apparently has little effect on the basal level of P_{89} and P_{7940} promoter activity in BEFs while strongly transactivating the heterologous SV40 early promoter (Fig. 3, p407). p1268, was strongly transactivated by E2 expressed from pC59, indicating that the P_{89} and P_{7940} promoter regions were intact and functional in this plasmid.

The constitutive enhancer is negatively regulated by the E2 repressor and is transactivated by the E2 transactivator. The deletion analysis of the constitutive enhancer presented above had mapped most of the activity between nt 7162 and 7275. This segment of the LCR contains an E2 binding motif at nt 7203 (37). To test whether E2 products or other early-region products could modulate the activity of the constitutive enhancer, LCR-CAT constructs were cotransfected with BPV-1 cDNA expression vectors that expressed various BPV-1 early-region genes. To control for nonspecific suppression of cotransfected CAT vectors by the Okayama-Berg expression vectors used to express the BPV-1 cDNAs, plasmid p1151 without a cDNA insert was used to equalize the amount of cotransfected cDNAs among all plates. Cotransfection with a plasmid that expressed the E2 repressor resulted in a six- to eightfold decrease in the BPV-1 constitutive enhancer activity (Table 2). Not all of the enhancer activity in p1534 or p1284 was eliminated by the E2 repressor: a residual enhancement of about seven- to ninefold was seen. Titration experiments (not shown) revealed that the amount of E2 repressor used in Table 2 was able to completely inhibit E2 transactivation of an E2-responsive enhancer, indicating that the residual enhancer activity seen in the presence of the E2 repressor was probably not due to insufficient expression of the repressor. cDNA vectors that expressed E6, E7, and E6/7 or only E3, E4, or E5 had no significant effect on the constitutive enhancer (data not shown). The two enhancer plasmids tested in this experiment differ in that p1534 contains two BPV-1 E2-binding sites, at nt 7203 and nt 7366, whereas p1284 has only one, at nt 7203 (also, p1284 is in the antisense orientation). Since the E2 repressor was effective on either plasmid, the repression observed was likely due to binding at the E2-binding site at nt 7203. Whereas the E2 repressor lowered constitutive enhancer activity of p1534 and p1284, the full-length E2 transactivator was capable of transactivating the enhancer (11- and 6-fold, respectively). Plasmid p1534 contains two E2-binding sites (at nt 7203 and nt 7366) and corresponds to a previously defined E2-responsive element (52). Plasmid p1284, which has only one E2-binding site (at nt 7203), was moderately (sevenfold) transactivated by E2, even after normalization for the nonspecific transactivating effects of E2 (Table 2). However, the ratio of the enhancer activity of p1284 in the presence of the transactivator versus the repressor was 56, illustrating that the constitutive enhancer could be modulated up or down over a wide range by the viral E2 transactivator or repressor proteins.

Mutational analysis of the constitutive enhancer. Since the E2 repressor was able to repress the constitutive enhancer in p1284 (Table 2), the possibility that the nt 7203 E2-binding site might overlap a binding site for a specific cellular protein was investigated. Mutations within or near the nt 7203 E2-binding site were generated and assayed for their effects on constitutive enhancer activity in BEFs. Synthetic oligodeoxynucleotides with site-directed mutations were used to reconstruct the region from BPV-1 nt 7143 to 7275 (Fig. 4). The mutated enhancer inserts were cloned into the enhancer-deleted CAT expression vector $pA_{10}CAT$ in the antisense orientation (to avoid any contribution due to promoter activity of the fragment), and the resulting mutants were transfected into early-passage BEFs. Each of the plasmids with a mutation within the nt 7203 E2-binding site resulted in lowered enhancer activity (p1390, p1420, p1391 versus p1397, and p1401 versus p1406; Fig. 4). These mutations

