JOURNAL OF VIROLOGY, July 1987, p. 2128–2137
0022-538X/87/072128-10$02.00/0

Vol. 61, No. 7

Bovine Papillomavirus Transcriptional Regulation: Localization of the E2-Responsive Elements of the Long Control Region

BARBARA A. SPALHOLZ,* PAUL F. LAMBERT, CAROLE L. YEE, AND PETER M. HOWLEY

Laboratory of Tumor Virus Biology, National Cancer Institute, Bethesda, Maryland 20892

Received 14 January 1987/Accepted 14 March 1987

The long control region (LCR) of the bovine papillomavirus type 1 genome can function as a conditional transcriptional enhancer which can be specifically *trans*-activated by the viral E2 gene product. To precisely map the target(s) of this *trans*-activation, BAL 31 exonuclease was used to generate two overlapping series of deleted DNA segments through the LCR. These fragments were assayed for their ability to activate transcription from the enhancer-deleted simian virus 40 early promoter of $pA_{10}CAT$ in the presence or absence of the viral E2 gene product. Two different E2-responsive elements were localized within the LCR. The major target for E2 *trans*-activation (E2-responsive element 1) was mapped to a 196-base-pair fragment between nucleotides 7611 and 7806, just upstream from promoters P_{7940} and P_{89}. Further deletions which destroyed or impaired enhancer function revealed that the $ACCN_6GGT$ sequence motifs at each end of E2-responsive element 1 are critical components of this element. Primer extension analysis of RNA extracted from acute transfections with plasmids containing the bovine papillomavirus type 1 LCR driving the CAT gene revealed that each of the P_{7940} and P_{89} promoters is responsive to E2 *trans*-activation.

Papillomaviruses are a group of small DNA viruses which cause benign epithelial tumors, or warts, in a variety of animal hosts. Some of these viruses are also associated with lesions which can progress to malignant carcinomas, although the mechanistic role of the papillomaviruses in such a progression to malignancy has not yet been determined. This group of viruses has been difficult to study because of the lack of a suitable tissue culture system capable of supporting viral replication. Bovine papillomavirus type 1 (BPV-1) causes cutaneous fibropapillomas in cattle and induces fibroblastic tumors in a variety of other hosts. BPV-1 readily transforms certain rodent cells in culture (14), a property which has facilitated the study of the papillomavriuses and which may provide some insight into the role of these viruses in cancer.

Transcriptional analysis of BPV-1-transformed mouse C127 cells (ID13) and of bovine fibropapilloma tissue has led to the characterization and mapping of the major viral RNA species present in these two types of BPV-1-infected cells (2, 6, 12, 28, 30). These studies established that the viral promoters utilized in transformed cells are a subset of those present in a productively infected fibropapilloma, that there are at least five BPV-1 transcriptional promoters active in transformed cells, and that various RNA species are generated by differential splicing. Electron microscopic examination and cDNA analysis located promoters which directed the synthesis of RNAs with 5′ ends mapping at nucleotides 89, 2443, and 3080 (28, 30). Analyses by primer extension and S1 exonuclease protection of RNA extracted from ID13 cells confirmed the presence of the P_{89}, P_{2443}, and P_{3080} promoters and established the existence of two new promoters, P_{7185} and P_{7940}, in the long control region (LCR) (2). The LCR (also referred to as the URR by some groups, for upstream regulatory region) maps just upstream of the eight designated open reading frames (ORFs) of the transforming region of the viral genome and contains important regulatory elements for viral transcription and replication (18, 24, 26, 27, 29). It is believed that the three promoters located in the LCR (P_{7185}, P_{7940}, and P_{89}) give rise to the transcripts encoding the gene products of the 5′ ORFs, including E6, E7, and E1. In addition, a wart-specific promoter, P_L, has been mapped from RNA extracted from fibropapilloma tissue which generates RNA species with heterogeneous 5′ ends in the vicinity of nucleotide 7240 (2). The P_{2443} promoter directs the synthesis of an RNA which could encode the full-length E2 gene product and a spliced mRNA which might represent the E5 transcript (30). The P_{3080} promoter can be predicted to direct the synthesis of an unspliced mRNA which would encode a carboxy-terminal domain of E2; a methionine codon (AUG) which could serve to initiate translation is present in the E2 translational reading frame at nucleotide 3091.

Clues to the complex regulation of these various promoters and their gene products come from several sources. We have previously reported the existence of a transcriptional enhancer in the LCR which is specifically *trans*-activated by the full-length E2 gene product, indicating positive regulation of viral transcription by a viral gene product (27, 31). Since the LCR is immediately upstream of the ORFs of the transforming region and contains at least four different transcriptional promoters, we postulated that E2 was involved in the regulation of the expression of other viral genes in vivo (27). Indeed the pleiotropic effects of mutations in the E2 gene on transformation and on plasmid maintenance (5, 11, 19, 23, 26) are most probably a consequence of the decreased transcriptional activity of the 5′ ORFs. The presence of the E2 gene is a feature of each of the papillomaviruses sequenced, and E2 *trans*-activation of an LCR enhancer is not a peculiarity of BPV-1 since this phenomenon has been demonstrated for human papillomavirus type 16 (22). This suggests that E2-mediated transcriptional regulation is a general property of papillomaviruses.

In addition to positive factors controlling transcription, it is also likely that there are cellular or viral factors which have a negative effect on transcription. Cycloheximide treatment of BPV-1-transformed cells increases the level of transcripts from the LCR promoters, P_{7185}, P_{7940}, and P_{89}, 10- to 100-fold (2, 15). This increase in transcription could

* Corresponding author.

