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Evidence for Cooperativity between E2 Binding Sites in E2 *trans*-Regulation of Bovine Papillomavirus Type 1

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The long control region of bovine papillomavirus type 1 (BPV-1) can function in an orientation- and position-independent manner as an E2-dependent enhancer. Dissection of the long control region has revealed two E2-responsive elements, $E2RE_1$ and $E2RE_2$, which map, respectively, between nucleotides 7611 and 7806 and between nucleotides 7200 and 7386 of the BPV-1 genome. In this study, we have carried out a detailed analysis of $E2RE_1$, which has previously been shown to be involved in the regulation of the BPV-1 promoters P_{89} and P_{7940}. One characteristic of $E2RE_1$ is the presence of a pair of $ACCN_6GGT$ motifs (E2 binding sites) at each end of the element. To determine the contribution of these sites, as well as other sequences within $E2RE_1$, to enhancer function, specific mutations and deletions were generated by oligonucleotide reconstruction. The functional analysis of these mutations confirmed that a pair of E2 binding sites was essential for E2-dependent enhancer activity but also indicated that cooperativity between the motifs at each end of $E2RE_1$ creates a highly responsive element. Isolated $ACCN_6GGT$ motif pairs could also act as E2-dependent enhancers but at a significantly reduced level in comparison to the intact element. The sequences between the E2 binding sites in $E2RE_1$ were not required for enhancer function and could actually block the enhancer activity of an isolated pair of E2 binding sites when positioned between the binding sites and the enhancer-deleted simian virus 40 early promoter.

Papillomaviruses are small DNA viruses which cause benign epithelial tumors or warts in a variety of animal hosts and are occasionally associated with lesions progressing to malignancy. Bovine papillomavirus type 1 (BPV-1) causes cutaneous fibropapillomas in its natural host, cattle, and induces fibroblastic tumors in a variety of other heterologous hosts. BPV-1 efficiently transforms certain rodent cells in tissue culture (for a review, see reference 18) and has served as the prototype for molecular and genetic analyses of the papillomaviruses. In these cells, the viral genome is maintained extrachromosomally (22). The amount of viral transcription is small in transformed mouse cells, and the transcripts are derived only from the early or transforming region of the genome (13). Viral late gene expression appears to be restricted to terminally differentiating keratinocytes in epithelial lesions (1, 3, 5), and to date, no tissue culture system exists to propagate papillomaviruses.

Transcription of the papillomaviruses is complex and appears to be regulated by a variety of *trans*-acting factors and *cis*-acting elements. Viral transcription is tightly regulated and results in a low level of transcription from the early region of the genome in transformed cells and a restriction of late gene transcription to terminally differentiated keratinocytes. One important regulatory circuit involves the interaction of the papillomavirus E2 gene products with enhancer elements in the viral long control region (LCR), also referred to as a noncoding region or upstream regulatory region. E2 *trans*-regulation was first described for BPV-1 (29) and has now been extended to other papillomaviruses (9, 15, 25, 31).

The E2 open reading frame (ORF) of BPV-1 encodes at least three transcriptional regulatory factors. The full-length E2 gene product is a transcriptional *trans*-activator that acts upon enhancer elements located in the LCR (29). The viral promoters P_{89} and P_{7940} located just downstream of the LCR are responsive to E2 (11, 28), as is the viral promoter P_{2443}, which is located just upstream of the E2 ORF (P. L. Hermonat, B. A. Spalholz, and P. M. Howley, EMBO J., in press). In addition to the *trans*-activator, the 3′ portion of the E2 ORF encodes a transcriptional repressor expressed from P_{3080} that can specifically repress transcriptional *trans*-activation by the full-length E2 gene product (20, 21). Genetic studies have indicated that an additional viral factor(s) is encoded in part by an exon of the E2 ORF downstream of the 3′ splice junction at nucleotide (nt) 3225 (14, 26). An RNA in which the E8 ORF is spliced to the E2 ORF through a 5′ splice site at nt 1234 and the 3′ splice junction at nt 3225 has been described previously (30), and recent studies have shown that the product of this RNA may represent an alternate E2 transcriptional repressor (P. Lambert, unpublished results). In addition, there is now biochemical evidence for the presence of three E2 proteins in BPV-1-transformed cells which are predicted to represent the E2 *trans*-activator, the E2 repressor, and this E8/E2 protein (N. L. Hubbert, J. T. Schiller, D. R. Lowy, and E. J. Androphy, Proc. Natl. Acad. Sci. USA, in press).

Experiments mapping the *cis*-responsive sequences for E2 *trans*-activation within the LCR have defined two E2-responsive elements, $E2RE_1$ and $E2RE_2$ (28). $E2RE_1$ (nt 7611 through 7806) strongly activates transcription from the enhancer-deleted simian virus 40 (SV40) promoter in the presence of the full-length E2 gene product and is required for the E2 activation of the BPV-1 promoters P_{89} and P_{7940}. The analysis of the *cis* sequences required for *trans*-repression by the 3′ E2 gene product showed that the site for *trans*-repression was coincident with $E2RE_1$ (20). $E2RE_1$ contains a pair of $ACCN_6GGT$ motifs at each end. This sequence motif is found in the control regions of each of the papillomaviruses sequenced to date and has been shown to be the binding site for the viral E2 proteins (2, 24). The domain of E2 responsible for this specific DNA binding maps to the carboxy-terminal 100 amino acids of the proteins (23), a region shared by the full-length *trans*-activator, the *trans*-

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repressor expressed from P_{3080}, and additional factors accessing the E2 ORF through the 3′ splice junction at nt 3225. Therefore, the transcriptional effects mediated by the full-length *trans*-activator and shorter E2 proteins with repressor activities, are likely to be mediated in part through competitive binding at these sequence motifs. Current evidence suggests that the E2 *trans*-activator, which can bind to $E2RE_1$ through the $ACCN_6GGT$ motifs via the carboxy-terminal DNA-binding domain, activates transcription through peptide determinants in the amino half of the E2 *trans*-activator protein. Repressor forms of E2, not containing the activator amino-terminal domain of E2, therefore could block this activation by direct competition for the $ACCN_6GGT$ binding sites in the enhancer.