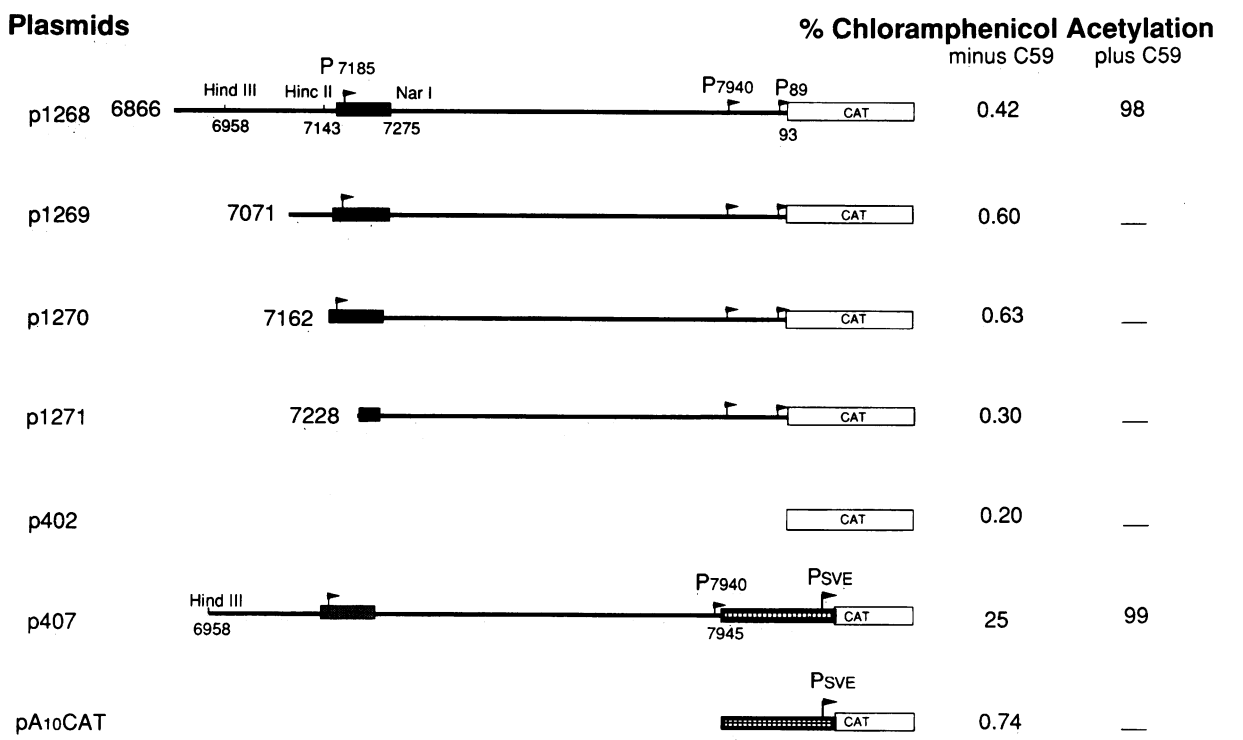

FIG. 3. The constitutive enhancer does not activate transcription from the major early promoter P_{89}. 5′ LCR BAL 31 deletions were reconstructed into the full-length LCR up to nt 93 as indicated in the figure and described in Materials and Methods. ▩, minimal BPV-1 constitutive enhancer region as defined in Fig. 2. The plasmids were transfected with or without the E2-expressing plasmid pC59 into early-passage BEFs. Plasmid p402 is a promoter-deleted CAT expression vector used as a negative control (52). $pA_{10}CAT$ and p407 contain the SV40 early promoter (▦). Data are expressed as percent chloramphenicol acetylation normalized to β-galactosidase activity. —, combinations of plasmids that were not tested.