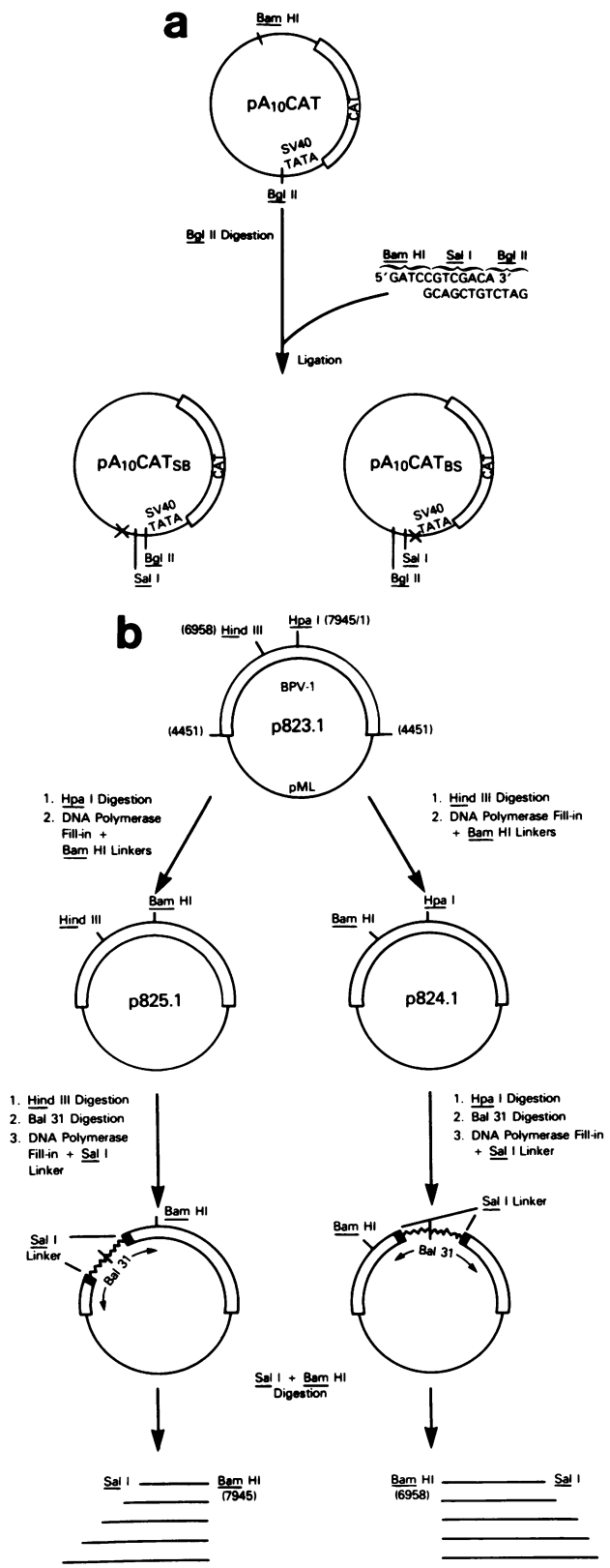


FIG. 1. (a) $pA_{10}CAT$-based vectors. A polylinker containing the recognition sites for *Bam*HI, *Sal*I, and *Bgl*II was inserted into the *Bgl*II site of $pA_{10}CAT$ to form $pA_{10}CAT_{BS}$ and $pA_{10}CAT_{SB}$. These vectors contain the CAT gene directed by the SV40 enhancer-deleted early promoter. (b) The generation of BAL 31 deletions through the LCR. BAL 31 exonuclease was used to generate deletion fragments of the LCR by digestion from either the *Hin*dIII site of p825.1 or the *Hpa*I site of p824.1. A *Sal*I linker was added to each digested endpoint, which allowed release of the fragments by digestion with *Bam*HI and *Sal*I.

indicate that a labile factor with the ability to negatively regulate the LCR promoters is not produced in the presence of cycloheximide. In addition, recent studies have indicated that BPV-1 encodes a negative transcriptional regulatory factor (P. F. Lambert, B. A. Spalholz, and P. M. Howley, Cell, in press). The regulation of papillomavirus transcription thus is apparently quite complex and appears to involve the interaction of both positive and negative factors.

In this study we extend our previous observations of the E2 *trans*-activation of the LCR enhancer function of BPV-1. We have localized two independent regions of the LCR which are responsive to E2. One of these is located in the vicinity of P_{7185} and P_L. The other region, which is the major responsive element ($E2RE_1$), is located between nucleotides 7611 and 7806. We also demonstrate that both P_{7940} and P_{89} are activated by E2 when linked to this responsive element.

MATERIALS AND METHODS

Construction of modified $pA_{10}CAT$ vectors. Bacterial transformations, recombinant screening, and nucleic acid manipulations were all performed by standard methods (20). Each strand of the partially double-stranded DNA linker depicted in Fig. 1a was separately synthesized. This linker contains a *Sal*I restriction enzyme site flanked by the complementary single-stranded sequences for a *Bam*HI site on one side and for a *Bgl*II site on the other. Ligation of this linker into a *Bgl*II or a *Bam*HI site regenerates the homologous site but not the heterologous site. This polylinker was ligated into the *Bgl*II site of $pA_{10}CAT$ (17) in either orientation to generate two plasmids, $pA_{10}CAT_{SB}$ and $pA_{10}CAT_{BS}$ (Fig. 1a). Another modified $pA_{10}CAT$ vector, p796, was generated by inserting a *Cla*I linker into the *Bgl*II site of $pA_{10}CAT$.

Generation of BAL 31 deletions within LCR. Figure 1b shows the scheme used to generate BAL 31 deletions in the LCR. Plasmid p823.1 contains the full-length BPV-1 genome cloned into the *Bam*HI site of pML2d, with each of the *Bam*HI sites having been destroyed. This plasmid contains a unique *Hin*dIII site and a unique *Hpa*I site in BPV-1 defining the ends of the LCR. A *Bam*HI linker was ligated into each of these sites independently to form p824.1 and p825.1, respectively. Plasmid p824.1 was linearized by *Hpa*I digestion, and p825.1 was linearized by *Hin*dIII digestion. Each linearized plasmid was separately digested with the exonuclease BAL 31 (Bethesda Research Laboratories, Inc.) at 30°C for 0.5 to 40 min. After digestion, the DNAs were incubated with DNA polymerase I, Klenow fragment, and deoxynucleoside triphosphates to generate flush ends before a *Sal*I linker was added to the ends by ligation with T4 DNA ligase. The array of deletion fragments for each plasmid was released by complete digestion with *Bam*HI and *Sal*I. The DNA fragments were separated by agarose gel electrophoresis, and those in the size range of 100 to 900 base pairs (bp) were isolated and purified from the agarose by electroelution. For ease in sequence analysis, the deletion fragments were initially cloned into the polylinker of M13mp18. The determination of the deletion endpoints was performed by the dideoxynucleotide chain termination method (25). The sequenced *Bam*HI-to-*Sal*I fragments were then cloned separately into both $pA_{10}CAT_{SB}$ and

$pA_{10}CAT_{BS}$ to examine both orientations of each LCR fragment. In addition to the BAL 31-generated fragments, three subgenomic restriction fragments were individually cloned into the *Cla*I site of p796, a $pA_{10}CAT$ derivative vector with the same requirements for CAT expression as $pA_{10}CAT$. These fragments were the 375-bp *Cla*I (nucleotide 6834)-to-*Hpa*II (nucleotide 7209) fragment (p797 and p801), the 156-bp *Hpa*II (nucleotides 7209 to 7365) fragment (p802), and the 123-bp *Hpa*II (nucleotides 7902 to 80) fragment (p1083).

Further fine mapping of the $E2RE_1$ was accomplished by BAL 31 digestion from the *Bam*HI site of the deletion fragment (nucleotides 7611 to 7946) derived from p825.1. The new ends were modified by the addition of a *Bgl*II linker; these new fragments were sequenced and inserted into $pA_{10}CAT_{SB}$ and $pA_{10}CAT_{BS}$ as described above.

Construction of BPV-1 LCR promoter CAT plasmids. Several approaches were used to generate CAT expression vectors directed by the BPV-1 LCR promoters. *Bgl*II linkers were inserted independently into each of the *Ava*II sites of the BPV-1 LCR in p824.1 after partial digestion with *Ava*II. Digestion of the resulting recombinant plasmids with *Bam*HI and *Bgl*II then generated fragments from the *Bam*HI at nucleotide 6958 to each of the former *Ava*II sites at nucleotides 7456, 7819, and 93. These three BPV-1 LCR fragments were cloned into the *Bgl*II site 5′ of the CAT gene of pCAT3M in the transcriptional sense orientation to generate p1066, p1067, and p1068, respectively. The plasmid pCAT3M is an enhancer- and promoter-deficient CAT vector (16).

To investigate the transcriptional activity of the P_{7940} promoter independently of the P_{89} promoter, a fragment containing the LCR from the *Hin*dIII site (nucleotide 6958) to the *Hpa*I site (nucleotide 7946/1), which was modified with *Bam*HI linkers at each end, was inserted in the sense orientation into the *Bgl*II site of pCAT3M, generating p1069.