There are, however, 12 E2 binding motifs located in the BPV-1 genome, with 10 in the LCR. Why then is $E2RE_1$ such a strong E2-responsive element? Functional *Bal* 31 exonuclease analysis of this element indicated that deletion of either pair of flanking $ACCN_6GGT$ motifs reduced the ability of this element to respond to E2. It was postulated that an E2-mediated interaction of these flanking binding sites was required for transcriptional activation. A similar study by Haugen et al. (11) reported that the two motifs between nt 7760 and nt 7795 were sufficient for some level of *trans*-activation of P_{89} and P_{7940}. Recent studies examining the inherent enhancer properties of the E2 binding site have established that although one binding site is not sufficient for enhancer activity, two or more tandem copies can act as an E2-dependent enhancer and that the E2 response increases with additional copies of the motif (12, 16). In this report, we have carried out a detailed mutational analysis of the $E2RE_1$ to determine what sequences are required for, or contribute to, enhancer activity; to determine what minimal element(s) will provide enhancer activity; and to determine the effect of other DNA sequences in $E2RE_1$ on these minimal elements. In agreement with other studies (10–12, 15), we find that a pair of $ACCN_6GGT$ motifs from either end of $E2RE_1$ is sufficient for E2 enhancer activity but that its activity is significantly lower than that of the intact element. Mutational analysis of the E2 binding sites suggests that the flanking pairs do interact in a cooperative manner to produce an enhanced E2 response, although the mechanism of this interaction is not yet understood. The sequences internal to the flanking motifs do not contribute any dramatic positive effect to enhancer activity. However, when these sequences are placed between a single pair of E2 binding sites and the SV40 promoter, they strongly abrogate E2-dependent enhancer activity, suggesting that these sequences may have inhibitory effects on E2 *trans*-activation.

MATERIALS AND METHODS

Oligonucleotide reconstruction of $E2RE_1$. The method described by Grundström et al. (7) was adapted to generate wild-type and mutated versions of $E2RE_1$ from synthetic oligonucleotides as outlined in Fig. 1. Specifically, oligonucleotides were synthesized by using an Applied Biosystems DNA synthesizer 380B by the phosphoramidite method. Oligonucleotides were dissolved in 98% deionized formamide and electrophoresed through 15 and 20% polyacrylamide sequencing type gels. Oligonucleotides were eluted from gel slices in elution buffer (0.5 M ammonium acetate, 20 mM magnesium acetate, 0.1 mM EDTA, 0.1% sodium dodecyl sulfate), fractionated through Sephadex G-25, and lyophilized to dryness. The purified oligonucleotides were phosphorylated at a concentration of 40 μM in polynucleotide

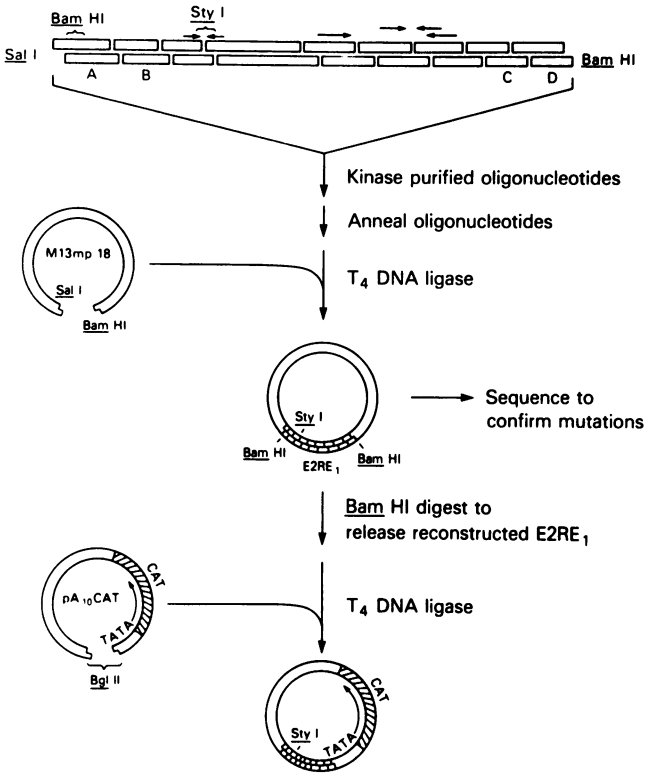


FIG. 1. Oligonucleotide reconstructions of wild-type and mutated versions of $E2RE_1$ were synthesized in overlapping segments (indicated by open boxes). The specific oligonucleotides were designed such that each component of interest was on a separate segment (see Fig. 2). The horizontal arrows indicate the location of inverted repeats within the sequence. A, B, C, and D indicate the positions of the four $ACCN_6GGT$ motifs in $E2RE_1$. *Sal*I and *Bam*HI restriction enzyme sites (as shown) were engineered at the ends of the element to facilitate cloning. In addition, oligonucleotides were made to link different components together while deleting others; no mutations were incorporated into these oligonucleotides.

kinase buffer (50 mM Tris [pH 9.0], 10 mM MgCl, 5 mM dithiothreitol, 0.1 mM spermidine, 0.1 mM EDTA) (3) with 0.1 mM ATP and 5 U of T4 polynucleotide kinase (P-L Biochemicals, Inc.) for 1 h at 37°C. The oligonucleotide solution was diluted to 5 μM in ligation buffer (50 mM Tris [pH 8.0], 10 mM MgCl, 10 mM dithiothreitol) before annealing.