involved both bases implicated as contact residues for E2 and as well as bases internal to the contact residues (Fig. 4) (37, 46). Just upstream of the nt 7203 E2-binding site is an inverted CCAAT motif (nt 7198 to 7201); however, mutations within this site resulted in variable alteration of enhancer activity (constructs p1377, p1383, and p1406). Within and downstream of the nt 7203 E2-binding site are two regions homologous to β-globin promoter elements (CACA motifs) (47) and SV40 enhancer elements (19). A mutation within the CACA motif internal to the E2-binding site (p1390) or a mutation within the downstream element (p1391) resulted in a slight, two- to threefold reduction in enhancer activity. A plasmid that mutated both CACA motifs (p1397) had lower enhancer activity than did plasmids containing individually mutated motifs (p1397 versus p1390 or p1391), although one caveat here is that the exact mutations differ between the double and single mutants. Thus, some mutations within and adjacent to the nt 7203 E2-binding site resulted in decreased constitutive enhancer activity. These results together with the repression of constitutive enhancer activity by the E2 repressor shown above suggested that a cellular factor(s) important for enhancer activity might bind to DNA sequences in this region.

TABLE 2. Modulation of the BPV-1 LCR constitutive enhancer by the E2 repressor

Transfected plasmid(s)[a]	Enhancer activity[b]
p1534 + p1151	46
p1534 + E2 transactivator	533
p1534 + E2 repressor	7.1
p1284 + p1151	76
p1284 + E2 transactivator	506
p1284 + E2 repressor	9.1
$pA_{10}CAT$	1.0

[a] E2 transactivator and E2 repressor were supplied by the cDNA vectors pC59 and p1565, respectively. p1151 is a control cDNA vector without an expressed insert used to equalize the amount of transfected cDNAs between plates.

[b] Expressed as fold enhancement relative to that of the enhancer-deleted plasmid $pA_{10}CAT$, normalized to cotransfected β-galactosidase activity. The actual percent chloramphenicol acetylation values for $pA_{10}CAT$ and p1534 plus E2 transactivator were 0.16 and 34%, respectively.

The constitutive enhancer contains elements necessary for P_{7185} promoter activity as well as an element unnecessary for P_{7185} activity. The region from nt 7143 to 7275 has been shown to have promoter activity in vivo in C127 and HeLa cells and to contain *cis* elements essential for P_{7185} promoter activity in vitro (55). Recently, it has been shown that promoter P_{7185} can be repressed by either the E2 repressor or the E2 transactivator in vitro (54). The bovine constitutive enhancer was therefore tested to see whether it functioned as a promoter element for P_{7185}. The mutated enhancer fragments shown in Fig. 4 were cloned into a promoter/enhancer-deleted CAT construct (p1550). This plasmid has an SV40 polyadenylation signal upstream of the promoter cloning site in order to reduce the background CAT activity of the vector (23). In this plasmid background, the wild-type nt 7143–7275 fragment had a small but reproducible promoter activity, typically three- to fivefold over background (Fig. 4, p1560 versus p1550). Most mutations that reduced