Additional LCR-and-CAT fusion plasmids were generated from the series of BAL 31 deletion fragments from p824.1 cloned into $pA_{10}CAT_{BS}$. These plasmids contain the BPV-1 LCR from the *Hin*dIII site at nucleotide 6958 to various downstream endpoints cloned in the transcriptional sense orientation relative to the CAT gene. Digestion of these plasmids with *Hin*dIII (a unique *Hin*dIII site lies just 5′ to the CAT AUG) and *Sal*I removed the simian virus 40 (SV40) promoter sequence upstream of the CAT gene. These plasmids were religated into closed circles after filling in the ends with DNA polymerase to regenerate the *Hin*dIII site. These plasmids, designated p1074, p1073, p1072, p1071, p1070, and p1075, have BPV-1 3′ LCR endpoints at nucleotides 7865, 7680, 7655, 7507, 7444, and 7385, respectively. An additional LCR-CAT plasmid (p1076) was generated by deleting the *Hin*dIII-to-*Mlu*I fragment of p407-1, which contains the entire BPV-1 LCR cloned in the transcriptional sense orientation 5′ to the SV40 promoter in $pA_{10}CAT$ (27). This deletion removes the SV40 promoter and sequences 3′ to nucleotide 7351 of the BPV-1 LCR. LCR upstream deletions in p1066 were constructed by deleting from the *Cla*I site of the vector polylinker to the *Nar*I site (nucleotide 7274), the *Cla*I site (nucleotide 7476), and the *Sty*I site (nucleotide 7656) of the LCR to generate p1078, p1077, and p1079, respectively.

Transient expression assays. African green monkey kidney CV-1 cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). CV-1 cotransfections for CAT expression analysis were performed as previously described (27). Briefly, 5 μg of the CAT-containing plasmid and 10 μg of either high-molecular-weight salmon sperm (ss) DNA or the E2-expressing plasmid, C59 (30), were coprecipitated with calcium phosphate by the method of Graham and van der Eb (10). The cells were incubated for 4 h before a 1-min 15% glycerol shock (7). The cells were incubated in media containing 5 mM sodium butyrate (8) until harvested 48 h posttransfection. Preparation of cellular extracts by freeze-thawing and CAT assays were performed as described previously (9, 27).

RNA extraction. RNA analysis was carried out on cells similarly cotransfected, except that for each sample, a 150-cm^2 flask of CV-1 cells received 60 μg of CAT plasmid and 120 μg of either ss DNA or C59 DNA. Cotransfected CV-1 cells were harvested 48 h posttransfection. A portion (1/20) of the cell pellet was removed and assayed for CAT expression. The remainder of the cell pellet was lysed in 4 M guanidine isothiocyanate–0.5% sodium *N*-lauroylsarcosine–25 mM sodium citrate (pH 7.0)–0.1 M 2-mercaptoethanol. The high-molecular-weight DNA was sheared by several passages through an 18-gauge needle. Total RNA was purified by pelleting through a cushion of 5.7 M cesium chloride and 0.1 M EDTA, pH 7.0, by centrifugation at 30,000 rpm in an SW41 rotor for 20 h (4). The pelleted RNA was suspended in distilled water and ethanol precipitated twice in the presence of 0.3 M sodium acetate before primer extension analysis.

Primer extension analysis. A 20-base oligonucleotide primer, 5′ TTGGGATATATCAACGGTGG 3′, complementary to CAT mRNA 25 nucleotides 3′ to the first AUG of the CAT message, was synthesized. The primer was 5′ end labeled by using T4 polynucleotide kinase and [γ-^{32}P]ATP (5,000 Ci/mmol) at a 2:1 molar ratio with the DNA. Approximately 4 ng of the labeled primer was annealed with 15 μg of total RNA in the presence of 100 mM NaCl–20 mM Tris hydrochloride (pH 7.0)–0.1 mM EDTA by denaturing the mixture at 90°C for 3 min, followed by a 10-min incubation at 55°C and gradual cooling to room temperature over a period of approximately 30 min. Under these conditions, there was a substantial molar excess of oligonucleotide to specific CAT mRNA, to ensure a quantitative analysis. The oligonucleotide primer annealed to the RNA was then extended with reverse transcriptase (cloned Moloney murine leukemia virus [Bethesda Research Laboratories]) in the presence of 50 mM Tris hydrochloride (pH 7.5)–100 μg of bovine serum albumin per ml–3 mM $MgCl_2$, 75 mM KCl–50 μg of actinomycin D per ml–0.5 mM dNTPs–10 mM dithiothreitol–4,000 U of reverse transcriptase per ml for 1 h at 37°C. The reaction was stopped by the addition of EDTA (final concentration, 25 mM) followed by extraction with phenol-chloroform. The primer extension products were precipitated with ethanol, electrophoresed on an 8% polyacrylamide-7 M urea gel and analyzed by autoradiography.

RESULTS

E2 *trans*-activation mediated at RNA level. In the initial studies describing *trans*-activation mediated by the E2-conditional enhancer within the BPV-1 LCR, the activation was stated to be transcriptional on the basis of the steady-state levels of CAT RNA measured by dot-blot analysis. Confirmation that the mechanism of E2 *trans*-activation is transcriptional was carried out by primer extension analysis using a 5′ end-labeled synthetic oligonucleotide complementary to a segment of the CAT-coding sequences. Since the analysis is done in vast primer excess, the quantity of primer

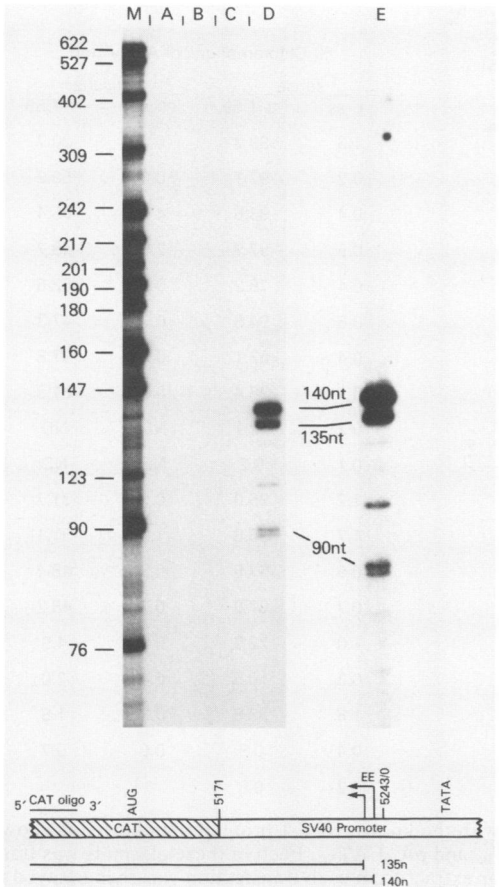
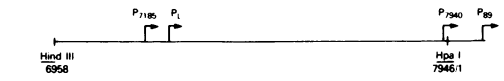