Equimolar amounts of the wild-type or mutated oligonucleotides were combined to reconstruct the wild-type, mutated, or deleted versions of $E2RE_1$ described in the text below. Various combinations of oligonucleotides were allowed to anneal for 1 h at room temperature before being added to purified *Bam*HI- and *Sal*I-digested M13mp18 DNA at a molar ratio of 20:1. The DNAs were ligated by the addition of ATP to 1 mM and 1 U of T4 DNA ligase for 5 h at room temperature prior to transformation using competent DH5α F′ cells (Bethesda Research Laboratories, Inc.). The resulting plaques were screened for the reconstructed $E2RE_1$ inserts, and the sequences of these reconstructed elements were verified by dideoxynucleotide sequencing (27). The reconstructed $E2RE_1$ inserts were released as a single fragment from the M13mp18 vector by *Bam*HI digestion and cloned in either orientation into the *Bgl*II or *Bam*HI site of the enhancer-dependent expression vector, $pA_{10}CAT$ (19).

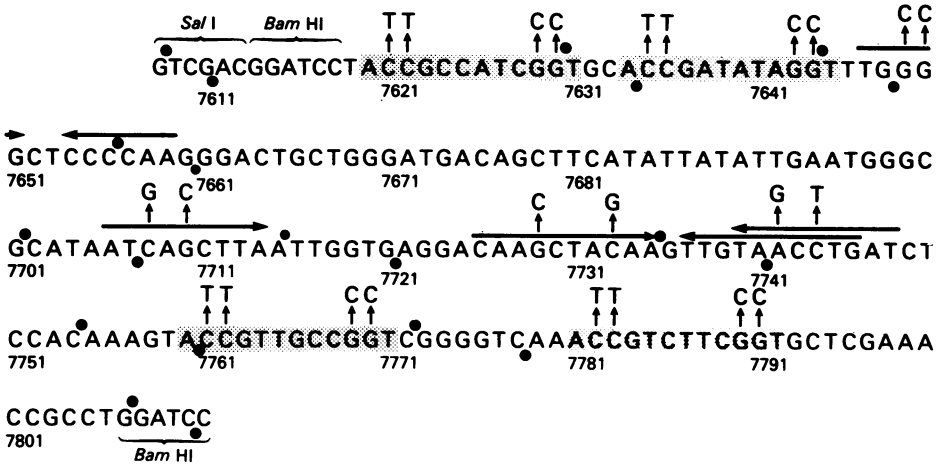


FIG. 2. Sequence of the reconstructed E2RE$_1$, showing wild-type sequence and mutational base changes. The ACCN$_6$GGT motifs are indicated by the shaded regions. The horizontal arrows show the position of three inverted repeat sequences. The vertical arrows and nucleotides above the continuous sequence indicate the mutational base changes investigated for enhancer activity. Dots above the sequence indicate the endpoints of the different synthesized oligonucleotides. Dots below the sequence indicate the endpoints of the complimentary synthesized strand. E2 binding sites: A, nt 7620 through 7631; B, nt 7634 through 7645; C, nt 7760 through 7771; and D, nt 7781 through 7792.

Transient expression assays. African green monkey kidney (CV-1) cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cotransfection of CV-1 cells for chloramphenicol acetyltransferase (CAT) expression analysis using 5 μg of the CAT-containing plasmid and 10 μg of high-molecular-weight salmon sperm DNA (ssDNA) or 8 μg of ssDNA and 2 μg of the E2 *trans*-activator-expressing plasmid, C59 (32), was performed as previously described (29). E2 repressor activity was assessed as described by Lambert et al. (20). The preparation of cellular extracts by freeze thawing and the CAT assays were performed as described previously (6, 29). The actual levels of CAT expression assayed and presented in this report varied, but the comparative *trans*-activations were representative of many experiments.

RESULTS

Rationale for mutations. In an initial study in which the viral LCR was dissected and analyzed for elements responsive to E2 *trans*-activation, a strong E2-responsive element (E2RE$_1$) was mapped between nt 7611 and 7806 (28). This element contains a total of four ACCN$_6$GGT motifs, with a pair of motifs at each end. This 12-base motif has been shown to be the binding site for the viral E2 protein (2, 24). In the initial study in which E2RE$_1$ was defined, it was found that the presence of these motifs was critical for E2RE$_1$ enhancer activity. The presence of at least one ACCN$_6$GGT binding motif at each end of the element was required for enhancer activity, and deletion of these motifs impaired the ability of E2RE$_1$ to function as an E2-dependent enhancer. These data suggested that an interaction between the ends of the element mediated by E2 binding at the ACCN$_6$GGT motifs was necessary for maximal enhancer activity of E2RE$_1$. To further investigate the effect of the E2 binding sites experimentally without perturbing surrounding sequences or disrupting adjacent motifs, point mutations were engineered into each of the four motifs of E2RE$_1$ by oligonucleotide reconstruction. The mutated motif, ATTN$_6$CCT, was chosen (see Fig. 2) because in vitro binding assays indicated that an E2 protein generated in a rabbit reticulocyte lysate does not bind to this sequence (23). We therefore reconstructed E2RE$_1$ with this mutation in each of the four motifs, singly or in a variety of combinations, to test the contribution of each motif to enhancer function. In these reconstructed elements, the wild-type E2 binding sites were designated A, B, C, and D, in order, where A was the most 5′ site with respect to the transcriptional direction of the genome. Mutated binding sites were correspondingly designated a, b, c, and d.

Several additional E2RE$_1$ mutations that did not involve the E2 binding sites were also investigated. These mutations were designed to disrupt possible secondary structures arising from several inverted sequence repeats present in E2RE$_1$ (Fig. 2). In particular one of these repeats TTGGGGCTCC CCAA, is almost identical to a site in the mouse *H-2K^b* major histocompatibility gene enhancer, which has been shown to bind a nuclear transcriptional factor (4). The mutations engineered into this sequence (GC transversions at nt 7649 and 7650) should disrupt this site. In addition, mutations were introduced into two other inverted repeat sequences to test their importance to enhancer function. Finally, several deletions of the sequences between the paired E2 binding sites were investigated to determine whether these internal sequences have a role in E2-dependent enhancer function and whether the spacing of the paired motifs is important in determining the strength of the enhancer.