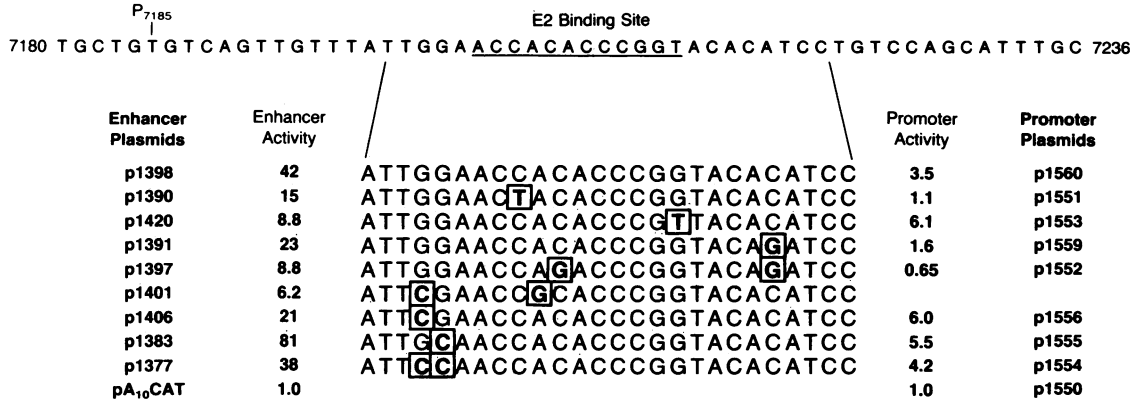

FIG. 4. Mutational analysis of enhancer and promoter activities within the 5′ LCR. The 5′ LCR from nt 7143 to 7275 was reconstructed with synthetic oligonucleotides as detailed in Materials and Methods. A portion of that sequence is shown, with the sequence of the mutants aligned below. The enhancer fragments were cloned in the antisense orientation to avoid any contribution due to promoter activity of the BPV-1 fragment. Results shown are the averages of duplicate plates normalized to β-galactosidase activity. Enhancer activity is relative to the activity of the enhancer-deleted construct $pA_{10}CAT$; actual percent chloramphenicol acetylation values for $pA_{10}CAT$ and the wild-type enhancer plasmid p1398 were 0.16 and 6.8, respectively. Promoter activity is relative to that of the promoter-deleted plasmid p1550.

enhancer activity also reduced promoter activity (p1390 versus p1551, p1391 versus p1559, p1397 versus p1552, and p1377 versus p1554). However, two mutants (p1420 and p1406) that had decreased enhancer activity had a slight increase in promoter activity (p1553 and p1556). Thus, analysis of these mutants defined an element(s) necessary for both the constitutive enhancer and promoter activities (p1551, p1559, p1397, and p1377) and an element(s) necessary for enhancer function that did not appear to be necessary for the low levels of promoter activity seen with this fragment (p1420 and p1406).

Nuclear extracts from BEFs contain a factor(s) that binds a site overlapping the nt 7203 E2-binding site. To test whether a DNA-binding protein necessary for both constitutive enhancer activity and P_{7185} activity bound at a site overlapping the nt 7203 E2-binding site, synthetic oligonucleotides from BPV-1 nt 7189 to 7227 were end labeled, incubated with an unfractionated nuclear extract, and subjected to nondenaturing gel electrophoresis to resolve DNA-protein complexes. In the presence of 500 ng of the nonspecific competitor poly(dI-dC), four retarded complexes, A1/A2 (a split pair of bands), B, C, and D, were observed with 1 ng of the labeled wild-type oligonucleotide and nuclear extract from BEFs (Fig. 5A, lane 2). Addition of increasing amounts of homologous cold competitor (Fig. 5A, lanes 3 to 5) competed for complexes A1/A2, B, and C, whereas addition of unlabeled nonhomologous competitor oligonucleotide wt. 7776-7798 competed only for complex C (oligonucleotide wt. 7776-7798 corresponds to BPV-1 nt 7776 to 7798 and includes an E2-binding site within the E2-responsive element E2RE1) (Fig. 5A, lanes 9 to 11). These data are consistent with complex C being a nonspecific retardation complex. A 100-fold molar excess of oligonucleotide wt. 1398 or wt. 7776-7798 failed to completely compete for complex D (Fig. 5A, lanes 5 and 11), suggesting that it too is either nonspecific or a very abundant factor. Additional experiments (not shown) have shown complex D to be competed for by salmon sperm DNA, indicating that it may be a nonspecific complex.

To test whether the retardation complexes A1/A2 and B were associated with the constitutive enhancer activity seen in Fig. 4, oligonucleotides containing mutations associated with the loss of enhancer activity were used in a competition binding assay. The wild-type oligonucleotide competed for the formation of the A1/A2 and B complexes (Fig. 5A, lanes 3 to 5; Fig. 5B, lanes 3 and 4). In contrast, mutated oligonucleotides mt. 1397 and mt. 1401 competed poorly for complexes A1/A2 and B, as did the unrelated oligonucleotide wt. 7776-7798 (Fig. 5). The mutation in oligonucleotide mt. 1397 is one of the mutations in the enhancer construct p1397 and promoter construct p1552, each of which had reduced activities. The mutation in oligonucleotide mt. 1401 (which is one of the mutations in enhancer construct p1401) competed only slightly at a 100-fold molar concentration. Thus, there appeared to be an association between retardation complexes A1/A2 and B and both P_{7185} activity and constitutive enhancer activity: mutations associated with loss of promoter activity also had a loss of constitutive enhancer activity and failed to compete for complexes A1/A2 and B. This defined a *cis* element necessary for both promoter and enhancer activity that we call the CE1 element.