FIG. 2. Primer extension analysis from CV-1 cotransfections of (A) $pA_{10}CAT$ plus ss DNA, (B) $pA_{10}CAT$ plus C59 DNA, (C) p407-1 plus ss DNA, (D) p407-1 plus C59, and (E) pSV2CAT. Lane M contains labeled marker fragments. The diagram of the SV40 promoter linked to the CAT gene depicts the expected primer extension products for the SV40 E_e promoter start sites. The CAT activities (percent chloramphenicol acetylated in a 30-min assay) measured in parallel from cellular extracts from these transfections were 0.2, 0.8, 0.5, 62.6, and 98.7% for lanes A, B, C, D, and E, respectively. n and nt, Nucleotides.

extension products generated is directly related to the abundance of the steady-state CAT RNA present in the cell. Additionally, size analysis of the products by polyacrylamide gel electrophoresis permits one to determine whether the authentic SV40 early promoter start sites are faithfully used. RNAs generated from the SV40 early promoter by using the early early (E_e) start sites will generate primed cDNA products 135 and 140 bases long. Transfections of the enhancer-deleted plasmid $pA_{10}CAT$ (Fig. 2, lanes A and B) failed to generate any detectable CAT RNA in the presence of ssDNA or a plasmid (C59) expressing the BPV-1 E2 gene product. Lanes C and D show the primer extension products of p407-1 cotransfections in the presence of ss or C59 DNA, respectively. The plasmid, p407-1, contains the entire BPV-1 LCR upstream of the SV40 enhancer-deleted early promoter and has been shown to express CAT when transfected with the BPV-1 E2 gene product (27). Lane E shows the products of a transfection with pSV2CAT, which contains the SV40 early promoter and enhancer directing synthesis of CAT (9, 16). No CAT-specific primer extension products were seen with p407-1 in the presence of the ss DNA; however, in the presence of E2 expressed from the C59 DNA, the two major primer extension products, 135 and 140 nucleotides in length, were generated (lane D). Primer extension products shorter in length than the expected products from the major E_e start sites were seen in both p407-1 and pSV2CAT transfections and may represent additional minor RNA start sites. These data confirm that the mechanism of E2 *trans*-activation is transcriptional and manifest at the steady-state levels of RNA.

Localization of the E2-responsive elements. To localize the region(s) of the LCR which contains the E2-responsive element(s), a series of deletion mutations of the LCR was generated by digestion with BAL 31 exonuclease from either end of the LCR. The resulting fragments were cloned in either orientation 5′ to the CAT gene in the $pA_{10}CAT$-based vectors, $pA_{10}CAT_{SB}$ and $pA_{10}CAT_{BS}$ depicted in Fig. 1a. Each of the resulting plasmids was transfected into CV-1 cells with either ss DNA alone or with C59 DNA. In each case, the cellular extracts from these transfections were assayed for CAT expression 48 h posttransfection. The data are presented in Fig. 3. Normalized amounts of each extract were incubated for 30 min with chloramphenicol and acetyl coenzyme A. Although these conditions were not always in the linear range for the CAT assay, they provided the conditions to identify E2-responsive elements. In all but two cases, the LCR inserts were assayed in the antisense orientation, as well as the sense orientation. Progressive deletion of the LCR from the *Hin*dIII site at nucleotide 6958 down to nucleotide 7634 did not eliminate E2-dependent enhancer activity. The fragment from nucleotide 7611 to the *Hpa*I site (nucleotide 7946) had enhancer activity equivalent to that of the entire LCR, and its activity was independent of orientation. Deletion to nucleotide 7634 (p973) from nucleotide 7611 (p977) resulted in a significant decrease in E2-conditional enhancer activity. A 124-bp *Hpa*II fragment containing only the downstream region from nucleotides 7903 to 80 cloned into the $pA_{10}CAT$-modified vector p796 (p1083) had no enhancer activity in response to E2. The series of deletion fragments generated from the *Hpa*I site at nucleotide 7946 shows that the sequences between nucleotide 7865 and the *Hpa*I site can be eliminated without decreasing enhancer activity (Fig. 3). Further deletion of sequences to nucleotide 7680 (p1000 and p1009) did reduce the activity of the LCR enhancer (Fig. 3). Deletion to nucleotide 7386 decreased, but did not eliminate, the E2-dependent enhancer activity of the fragment. Since two nonoverlapping LCR fragments contain E2-conditional enhancer activity, it is clear that there must be at least two E2-responsive elements (E2REs) located in the LCR. From the overlapping sets of fragments which contain full E2-dependent enhancer activity, the major E2-responsive element ($E2RE_1$) could be preliminarily mapped between nucleotides 7611 and 7865.

As noted above, a second E2-responsive element ($E2RE_2$) is present further upstream in the LCR. The activity of this element can be seen in the deletions from the *Hpa*I site which eliminate the enhancer between nucleotides 7611 and 7865. Some plasmids which were deleted of $E2RE_1$ (Fig. 3) still retained substantial E2-dependent activity and expressed approximately 20-fold-higher levels of CAT in the presence of E2 than in the absence of E2. Comparison of the activities obtained from p1005 and p797 transfections (Fig. 3) suggests that the sequences sufficient for partial activity are located between nucleotides 7209 and 7386, although the upstream endpoint needs to be further defined. An *Hpa*II fragment from nucleotides 7209 to 7365 was therefore cloned into p796 and found to activate CAT expression fivefold in

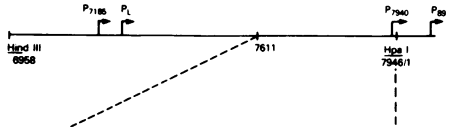


Plasmids		% Chloramphenicol Acetylated			
		sense		antisense	
sense	antisense	+ ss	+ C59	+ ss	+ C59
p407-1	p407-2	1.3	93.2	0.2	20.7
p983	p993	0.2	97.1	0.6	50.2
p982	p992	0.4	93.6	4.4	85.4
p981	p991	0.5	97.7	2.5	48.7
p978	p988	0.4	78.2	0.6	62.6
p979	p989	0.6	94.5	0.7	47.3
p975	p976	0.4	62.1	0.2	37.8
p977	p987	0.3	96.4	0.3	81.3
p973		0.2	42.1	ND	ND
p1083		0.1	0.2	ND	ND
p998	p1007	1.2	95.0	0.5	31.7
p997	p1006	1.7	96.8	1.0	51.1
p1000	p1009	0.9	57.5	0.8	48.4
p1001	p1010	0.7	67.0	0.7	48.2
p1002	p1011	1.0	22.7	0.7	4.9
p1004	p1030	0.3	16.5	0.4	2.0
p1005	p1031	1.8	38.8	0.3	1.8
p797	p801	0.4	0.9	0.6	0.7
$pA_{10}CAT$		0.2	0.8		

FIG. 3. Localization of the E2-responsive elements. A diagram of the LCR with the known BPV-1 promoters indicated is shown at the top for reference. Each of the fragments depicted was cloned into both $pA_{10}CAT_{BS}$ and $pA_{10}CAT_{SB}$. Each of these plasmids was transfected into CV-1 cells with either ss DNA or C59 DNA. A normalized amount of cellular extract from each transfection was then assayed for CAT activity (percent chloramphenicol acetylated in a 30-min assay). The data represent the average of three experiments. The sequenced endpoint for each BAL 31 deletion fragment is indicated.

the presence of E2 (p802; data not shown). In contrast to $E2RE_1$, $E2RE_2$ had significant E2-dependent activity only in the transcriptional sense orientation (Fig. 3, plasmids p1002, p1011, p1004, p1030, p1005, and p1031).