ACCN$_6$GGT motif mutational analysis. Each of the reconstructed and mutated E2RE$_1$s was cloned into the enhancer-dependent expression vector, pA$_{10}$CAT, and transfected into CV-1 cells in the absence or presence of an E2 expression plasmid. The levels of CAT expressed were assayed from cell extracts and were normalized for protein concentration. The data presented in Fig. 3 were obtained from extracts assayed within the linear range of CAT enzyme activity, and thus, comparison of activities should accurately reflect the enhancer strengths of the mutated elements. The activity of p964/p968, which contains the genomic E2RE$_1$ fragment (nt 7611 to nt 7805), is presented on the top line. The wild-type reconstructed fragment for E2RE$_1$ (2nd line) had an orientation-independent, E2-dependent enhancer ac-

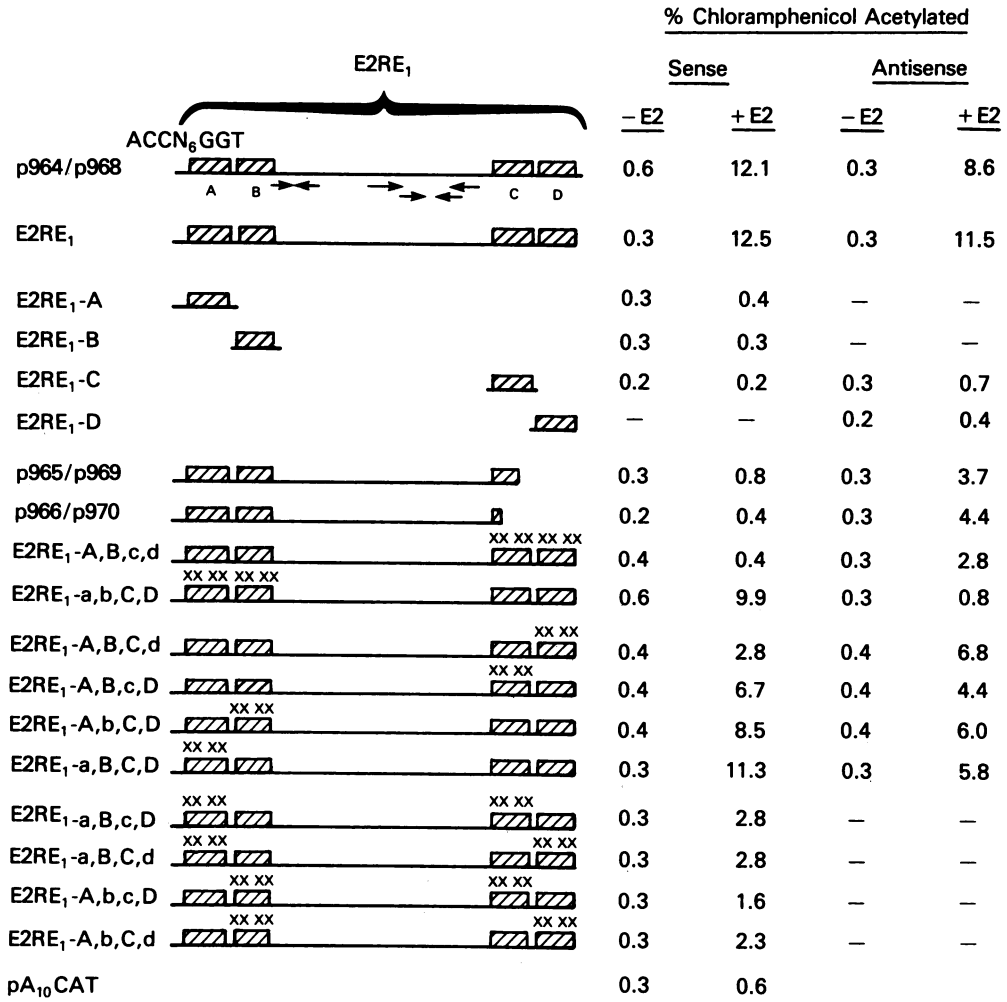


FIG. 3. Effects of $ACCN_6GGT$ mutation on $E2RE_1$ activity. A diagram of $E2RE_1$ showing motifs A, B, C, and D and the various inverted repeat sequences is shown for the original fragment defining $E2RE_1$ found in p964/p968 (28). $E2RE_1$ is the wild-type element reconstructed from oligonucleotides. Reconstructed elements containing mutations of $E2RE_1$ are indicated as follows. A, B, C, or D represents an unmutated motif; a, b, c, or d represents a mutated motif. ×× above an element indicates the mutation shown in Fig. 2 for that element. Plasmids p965/p969 and p966/p970 contain previously described deletion fragments (28). Elements were cloned into $pA_{10}CAT$, in the sense or antisense orientation, as indicated, and were assayed in the absence or presence of E2 *trans*-activator. In this representative experiment, 5 μg of protein from the cellular extracts was incubated in the presence of [^{14}C]chloramphenicol and acetyl coenzyme A for 30 min. Acetylated products were separated by thin-layer chromatography and quantitated by scintillation counting.