One mutation disassociated the constitutive enhancer activity from the promoter activity measured in the CAT transfection assays. This mutation was a G-to-T change at position 7213 in constructs p1420 and p1553 (Fig. 4). An unlabeled oligonucleotide containing this mutation (mt. 1420) did compete for complexes A1/A2 and B in a gel retardation assay, as demonstrated by the fact that the wild-type oligonucleotide (Fig. 5B, lanes 3 and 4) and mutated oligonucleotide mt. 1420 (lanes 8 to 10) competed for complexes A1/A2 and B.

The mutation at nt 7213 (constructs p1420 and p1553 and competing oligonucleotide mt. 1420) may have defined a second element(s) that was necessary for constitutive enhancer activity but was apparently not necessary for promoter activity; we call this element CE2.

DNA binding by the BPV-1 E2 product and binding by the cellular CE1-binding factor(s) are mutually exclusive. Since the nt 7203 E2-binding site overlaps the CE1 cellular factor site, it seemed possible that the E2 repressor might compete for binding of the CE1 factor(s). If the CE1-binding cellular factor were necessary for promoter and enhancer activity, its displacement by the E2 repressor would explain the repression of enhancer activity observed in Table 2. Alternatively, both factors might bind at the same time, forming a new complex that would repress the function of the CE1