To further define the minimal sequences comprising $E2RE_1$, the deletion fragment from nucleotides 7611 to 7945 was digested with BAL 31 exonuclease from the right-hand side (nucleotide 7945) to generate the fragments depicted in Fig. 4. Each of these fragments was cloned into $pA_{10}CAT_{SB}$ and into $pA_{10}CAT_{BS}$, and the resulting recombinants were assayed for CAT expression in cotransfections of CV-1 cells as above. The plasmids p964 and p968 had full activity in the presence of E2, mapping $E2RE_1$ to between nucleotides 7611 and 7806 (Fig. 4). Further deletion to nucleotide 7769 reduced enhancer activity by at least 50%; deletion to nucleotide 7762 virtually eliminated the ability of this fragment to respond to E2. We therefore localized $E2RE_1$, as assayed by its ability to activate the SV40 enhancer-deleted early pro-



Plasmids		% Chloramphenicol Acetylated			
		sense		antisense	
sense	antisense	+ ss	+ C59	+ ss	+ C59
p977	p987	0.3	96.4	0.3	81.3
p963	p967	0.4	85.3	0.3	58.8
p964	p968	0.8	95.8	0.7	96.1
p965	p969	0.7	48.6	0.2	63.8
p966	p970	0.5	2.7	0.2	2.7
$pA_{10}CAT$		0.5	1.1		

FIG. 4. Fine mapping of $E2RE_1$. The BPV-1 LCR fragment from nucleotides 7611 to 7946 was progressively digested with BAL 31 exonuclease from nucleotide 7946 to generate the indicated fragments. A diagram of the LCR is shown above as a reference. Each of the new endpoints was determined by sequencing, and the fragments were cloned into the $pA_{10}CAT$-based vectors. Each of these plasmids was transfected into CV-1 cells in the presence of ss DNA or C59 DNA and was then assayed for CAT expression (percent chloramphenicol acetylated in a 30-min assay).

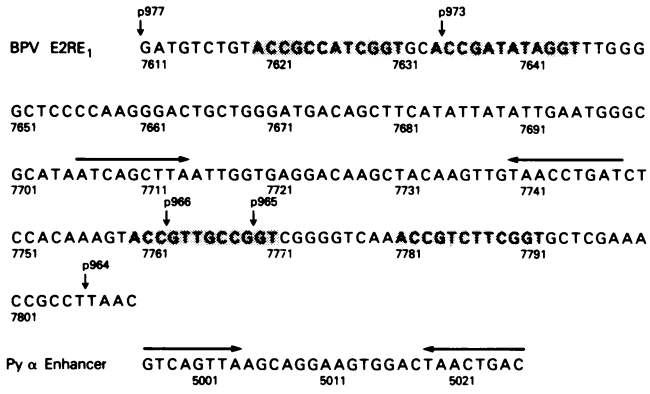


FIG. 5. The sequence of the BPV-1 E2RE$_1$. The locations of the endpoints of BAL 31 deletion fragments for some of the plasmids are indicated by vertical arrows. The sequences shown in shading represent the ACCN$_6$GGT motif conserved among the papillomavirus genomes. An additional C at nucleotide 7761 was confirmed during sequence analysis and is included in this figure. The horizontal arrows indicate an inverted repeat in BPV-1 E2RE$_1$ which is very similar to an inverted repeat in the polyomavirus alpha enhancer. The sequence of this region in the polyomavirus genome is shown below for comparison.

moter between nucleotides 7611 and 7806. To assess whether the mechanism of activation of E2RE$_1$ was identical to that of the full LCR fragment, we analyzed the RNA from cotransfections of CV-1 cells with pA$_{10}$CAT-based plasmids containing only E2RE$_1$ as an enhancer by primer extension analysis with the CAT oligonucleotide primer described above. Cotransfections of p977, p963, and p964 with C59 expressing E2 resulted in a marked increase in the levels of CAT mRNA identical to that shown for p407-1 in Fig. 2 (data not shown); the increase in steady-state levels of CAT mRNA was therefore due to the activation of the SV40 early promoter utilizing the E$_e$ start sites.

The sequence of the E2RE$_1$ (nucleotides 7611 to 7806) is shown in Fig. 5. An additional C in the BPV-1 LCR at nucleotide 7761 was noted during sequencing of the deletion fragments which contain this region. This correction of the published sequence (3) adds an additional base to the sequence of the BPV-1 genome. The location of various BAL 31 deletion endpoints are marked in this figure. Comparison of the location of each of the deletion fragment endpoints with the CAT expression levels obtained with each deletion fragment (Fig. 3 and 4) suggests that the ACCN$_6$GGT sequence motifs are a critical component of this conditional enhancer. In each of the LCR fragments in p965 and p969, one complete motif, as well as some flanking sequences, has been removed, resulting in a significant reduction in enhancer activity. A deletion which extended an additional seven nucleotides into the second motif (p966 and p970) effectively eliminated the E2-dependent enhancer function. Thus the presence of the ACCN$_6$GGT motifs alone in the LCR fragment was not sufficient for E2-dependent enhancer activity, since p966 and p970 still contained the two upstream motifs, yet were unresponsive to E2-mediated *trans*-activation. Deletion of the first of the upstream motifs (p973) as shown in Fig. 3 also reduced the E2-dependent enhancer activity of the LCR. As noted below, in experiments assessing the BPV-1 promoter response to E2 *trans*-activation, a deletion in E2RE$_1$ to nucleotide 7656 which deleted both upstream ACCN$_6$GGT motifs virtually eliminated the activity of the fragment. Thus, the paired motifs at each end of E2RE$_1$ appear to be required for the full E2-dependent enhancer activity. Deletion of one motif at either end results in a significant decrease in activity. Deletion of both motifs at either end eliminates the activity.

In addition to these motifs, this sequence also contains an inverted sequence repeat which is very homologous to an inverted repeat in the alpha enhancer of polyomavirus (13). The sequence of this region in polyomavirus is shown at the bottom of Fig. 5 for comparison. The presence of this sequence in the BPV-1 E2RE$_1$ suggests that factors other than E2, presumably cellular factors, are likely to be involved in its activity.

Analysis of BPV-1 promoters responsive to E2. To determine whether the BPV-1 promoters downstream of E2RE$_1$ (i.e., P$_{7940}$ and P$_{89}$) are regulated by the *trans*-activation of the E2-responsive elements mapped above, the LCR was tested for its ability to directly promote CAT expression, independent of the SV40 promoter. For these experiments, the series of plasmids containing the BPV-1 LCR driving the CAT gene shown in Fig. 6 was constructed. To generate each of these plasmids, either the SV40 promoter was removed from the original plasmid or an LCR fragment was inserted into a promoter-deleted CAT vector as described in Materials and Methods. In the schematic diagram at the top of Fig. 6, the positions of the LCR promoters (2) and the E2-responsive elements are noted. None of the plasmids expressed levels of CAT substantially above the background (pCAT3M) in CV-1 cells in the absence of E2. However, plasmids containing the downstream sequences of the LCR, including E2RE$_1$ expressed measurable levels of CAT in the presence of E2. Although the levels of CAT expression from plasmids with different BPV-1 sequences 5′ to the CAT gene do not necessarily reflect an accurate measure of promoter strength, expression of CAT per se is reflective of an active promoter. Plasmids p1066 and p1069, which contained E2RE$_1$ and at least one of the downstream LCR promoters, expressed CAT at a level 20- to 100-fold above the background in the presence of E2. The plasmid p1069 contains only the P$_{7940}$ promoter, suggesting that this promoter is responsive to E2. Both the P$_{89}$ and P$_{7940}$ promoters are present in p1066 so analysis of the 5′ ends of the CAT mRNA from this plasmid was necessary to determine whether P$_{89}$ was independently responsive to E2 (see below).