tivity equivalent to that of the genomic fragment. Analysis of the reconstructed elements containing mutated E2 binding sites leads to several conclusions. Elements containing only one intact $ACCN_6GGT$ motif were inactive as E2-dependent enhancers ($E2RE_1$-A, -B, -C, and -D). Elements which retained an intact $ACCN_6GGT$ motif pair but which had lost the other pair either through deletion or mutation, activated transcription in the presence of E2, but only when the intact pair of motifs was proximal to the promoter. For example, an element containing the C and D motifs intact but which was mutated in the A and B motifs responded to E2 but only in the sense orientation ($E2RE_1$-abCD). Its activity was decreased 10-fold in the opposite orientation. Similarly, if motifs A and B were retained and C and D were mutated ($E2RE_1$-ABcd), then the element served as an E2-responsive element only in the antisense orientation. A similar orientation dependence of BPV-LCR deletion fragments was observed by Harrison et al. (10). The deletion mutations represented in plasmids p965/p969 and p966/p970 had activities consistent with these observations. These data are also in agreement with our previous data showing the importance of a pair of motifs in $E2RE_1$ for full E2-dependent enhancer activity (28). These data are presented here because the results for p965 and p970 differ from those previously reported (28), in which the plasmids were inadvertently switched, as confirmed by sequence analysis. On the basis of the previous data, it was suggested that deletion of one of the pairs of motifs eliminated the E2 responsiveness of the fragment. It is now apparent that although deletion or mutation of either flanking motif pair reduces E2 responsiveness and destroys the orientation independence of the element, it does not totally eliminate E2 responsiveness. The deletions represented in p965/p969 and p966/p970 each destroy both of the downstream E2 binding sites of $E2RE_1$. Each deletion removes motif D completely and critical sequences in motif C (28).

In mutated elements in which only one $ACCN_6GGT$ motif is mutated so that the element contains an intact pair of

TABLE 1. Activity of isolated $ACCN_6GGT$ motif pairs

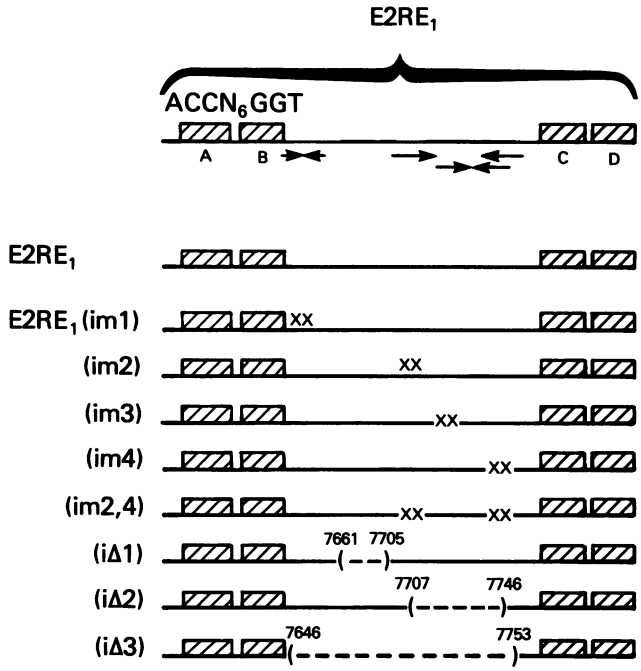
Enhancer element	% Chloramphenicol acetylated			
	Sense		Antisense	
	Without E2	With E2	Without E2	With E2
$E2RE_1$ (wild type)	0.6	36.1	0.3	25.4
Motifs A+B	0.2	3.3	0.2	4.3
Motifs C+D	0.3	4.8	0.3	6.7
Motifs B+C	0.2	3.6		
$pA_{10}CAT$	0.2	0.5		

motifs at one end and one wild-type motif at the other end, orientation-independent E2 enhancer activity was retained. $E2RE_1$ mutations ABCd, ABcD, AbCD, and aBCD clearly demonstrated E2-dependent enhancer activity in an orientation-independent manner. Furthermore, the E2-dependent enhancer strength of the elements mutated at a single binding site was greater than the strength of those elements mutated in both motifs of a pair. The absolute level of E2 response for this class of mutation depended on which motifs were mutated. Elements containing motifs C and D intact generally were stronger E2-dependent enhancers than elements in which these binding sites were mutated. Such variations in enhancer strength could reflect the relative contribution of the different $ACCN_6GGT$ motifs to enhancer activity. Motifs A, C, and D contain the sequence $ACCGN_4CGGT$, which has been reported to bind E2 more avidly than $ACCN_6GGT$ (12). The strength of E2 binding therefore seems to correlate with E2-responsive behavior, although the actual configuration of the motifs within a pair has not been addressed. The final class of $ACCN_6GGT$ mutations analyzed contained elements in which one motif of each pair was mutated and one was retained ($E2RE_1$-aBcD, -aBCd, -AbcD, and -AbCd). In each case, the E2 response was dramatically reduced but was still significant relative to the elements containing only one motif intact. Thus, it appears that the $E2RE_1$ E2 binding sites interact cooperatively to augment enhancer activity and to ensure orientation-independent activity.

Enhancer activity of isolated E2 binding sites. The data from the mutations of $ACCN_6GGT$ motifs within $E2RE_1$ indicated that at least two E2 binding sites are required for the element to act as an E2-dependent enhancer. We therefore tested the ability of the paired E2 binding sites in $E2RE_1$ to act independently of the remaining sequences in $E2RE_1$ as E2-responsive enhancers (Table 1). Either flanking pair AB or CD was sufficient to act as an E2-dependent enhancer but at a level approximately 5- to 10-fold lower than that of the intact element. Also, a pair reconstructed from only motifs B and C had similar E2-dependent enhancer activity levels. The CD pair was the most active but was still less active than the mutated element $E2RE_1$-abCD, which contains only the C and D binding sites intact and the remaining internal $E2RE_1$ sequences. Unlike the mutated $E2RE_1$-abCD and $E2RE_1$-ABcd elements, the isolated $ACCN_6GGT$ pairs could respond to E2 in an orientation-independent manner. This result suggested that the $E2RE_1$ internal sequences can affect enhancer activity. In $E2RE_1$-abCD, $E2RE_1$-ABcd, p965/p969, or p966/p970, in which the intact motif pair is adjacent to the SV40 promoter, the internal sequences have a positive effect on E2-dependent enhancer activity. In the opposite orientation, in which the internal sequences are interposed between the pair of intact E2 binding sites and the promoter, they appeared to block enhancer activity.