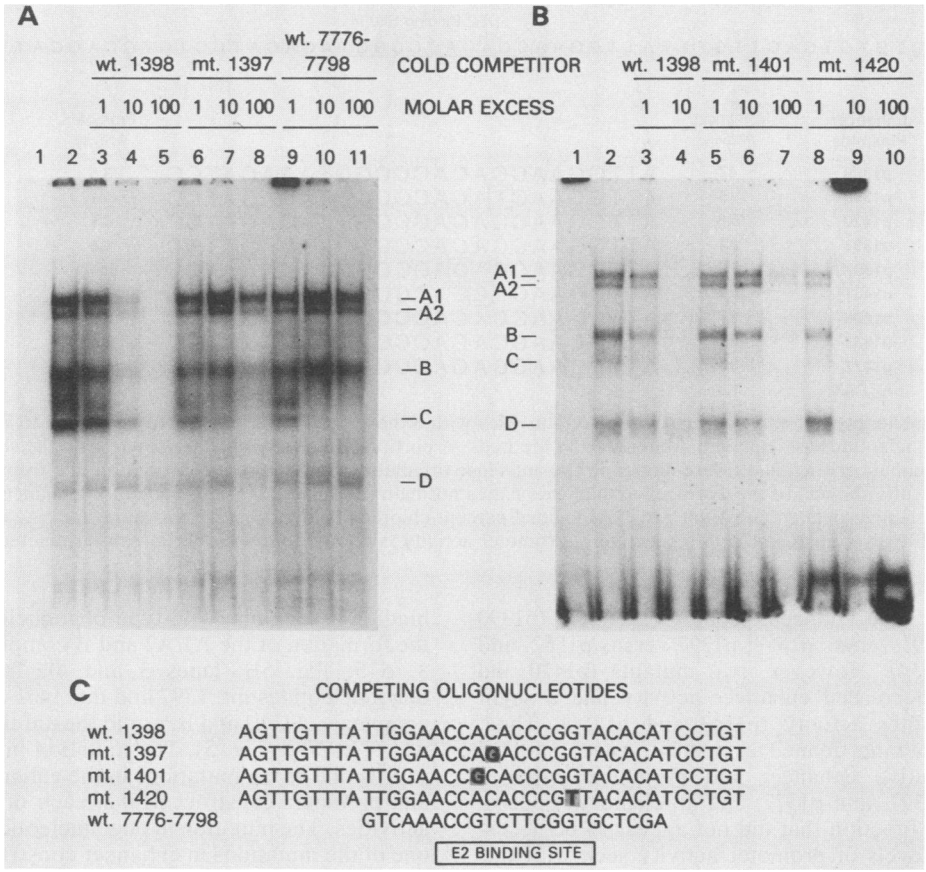

FIG. 5. (A and B) Gel retardation assay for protein-DNA complexes. Labeled wild-type sequence double-stranded oligonucleotides were incubated with BEF nuclear extract (panel A, lanes 2 to 11; panel B, lanes 2 to 10) and subjected to nondenaturing gel electrophoresis as described in Materials and Methods. Lanes 1 contained labeled oligonucleotide without nuclear extract. The identities and molar excesses of competing unlabeled oligonucleotides are shown above the lane numbers. (C) Complete sequences of unlabeled competitor oligonucleotides.

element. To distinguish between these two possibilities, labeled oligonucleotides were incubated with BEF nuclear extract and with an in vitro reticulocyte lysate-translated E2 product. The resulting protein-DNA complexes were resolved by gel retardation electrophoresis. If both E2- and the CE1-binding factor(s) bind the labeled oligonucleotide, a new gel retardation complex should be resolved from the A1/A2, B, and E2 protein-DNA complexes. Alternatively, if E2 and CE1 factor(s) binding were mutually exclusive, then the addition of increasing amounts of E2 protein would result in less of retardation complex A1/A2 or B being formed, and no new complex should be observed. With increasing amounts of in vitro-translated E2 lysate, the amounts of complexes A1/A2 and B decreased (Fig. 6, lanes 5 to 9). No new complexes resulting from both cellular factors binding together with E2 were observed. A comparison of lanes 2 and 4 in Fig. 6 revealed no significant inhibitory effect of the reticulocyte lysate on formation of retardation complex A1/A2 or B, although the nonspecific complex C became more prominent when untranslated lysate was present. These results indicated that E2 competed with the CE1 factor(s) for binding at their respective overlapping binding sites.

DISCUSSION

This study defined a strong constitutive enhancer element that has not been previously recognized and mapped it to a 113-bp fragment in the 5′ LCR of the BPV-1 genome. This enhancer was strongly active in primary bovine fibroblasts and epithelial cells but was only weakly active in primary human fibroblasts, mouse cells, or monkey kidney cells (Table 1). The E2 repressor could repress enhancer activity about seven- to eightfold, with a residual seven- to ninefold activity remaining. Clearly, additional elements in the 113-bp fragment that contribute to the constitutive enhancer activity and are not repressed by the E2 repressor remain to be defined. In contrast, the E2 transactivator caused a transactivation (7- to 11-fold; Table 2) of the constitutive enhancer. It is unlikely that the E2 transactivation observed in Table 2 is nonspecific, since the experiments were internally controlled with a cotransfected β-galactosidase expression vector, which should account for the nonspecific transactivation of the SV40 promoter by E2. The amount of transactivation observed in Table 2 was greater than that previously observed, with only one E2-binding motif (51). It is possible that the full-length E2 product transactivated the constitutive enhancer by synergistically interacting with those elements that were not repressed by the E2 repressor. The locations and identities of enhancer elements that may interact with the E2 transactivator or escape repression by the E2 repressor are currently under investigation; inspection of the BPV-1 sequence from nt 7162 to 7275 reveals potential sites for other DNA-binding proteins, including AP1 and octamer motifs. It is noteworthy that BPV-1 has