The upstream region of the LCR contains E2RE$_2$, the P$_{7185}$ promoter, which is active at low levels in BPV-1 transformed cells, and the late promoter, P$_L$, which is wart specific and directs the synthesis of RNAs with 5′ ends mapping heterogeneously between nucleotides 7210 and 7250 (2). Thus, as assayed by CAT expression in CV-1 cells, no evidence of E2 stimulation of these two promoters was noted (Fig. 6). To determine whether E2RE$_1$ is sufficient for E2 activation of the downstream promoters, P$_{7940}$ and P$_{89}$, deletions of sequences upstream of the *Nar*I site at nucleotide 7274 (p1078), the *Cla*I site at nucleotide 7476 (p1077), and the *Fok*I site at nucleotide 7610 (p1080) were made. Each of these plasmids was still able to express CAT in the presence of E2, indicating that E2RE$_2$ is not required for activation of the downstream promoters. The level of expression was slightly lower for p1078 than for the parent plasmid, p1066, suggesting that E2RE$_2$ might be important for full expression of the downstream promoters in the context of the entire LCR. An additional 5′ LCR deletion to the *Sty*I site at nucleotide 7656 (p1079) differs from p1080 by a deletion of 35 nucleotides, which includes the two upstream ACCN$_6$GGT motifs of E2RE$_1$. This deletion reduced CAT expression to near background levels indicating that the sequences be-

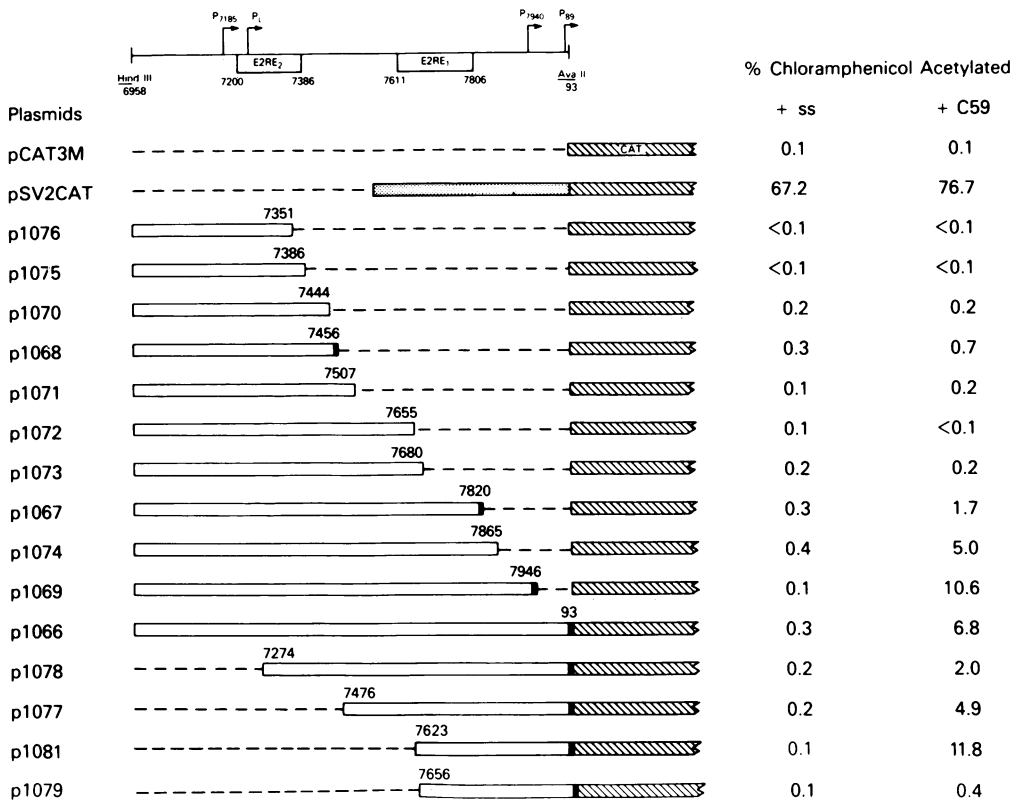


FIG. 6. The BPV-1 LCR or fragments of the LCR were assayed for their ability to promote the expression of CAT directly. Portions of the BPV-1 LCR (□) were cloned, as described in Materials and Methods, 5′ to the CAT gene (a linker indicated by ■), and these plasmids were then assayed for CAT expression with or without C59 DNA. CAT activity is expressed as the percent chloramphenicol acetylated in a 30-min assay. The plasmid pCAT3M is an enhancer- and promoter-deleted CAT vector, and pSV2CAT is a CAT expression vector containing both the SV40 enhancer and promoter (indicated by ▨). The locations of the LCR promoters and the E2-responsive elements are shown above for reference.

tween nucleotides 7611 and 7656 containing two AC CN_6GGT motifs are involved in the $E2RE_1$-mediated activation of the P_{7940} and P_{89} promoters (see below).

BPV-1 promoters P_{7940} and P_{89} activated in response to E2. To determine whether the increased CAT expression in p1066 in the presence of E2 was due to an increase in CAT RNA and to determine the 5′ ends of the RNAs, primer extension of the RNAs was done with the CAT oligonucleotide described above. RNA from CV-1 cells transfected with the promoter-deficient CAT vector, pCAT3M, in the absence or presence of C59 DNA, respectively, expressed no detectable CAT RNA (Fig. 7, lanes A and B). RNA from cells transfected with p1066 in the absence or presence of C59 was also analyzed, and the oligonucleotide-primed cDNAs are presented in lanes C and D. A diagram of the LCR is shown below the autoradiograph indicating the predicted sizes of the primed cDNAs for each of the two viral promoters mapped to this region of the LCR. No CAT RNA was found in the cells transfected with p1066 alone (lane C). In the presence of the expressed E2, however, both P_{7940} and P_{89} were active in p1066. The prominent band at 185 nucleotides corresponds to P_{7940}, and the band at 88 nucleotides corresponds to a start site for the CAT RNA at the P_{89} promoter. Thus, both downstream promoters are responsive to E2 *trans*-activation mediated by $E2RE_1$. A third prominent primer extension product was also noted at approximately 155 nucleotides and, if unspliced, would correspond to a start site at nucleotide 25 within BPV-1. Analysis of RNAs from transformed cells and from productively infected cells has not identified a transcriptional start site at nucleotide 25; thus, the physiologic significance of this RNA species is unclear.

Additional analysis was also performed on RNA extracted from CV-1 cells transfected with p1069 which contain the P_{7940} promoter, but not the P_{89} promoter. A prominent primer extension product corresponding to a start site at P_{7940} was present in the analysis of RNAs in cells cotransfected with C59 expressing E2, but not in cells cotransfected with ss DNA (data not shown). Primer extension analysis of RNA extracted from p1077 transfections in the presence or absence of C59 DNA showed no appreciable difference in comparison with the p1066 cotransfections (data not shown). Thus sequences upstream of the *Cla*I site at nucleotide 7476 are not strictly required for the E2-mediated *trans*-activation of the two downstream promoters through $E2RE_1$.