Analysis of mutations internal to the $ACCN_6GGT$ motifs. The region of $E2RE_1$ internal to the paired E2 binding sites was examined by analysis of point mutations and deletion mutations (Fig. 4). None of the mutations generated had any dramatic effect on enhancer activity. Elements with point mutations in the inverted repeat sequences of this region varied only slightly in enhancer activity from the wild-type $E2RE_1$, indicating that either the mutations did not sufficiently disrupt the inverted repeat sequence or that as individual components, these sequences were not required for E2-dependent enhancer activity. In addition reconstructed elements deleting parts or all of the internal segments of $E2RE_1$ (iΔ1 through iΔ3) were only slightly less active (if at all) than the intact element. This result suggested that the internal sequences may contribute some positive effects to the E2 response but also indicated that there was no augmentation of E2 responsiveness by having the paired motifs brought closer together. Even in the case in which all the internal sequences were removed ($E2RE_1$ [iΔ3]), which left four E2 binding sites in tandem, the activity was no greater than that of the wild-type $E2RE_1$.

E2 *trans*-repressor activity and $E2RE_1$ mutations. We next measured the ability of the E2 repressor encoded by the carboxy-terminal two-thirds of the E2 ORF to repress the E2-dependent enhancer activity of different $E2RE_1$ reconstructions. Repression of E2 *trans*-activation in $E2RE_1$ by the E2 repressor has been described previously (20). Various amounts of plasmid pCW1-28, which expresses the E2 repressor, were transfected into CV-1 cells with a constant amount of plasmid C59, which expresses the E2 *trans*-activator, and different mutated $E2RE_1$/CAT reporter plasmids. CAT expression resulting from these transfections was assayed and plotted against the amount of E2 repressor plasmid added (Fig. 5). The $E2RE_1$ internal deletion mutations and the isolated CD pair of E2 binding motifs were compared with the wild-type element as substrates for E2 repression. A study of these mutations was undertaken here, to determine the minimal unit upon which E2 repression could occur, as well as whether the sequences between the $ACCN_6GGT$ pairs affect repressor activity. The repressor was most active in suppressing *trans*-activation of the wild-type $E2RE_1$. The elements reconstructed with internal deletions could also be repressed but not as effectively as the wild-type $E2RE_1$. The deletion removing all the internal sequences was approximately half as sensitive to E2 *trans*-repression as the wild-type $E2RE_1$, as judged by the degree of repression in this assay. The E2 repressor was also able to repress enhancer activity from the isolated CD motif pair. Although the initial level of *trans*-activation from this element was much lower than the intact $E2RE_1$, it was still reduced efficiently by the E2 repressor. The degree of repression indicated that the isolated E2 binding site pair was highly sensitive to repression and suggested that it is sufficient for E2 repression. Therefore, it would appear that in elements in which internal sequences were deleted but in which two pairs of binding sites remained, the reduced sensitivity to E2 repression resulted from the binding sites being brought closer together. This result could support the hypothesis that cooperative interactions between E2 *trans*-activator molecules bound to the $ACCN_6GGT$ motifs are important in *trans*-activation. Such cooperative effects could involve the amino portion of the full E2 *trans*-activator protein and as such would not be expected for the *trans*-repressor in which the amino-terminal portion of E2 is absent. Therefore, bringing the E2 binding sites closer



	% Chloramphenicol Acetylated			
	Sense		Antisense	
	−E2	+E2	−E2	+E2
$E2RE_1$	1.4	77.8	1.1	58.6
$E2RE_1$ (im1)	2.0	40.1	1.3	78.9
(im2)	1.3	68.4	1.2	59.4
(im3)	1.0	78.1	1.9	80.6
(im4)	1.5	61.3	—	—
(im2,4)	2.3	69.5	—	—
(iΔ1)	0.8	36.5	—	—
(iΔ2)	0.7	58.9	0.6	35.8
(iΔ3)	1.2	57.6	0.8	65.9

FIG. 4. Effects of $E2RE_1$ internal mutations. A diagram of the components in $E2RE_1$ is shown at the top of the figure. $E2RE_1$ is the wild-type reconstructed element. $E2RE_1$ (im$x$) indicates internal mutation of $E2RE_1$ ([iΔx] indicates internal deletion). Mutations in sequences are denoted by ×'s above a component: im1, mutations at nt 7649 and 7650; im2, mutations at nt 7708 and 7710; im3, mutations at nt 7729 and 7733 ; and im4, mutations at nt 7742 and 7744. The actual base changes are shown in Fig. 2. The endpoints and deletions of sequences (-----) are indicated. Elements were assayed as described in the legend to Fig. 3, except that 30 μg of cellular extract protein was used.

together could increase the effectiveness of the E2 *trans*-activator to *trans*-activate in the presence of repressor.