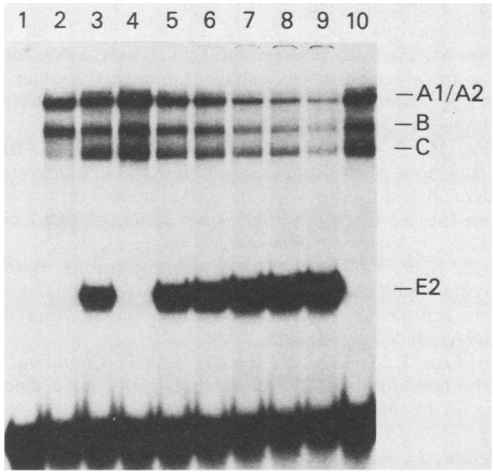

FIG. 6. In vitro-translated E2 inhibits formation of the cellular A1/A2 and B complexes. The 121-amino-acid C-terminal DNA-binding domain, termed $E2_{290-410}$, was in vitro transcribed and translated as previously described (44). Reticulocyte lysate (2 μl) was added to reaction mixtures in lanes 3 to 10, with different amounts of otherwise identical translated or mock-translated lysate added to keep the total amount constant at 2 μl per lane; 1 ng of end-labeled oligonucleotide wt. 1398 (sequence shown in Fig. 5) and 500 ng of nonspecific competitor poly(dI-dC) were added to each lane. A 2-μl sample of BEF nuclear extract was added to lanes 2 to 10. Lanes: 1, labeled oligonucleotide alone; 2, nuclear extract; 3, 0.25 μl of translated $E2_{290-410}$ plus nuclear extract; 4, mock-translated reticulocyte lysate plus nuclear extract; 5, 0.5 μl of $E2_{290-410}$ plus nuclear extract; 6, 0.75 μl of $E2_{290-410}$ plus nuclear extract; 7, 1.0 μl of $E2_{290-410}$ plus nuclear extract; 8, 1.5 μl of $E2_{290-410}$ plus nuclear extract; 9, 2 μl of $E2_{290-410}$ plus nuclear extract; 10, mock-translated lysate plus nuclear extract.

provided a mechanism for both positive and negative modulation of a constitutive enhancer with viral *trans* factors. This is in contrast to the human genital papillomaviruses, in which constitutive enhancers drive expression of the major early promoters and E2 products repress promoter activity by apparently competing or interacting with proximal promoter factors (6, 10, 11, 20, 49, 57).

Two *cis* elements within the BPV-1 constitutive enhancer, CE1 and CE2, that overlap the BPV-1 nt 7203 E2-binding site were genetically defined. The CE1 element was necessary for both enhancer and promoter activity, whereas the CE2 element appeared to be necessary only for enhancer activity. Cell factors were detected that bound the CE1 element, and this binding was competed for in vitro by the E2 DNA-binding C-terminal fragment. It is likely that the E2 transactivator would compete for binding of the CE1 factor(s) in a similar fashion. However, in vitro-translated full-length E2 transactivator has not been a satisfactory reagent for in vitro binding studies for technical reasons, since it produces unsatisfactory gel retardation assays. Unlike the case for the E2 repressor, our model would predict that the transactivator might be able to cooperatively interact with additional, not yet defined cellular factors (which specifically interact with sequence elements in the nt 7162–7275 fragment), to result in transactivation of enhancer activity above the basal constitutive enhancer level.

Other workers have previously described weak enhancer or promoter activity in the 5′ LCR of the BPV-1 genome. Campo et al. (9) found evidence of weak promoter but not enhancer activity when the 5′ LCR was used to stably express thymidine kinase in thymidine kinase-negative L cells. Sowden et al. (50) found weak enhancer activity in a 5′ LCR fragment from nt 7215 to 7391 in C127 cells; however, this does not represent the same activity studied here, since that fragment does not contain the CE1 or CE2 element. Stenlund et al. (55) demonstrated the BPV-1 nt 7143–7275 fragment to have promoter activity in vivo and in vitro. Furthermore, they showed that a HeLa cell extract produced a DNase I-resistant footprint over the nt 7203 E2-binding site. Recently, Stenlund and Botchan (54) showed that E2 repressed P_{7185} transcription in vitro and that both the E2 repressor and transactivator repressed promoter activity of the nt 7143–7275 fragment in transfections of HeLa cells. In their study, mutations that abrogated promoter activity in this fragment mapped to a similar domain as the CE1 element defined in our study, and a deletion at nt 7213 did not alter promoter activity in HeLa cells. In this study, a G-to-T mutation at nt 7213 defined the CE2 element, an element necessary for enhancer but not promoter activity (Fig. 4).