DISCUSSION

The studies described in this paper were undertaken to further an understanding of the regulation of papillomavirus gene expression. We have previously shown that the E2 gene product of BPV-1 is a transcriptional *trans*-activator and that the target of this *trans*-activation mapped within the 1-kilobase *Hin*dIII-to-*Hpa*I LCR fragment (27). This LCR element has properties of a classical enhancer, in that it activates gene expression from the enhancer-deleted SV40

promoter in an orientation- and position-independent manner. In this paper we have confirmed that the *trans*-activation is mediated at the level of transcription by direct measurement of the levels of CAT RNA by quantitative primer extension analysis. The increased gene expression of plasmids containing the LCR in the presence of the E2 gene product is due to an increase in the steady-state level of mRNA. Since the target for this *trans*-activation is not represented in the RNA transcript, and since the activation is independent of the orientation and position of the element, we interpret the data to indicate that the mechanism of E2 *trans*-activation is through an increase in the rate of transcription, rather than through message stability. The generality of E2 *trans*-activation among the papillomaviruses has recently become apparent. Several other papillomaviruses have now been shown to contain enhancers in presumed control regions corresponding to the BPV-1 LCR (22; H. Hirochika, T. Broker, and L. Chow, personal communication). These elements are conditional enhancers, in that they can be specifically *trans*-activated by either their homologous E2 gene product or the BPV-1 E2 gene product. In our laboratory, the human papillomavirus type 16 LCR enhancer has been analyzed and shown to respond to either the human papillomavirus type 16 or BPV-1 E2 gene product (22). The fact that different papillomaviruses contain this similar viral control mechanism suggests that it must play a critical role in transcriptional regulation during the life cycle of the virus.

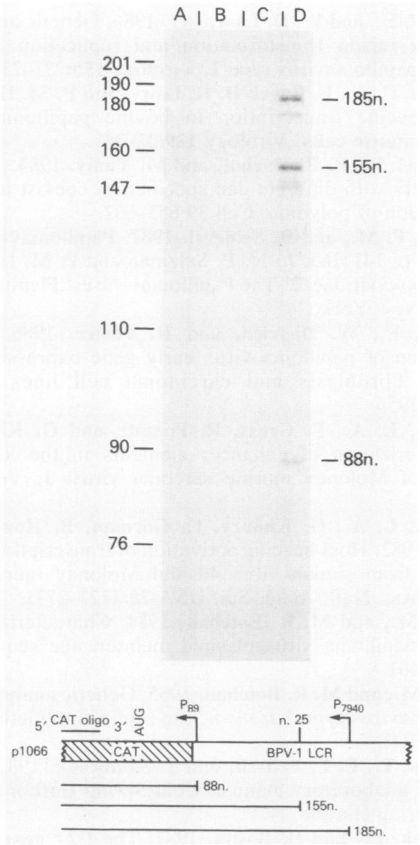


FIG. 7. Primer extension analysis of the CAT RNA extracted from CV-1 cells after cotransfection: (A) pCAT3M plus ss DNA, (B) pCAT3M plus C59 DNA, (C) p1066 plus ss DNA, and (D) p1066 plus C59 DNA. The positions of marker band lengths are shown at the left, and the calculated lengths of prominent cDNAs generated by this primer extension analysis are shown at the right of the autoradiograph. A diagram of p1066 is shown below which locates the positions of the primer extension products relative to the known BPV-1 promoters. The 88-nucleotide (88n.) and 185-nucleotide (185n.) products correspond to the major 5′ ends for P_{89} and P_{7940}, respectively. The 155-nucleotide (155n.) product does not correspond with the 5′ end of any BPV-1 promoter yet described.

The data presented in Fig. 3 and 4 clearly show the existence of a strong conditional enhancer near the 3′ end of the LCR. We have localized this enhancer to between nucleotides 7611 and 7806 and have designated this region $E2RE_1$. The deletion analysis also revealed the presence of a second E2-responsive region in the vicinity of the late promoter and the P_{7185} promoter. This activity maps between nucleotides 7200 and 7386 and is designated $E2RE_2$. The activity of this element differs from that of $E2RE_1$, in that it is stronger in one orientation than the other, and it is severalfold lower in activity. We have not studied this element in great detail, but it is possible that it plays some role in the regulation of the P_L or of the P_{7185} promoter. Additional variability was noted for the basal and the E2-*trans*-activated enhancer levels of the different LCR fragments analyzed, suggesting that *cis* elements are located within this region which are also involved in the transcriptional regulation.

The anatomy of $E2RE_1$ reveals several features which may be critical to its function in transcriptional activation. At each end of this element are two repeated motifs, $ACCN_6GGT$. Their presence correlated well with the ability of the fragment to function as an E2-dependent enhancer. Deletion of one complete copy on either side (p965, p969, or p973) reduced the enhancer activity of this element at least 50%. Deletion of both copies on the downstream side of the enhancer (p966 and p970) correlated with the inability of this fragment to activate transcription even though the two upstream motifs were still present. Similarly, deletion of both of the upstream motifs in p1079 eliminated the ability of $E2RE_1$ to activate the authentic BPV-1 promoters, P_{7940} and P_{89}, in the presence of E2 (Fig. 6). The observation that the E2-dependent activation of transcription from $E2RE_1$ appeared to require at least one (but preferably two) motifs at each end of the element suggests that some cooperativity between the two ends of the element is involved in the activation mechanism. One obvious possibility is that these motifs are the binding site for a multimeric protein which binds both ends of this element at the same time.

Recently several groups have expressed the E2 gene product in bacteria (1, 21) and in mammalian cell extracts (A. McBride and P. M. Howley, manuscript in preparation). In vitro studies have shown these E2 proteins can bind DNA and can specifically bind to fragments of the BPV-1 genome containing $E2RE_1$. The fragment with the highest affinity for E2 is that which contains $E2RE_1$ (1, 21). Most recently it has been shown that synthetic oligonucleotides containing the $ACCN_6GGT$ motif can specifically compete for the E2 binding of the BPV-1 $E2RE_1$ sequences (1; McBride and Howley, in preparation). Given that the presence and specific location of the $ACCN_6GGT$ motifs in $E2RE_1$ correlate with the E2-dependent activation of transcription and that E2 has been shown to bind these sequences directly in vitro, we postulate that direct E2 binding to the $ACCN_6GGT$ motifs in $E2RE_1$ is critical for the transcriptional activation of the BPV-1 P_{7940} and P_{89} promoters. On the basis of the twofold symmetry of the double-stranded $ACCN_6GGT$ motif, it is possible that E2 exists in a multimeric form when binding to the motif. We can also hypothesize that there is interaction between complexes of the E2 multimeric protein

binding at the different $ACCN_6GGT$ motifs. This interaction could involve direct binding through domains of the E2 protein other than the DNA-binding domain or could be indirect through association with cellular factors. Through such interactions, the E2 protein binding to the motifs at each end of $E2RE_1$ might alter the tertiary structure of this regulatory element into a configuration which may then allow the interaction of other transcription factors.