DISCUSSION

The functional analysis of the components of $E2RE_1$ through the mutations and deletions described in this report leads to several conclusions. On the simplest level, the E2 binding site ($ACCN_6GGT$ motif) can act as an E2-dependent enhancer when present in at least two tandem copies. These binding sites do not have to be identical in positions 4 through 9, nor do they have to be paired as they normally are in $E2RE_1$ (a pair made up of motifs B and C is also active). This result is consistent with other recent studies which have shown that consensus E2 binding site sequences in two or more copies can act independently as E2-dependent enhancers (10–12, 15). The level of transcriptional activation in response to E2 provided by these isolated tandem E2 binding sites, however, is significantly lower than the activity seen from the full $E2RE_1$ element. When assayed independently, the binding site pairs in $E2RE_1$ show only 5 to 20% of the E2-dependent enhancer activity of the entire element. If the four binding sites of $E2RE_1$ were to interact additively, this activity could account for up to 40% of the total activity. The activity of $E2RE_1$ internal deletions and of $E2RE_1$ mutations ABcd and abCD suggest that the internal sequences may contribute somewhat to the activity of this element; however, none of the internal mutations created was able to identify additional elements required for E2-dependent enhancer activity, which could account for the additional activity in the element. (It should be noted, however, that the mutations which were investigated disrupted sequences of possible secondary structure; it is not known if any factors actually interact at these sites.) It is also possible that other sequences within this region which were not investigated in this study are important to the E2 response. Although the presence of these internal sequences could contribute some positive effect to the E2 response, as seen in $E2RE_1$-abCD and $E2RE_1$-ABcd in the sense and antisense orientations, respectively, they also have a strong inhibitory effect when placed between the E2 binding sites and the SV40 promoter. That the inhibitory effect could be due simply to an increased distance between the enhancer and the promoter seems unlikely, since tandem E2 binding sites can still function as E2-dependent enhancers (albeit at a lower level) when placed 3′ to the CAT gene approximately 2 kilobases away (data not shown; 12). Also, the enhancement of activity by a distal pair of motifs is clearly evident in the activity of the entire element when compared with the activity of the isolated motif pairs in $E2RE_1$-abCD or ABcd, assayed in the sense and antisense orientation, respectively.

An hypothesis that the ends of $E2RE_1$ interact with each other through the binding of E2 is consistent with results from all the mutations studied. Cooperative interaction would account for the rescue by a single binding site of the E2-dependent enhancer activity of a distal pair of E2 binding sites when it was separated from the SV40 early promoter by the internal sequences of $E2RE_1$. The additional single motif not only rescued the orientation independence of a pair of motifs positioned away from the promoter but increased the E2 response of the element when the paired motifs were adjacent to the promoter, even though a single motif does not act as an E2-dependent enhancer. Since the E2 proteins can bind to single copies, as well as pairs, of the $ACCN_6GGT$ motif (2, 23) (but the *trans*-activator activates transcription only when interacting with two or more tandem copies of the motif), the ends of the element must be

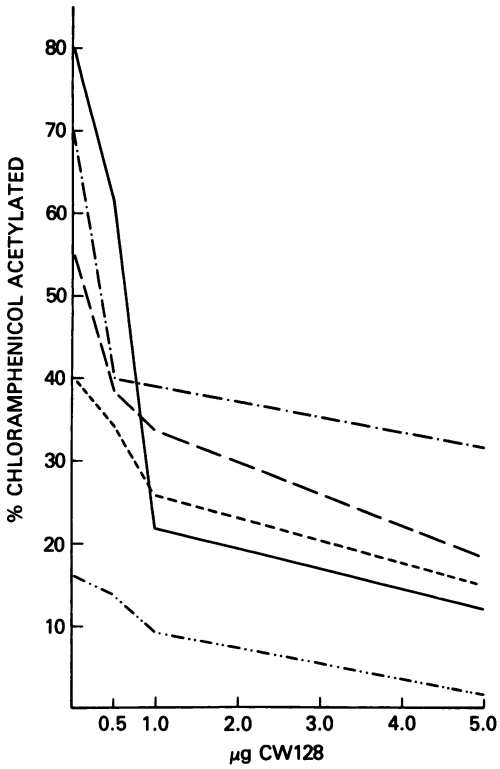


FIG. 5. Repression of E2 *trans*-activation of $E2RE_1$ by the *trans*-repressor encoded by pCW1-28 using various $E2RE_1$ (iΔ) mutations. Increasing amounts of pCW1-28 were added to 5 μg of $E2RE_1$-CAT plasmid and 2 μg of C59, a plasmid expressing the E2 *trans*-activator. Total DNA concentration in all the calcium phosphate coprecipitates was made 15 μg with the addition of carrier DNA. Transfected CV-1 cells were harvested 48 h posttransfection, and extracts were prepared. CAT assays were normalized for protein concentration. ——, $E2RE_1$ (wild type); - - - -, $E2RE_1$ with an internal deletion from nt 7661 through 7705; — — —, $E2RE_1$ with an internal deletion from nt 7707 through 7746; -·-·-·-, $E2RE_1$ with an internal deletion from nt 7653 through 7754; -··-··-, $E2RE_1$ with motif pair C and D. All assays in the absence of E2 *trans*-activator resulted in 0.4 to 0.8% chloramphenicol acetylated.

interacting through the E2 *trans*-activator protein to create functional binding sites. This interaction eliminates the directional negative effects of the internal sequences and explains why the intact element was as active as four tandem motifs. It also explains why mutated elements containing only one functional E2 binding site, at each end of the element, responded to E2 at levels comparable with that of an isolated pair of binding sites.

What function the internal sequences of $E2RE_1$ may have remains unclear. It is possible that other modulating factors bind in this region which might augment the E2 response or block transcription in the absence of E2. It is also possible that when the ends of $E2RE_1$ cannot interact through E2 binding, these factors block the effect of the E2 *trans*-activator at a distal motif pair on the promoter. Alternatively, it is possible that sequence secondary structure could account for all or some of these effects. The internal mutations selected for study here did not localize any functionally important sites; however, a complete investigation of this question will require analysis of DNA protein interactions, which is now in progress.