The identity of the cellular factor(s) that binds the CE1 element is not yet known, but sequences surrounding the CE1 element are homologous to those of other previously described promoter and enhancer elements. In particular, a homologous CACACCC motif (termed GT-I) and an associated DNA-binding factor (termed TEF-2) have been described in the SV40 enhancer (13, 19), and a similar sequence is present in the BPV-1 distal enhancer (39). Many β-globin promoters contain a CACA element that is developmentally regulated (47). The CACACCC motif is contained within the nt 7203 E2-binding, site and a similar sequence is repeated just downstream (Fig. 4). Mutations within the first three nucleotides of this motif are associated with loss of enhancer and promoter activity in BEFs in this study (Fig. 4) and with loss of promoter activity in HeLa cells (54). Recent experiments in this laboratory have shown that purified human transcription factor Sp1 could bind to the nt 7203 E2-binding site. Furthermore, oligonucleotides containing the same mutations within the CACA CE1 motif that result in loss of promoter and enhancer activity (Fig. 4) failed to compete for Sp1 binding to the wild-type sequence (S. Vande Pol and P. M. Howley, unpublished data). Should the CE1-binding factor prove to be Sp1, this finding would imply that the cell type specificity seen in this enhancer resides elsewhere within the 113-bp enhancer fragment (Sp1 is a ubiquitous transcription factor), although Sp1 could still be a necessary component of the enhancer and the site of E2 regulation. However, Sp1 may not be the only cellular factor binding at this site. There are at least two cellular factors that can bind to the CACA motif within the β-globin promoter (17, 30), and avian Sp1 has been shown to bind to CACA motifs in the chicken β-globin promoter (36). In addition, a cellular factor that binds to a subset of Sp1-binding sites and suppresses transcription has been cloned (31). Regulation of the constitutive enhancer and P_{7185} appears be quite complex, with competition between the viral E2 products as well as other positively or negatively acting cellular factors (possibly Sp1) binding to the CACA motif. We are currently attempting to identify additional cellular factors that may be important in regulating the constitutive enhancer and P_{7185}.

Additional work is needed to identify factors that may bind to the CE2 element. The CE2 element was defined by a mutation that resulted in loss of constitutive enhancer activity with a heterologous promoter while slightly increasing P_{7185} activity (Fig. 4). It has been assumed that the in vivo promoter activity from the *Hin*cII (nt 7143)-to-*Nar*I (nt 7275)

fragment observed in this and other studies represents promoter activity from P_{7185}. However, the low levels of RNA produced from these constructs in vivo has thus far precluded confirmation of this assumption. It is possible that the viral late promoter accounts for some of the promoter activity in these constructs or in the mutants. Little is known about *cis* or *trans* factors necessary for P_L function except that it is active only in the differentiated keratinocytes of a bovine fibropapilloma.

The role of either the constitutive enhancer or P_{7185} in the life cycle of BPV-1 infection is not known. The constitutive enhancer did not activate promoter P_{89} or P_{7940} in the absence of the E2 transactivator (Fig. 3). We are currently investigating the influence of the BPV-1 constitutive enhancer on other BPV-1 promoters. Another possible role for the constitutive enhancer may be in viral DNA replication. It is intriguing that the cell type strength of the constitutive enhancer studied here roughly correlates with the capacity of the cells to support viral DNA replication as measured by the plasmid copy number. Bovine fibroblasts support a high copy number (8; data not shown), versus an intermediate copy number in C127 cells and the lack of replication in CV-1 cells. There is evidence that *cis* elements in this region are necessary for replication and plasmid maintenance, since linker mutants through this region have been reported to be unable to replicate in transient replication assays (41). Experiments are in progress to further investigate a role for this enhancer element by constructing complete BPV-1 genomes that are defective for the constitutive enhancer and to assess its role in transformation, transcription, and DNA replication.

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