The possibility of an interaction of $E2RE_1$ with cellular transcription factors, in addition to the viral E2 protein, is supported by the presence of inverted sequence repeats in the middle of $E2RE_1$ which are quite similar to repeats in the alpha enhancer of polyomavirus. These sequences might serve as binding sites for other viral or cellular transcription factors. Mutational analysis of $E2RE_1$ will be necessary to identify other critical *cis* elements. It will be of interest to determine whether E2 binding to sequences in $E2RE_1$ may play a role in determining the availability of such sites for interaction with cellular factors.

We have not done extensive mapping of $E2RE_2$ to determine whether the $ACCN_6GGT$ motifs are essential for the E2 response of this element, but two differences between p1005 (Fig. 3) and p802 (a plasmid containing the *Hpa*II fragment from nucleotides 7209 to 7365 cloned into p796) are the loss of sequences between nucleotides 7365 and 7386 which contains one copy of this motif and the interruption of a second motif (nucleotides 7200 to 7211) 150 nucleotides upstream. This fragment is fourfold less active in response to E2 than the fragment in p1005, suggesting that these motifs are also important elements in the E2-mediated response of $E2RE_2$. We do not know yet what role $E2RE_2$ plays in the control of BPV-1 transcription or if the presence of the putative E2 binding sites near P_{2443} and P_{3080} is important in the control of viral transcription; however, these studies are currently in progress.

E2 *trans*-activation is likely to be important in controlling the expression of the genes of the transforming region. The fact that E2 is apparently required for the activation of the P_{7940} and P_{89} promoters would explain the pleiotropic effects of mutations in the E2 gene (5, 11, 23, 26) since genes involved in replication, plasmid maintenance, and transformation are under control of the LCR promoters. In a model temporally ordering the events in the life cycle of BPV-1, the expression of the full-length E2 ORF would occur early in infection. The expression of E2 would then lead to the activation of P_{89} and P_{7940}, leading in turn to the expression of the products of the 5′ ORFs of the transforming region. Studies of the temporal expression of the known BPV-1 promoters to explore this model are currently in progress.

ACKNOWLEDGMENTS

We thank C. Baker and W. Phelps for critical review of this manuscript.

This investigation was supported in part by Public Health Service grants CA 07557 to B.A.S. and CA 07715 to P.F.L. from the National Cancer Institute. This work was also supported in part by grant 1760 from the Council for Tobacco Research.

LITERATURE CITED

1. **Androphy, E. J., D. R. Lowy, and J. T. Schiller.** 1987. A peptide encoded by the bovine papillomavirus E2 *trans*-acting gene binds to specific sites in papillomavirus DNA. Nature (London) **325**:70–73.
2. **Baker, C. C., and P. M. Howley.** 1987. Differential promoter utilization by the bovine papillomavirus in transformed cells and productively infected wart tissue. EMBO J. **6**:1027–1035.
3. **Chen, E. Y., P. M. Howley, A. D. Levinson, and P. H. Seeburg.** 1982. The primary structure and genetic organization of the bovine papillomavirus type 1 genome. Nature (London) **299**:529–534.
4. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**: 5294–5499.
5. **DiMaio, D.** 1986. Nonsense mutation in open reading frame E2 of bovine papillomavirus DNA. J. Virol. **57**:475–480.
6. **Engel, L. W., C. A. Heilman, and P. M. Howley.** 1983. Transcriptional organization of bovine papillomavirus type 1. J. Virol. **47**:516–528.
7. **Frost, E., and J. Williams.** 1978. Mapping temperature-sensitive and host-range mutations of adenovirus type 5 by marker rescue. Virology **91**:39–50.
8. **Gorman, C. M., B. H. Howard, and R. Reeves.** 1983. Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate. Nucleic Acids Res. **11**:7631–7648.
9. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
10. **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.
11. **Groff, D. E., and W. D. Lancaster.** 1986. Genetic analysis of the 3′ early region transformation and replication functions of bovine papillomavirus type 1. Virology **150**:221–230.
12. **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.
13. **Herbomel, P., B. Bourachot, and M. Yaniv.** 1984. Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. Cell **39**:653–662.
14. **Howley, P. M., and R. Schlegel.** 1987. Papillomavirus transformation, p. 141–163. *In* N. P. Salzman and P. M. Howley (ed.), The Papovaviridae. 2. The Papillomaviruses. Plenum Publishing Corp., New York.
15. **Kleiner, E., W. Dietrich, and H. Pfister.** 1986. Differential regulation of papilloma virus early gene expression in transformed fibroblasts and carcinoma cell lines. EMBO J. **6**:1945–1950.
16. **Laimins, L. A., P. Gruss, R. Pozzati, and G. Khoury.** 1984. Characterization of enhancer elements in the long terminal repeat of Moloney murine sarcoma virus. J. Virol. **49**:183–189.
17. **Laimins, L. A., G. Khoury, C. Gorman, B. Howard, and P. Gruss.** 1982. Host-specific activation of transcription by tandem repeats from simian virus 40 and Moloney murine sarcoma virus. Proc. Natl. Acad. Sci. USA **78**:2727–2731.
18. **Lusky, M., and M. R. Botchan.** 1984. Characterization of the bovine papilloma virus plasmid maintenance sequences. Cell **36**:391–401.
19. **Lusky, M., and M. R. Botchan.** 1985. Genetic analysis of bovine papillomavirus type 1 *trans*-acting replication factors. J. Virol. **53**:955–965.
20. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Moskaluk, C., and D. Bastia.** 1987. The E2 "gene" of bovine papilloma virus encodes an enhancer-binding protein. Proc. Natl. Acad. Sci. USA **84**:1215–1218.
22. **Phelps, W. C., and P. M. Howley.** 1987. Transcriptional transactivation by the human papillomavirus type 16 E2 gene product. J. Virol. **61**:1630–1638.
23. **Rabson, M. S., C. Yee, Y.-C. Yang, and P. M. Howley.** 1986. Bovine papillomavirus type 1 3′ early region transformation and plasmid maintenance functions. J. Virol. **60**:626–634.
24. **Rösl, F., W. Waldeck, and G. Sauer.** 1983. Isolation of episomal bovine papillomavirus chromatin and identification of a DNase I-hypersensitive region. J. Virol. **46**:567–574.
25. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequenc-

ing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
26. **Sarver, N., M. S. Rabson, Y.-C. Yang, J. C. Byrne, and P. M. Howley.** 1984. Localization and analysis of bovine papillomavirus type 1 transforming functions. J. Virol. **52**:377–388.
27. **Spalholz, B. A., Y. C. Yang, and P. M. Howley.** 1985. Transactivation of a bovine papilloma virus transcriptional regulatory element by the E2 gene product. Cell **42**:183–191.
28. **Stenlund, A., J. Zabielski, H. Ahola, J. Moreno-Lopez, and U. Pettersson.** 1985. Messenger RNAs from the transforming region of bovine papilloma virus type 1. J. Mol. Biol. **182**:541–554.
29. **Waldeck, S., F. Rösl, and H. Zentgraf.** 1984. Origin of replication in episomal bovine papilloma virus type 1 DNA isolated from transformed cells. EMBO J. **3**:2173–2178.
30. **Yang, Y.-C., H. Okayama, and P. M. Howley.** 1985. Bovine papillomavirus contains multiple transforming genes. Proc. Natl. Acad. Sci. USA **82**:1030–1034.
31. **Yang, Y.-C., B. A. Spalholz, M. S. Rabson, and P. M. Howley.** 1985. Dissociation of transforming and *trans*-activation functions for bovine papillomavirus type 1. Nature (London) **318**:575–577.