The possibility of other factors interacting in this control element highlights the complexity of transcriptional regulation in the LCR. The role of E2 *trans*-activation in the life cycle of the virus is probably critical but not yet fully understood. Two $ACCN_6GGT$ binding sites are required to create an E2-dependent enhancer, suggesting that the E2 *trans*-activator protein most likely functions as a multimer. Furthermore, the dyad symmetry within the E2 binding site would suggest that dimers of the E2 proteins may interact with individual sites. This type of interaction has been described for GCN4, another eucaryotic enhancer-binding protein (17), and recent evidence from our laboratory has confirmed that the E2 proteins bind as dimers to the $ACCN_6GGT$ motifs (A. McBride, personal communication). The requirement for two binding sites could indicate that a minimum of a tetrameric complex is required for *trans*-activation. These E2 multimers might then interact with additional transcription factors to elicit transcription. Interaction of enhancer-binding proteins with DNA, which then activates the transcriptional machinery of the cell to initiate transcription from particular promoters, has been discussed for other systems (8). The mechanism by which the E2 *trans*-repressor acts antagonistically to suppress E2 *trans*-activation is not entirely clear. From this study, we know that E2 repression functions efficiently even when just the isolated E2 binding site pairs are used as the E2-dependent enhancer. This result would be consistent with a mechanism involving competitive binding between the *trans*-repressor and the *trans*-activator at the $ACCN_6GGT$ motif to regulate transcriptional levels. Other mechanisms involving subunit mixing in the formation of heterodimers or competition for binding to other cellular factors are still possible, however. In addition to the E2 *trans*-activator and -repressor, it is clear that additional E2 regulatory factors exist. A splice acceptor site at nt 3225 makes it possible for regions from other upstream ORFs to splice into the 3′ region of the E2 ORF. Any protein containing the carboxy-terminal 100 amino acids of E2 will have the capacity to competitively bind at the $ACCN_6GGT$ motifs (23). An mRNA species that has been described previously (30) has been predicted to encode an E8/E2 fusion protein (21). There is evidence that an E2 protein of this predicted size exists in BPV-1-transformed cells (Hubbert et al., in press), as well as preliminary evidence that this protein functions as an E2 repressor (P. Lambert, personal communication).

In looking at the *cis* elements involved in E2 *trans*-activation, $E2RE_1$ has been shown to be required for expression from the BPV-1 promoters P_{89} and P_{7940} (11, 28) and the LCR has also recently been shown to be involved in the E2-activated expression of P_{2443} (Hermonat et al., in press). The contribution of E2 regulation through $E2RE_1$ in the context of the entire viral genome, however, has not yet been addressed. In BPV-1, there are 10 E2 binding sites in the LCR, 4 copies in $E2RE_1$, 2 copies in $E2RE_2$ (28), and 4 others. In addition to the LCR sites, there is a copy near each of two downstream promoters (P_{2443} and P_{3080}), suggesting that these promoters may also be regulated by E2 gene products. In this manuscript, we have shown that the E2 binding sites of $E2RE_1$ can cooperatively interact to create a highly E2-responsive enhancer, but also that the arrangement of binding sites, as well as the sequence of binding sites, can affect transcriptional activation.

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LITERATURE CITED

1. **Amtmann, E., and G. Sauer.** 1982. Bovine papillomavirus transcription: polyadenylated RNA species and assessment of the direction of transcription. J. Virol. **43**:59–66.
2. **Androphy, E. J., 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.
3. **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.
4. **Baldwin, A. S., Jr., and P. A. Sharp.** 1987. Binding of a nuclear factor to a regulatory sequence in the promoter of the mouse *H-2k*^{b} class 1 major histocompatibility gene. Mol. Cell. Biol. **7**: 305–313.
5. **Engel, L. W., C. A. Heilman, and P. M. Howley.** 1983. Transcriptional organization of the bovine papillomavirus type 1. J. Virol. **47**:516–528.
6. **Gorman, C. M., L. V. Moffat, and B. H. Howard.** 1982. Recombinant genomes that express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
7. **Grundström, T., W. M. Zenke, M. Wintzerith, H. W. D. Matthes, A. Staub, and P. Chambon.** 1985. Oligonucleotide-directed mutagenesis by microscale 'shot-gun' gene synthesis. Nucleic Acids Res. **13**:3305–3316.
8. **Guarente, L.** 1988. UASs and enhancers: common mechanism of transcriptional activation in yeast and mammals. Cell **52**:303–305.
9. **Guis, D., S. Grossman, M. A. Bedell, and L. A. Laimins.** 1988. Inducible and constitutive enhancer domains in the noncoding region of human papillomavirus type 18. J. Virol. **62**:665–672.
10. **Harrison, S. M., K. L. Gearing, S.-Y. Kim, A. J. Kingsman, and S. M. Kingsman.** 1987. Multiple *cis*-active elements in the long control region of bovine papillomavirus type 1 (BPV-1). Nucleic Acids Res. **15**:10267–10284.
11. **Haugen, T. H., T. P. Cripe, G. D. Ginder, M. Karin, and L. P. Turek.** 1987. Transactivation of an upstream early gene promoter of bovine papillomavirus-1 by a product of the viral E2 gene. EMBO J. **6**:145–152.
12. **Hawley-Nelson, P., E. Androphy, D. R. Lowy, and J. T. Schiller.** 1988. The specific DNA recognition sequence of the bovine papillomavirus E2 protein is an E2-dependent enhancer. EMBO J. **7**:525–531.
13. **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.
14. **Hermonat, P. L., and P. M. Howley.** 1987. Mutational analysis of the 3′ open reading frames and the splice junction at nucleotide 3225 of bovine papillomavirus type 1. J. Virol. **61**:3889–3895.
15. **Hirochika, H., T. R. Broker, and L. T. Chow.** 1987. Enhancers and *trans*-acting E2 transcriptional factors of papillomaviruses. J. Virol. **61**:2599–2606.
16. **Hirochika, H., R. Hirochika, T. R. Broker, and L. T. Chow.** 1988. Functional mapping of the human papillomavirus type 11 transcriptional enhancer and its interaction with the trans-acting E2 proteins. Genes Devel. **2**:54–67.
17. **Hope, I. A., and K. Struhl.** 1987. GCN4, a eukaryotic transcriptional activator protein, binds as a dimer to target DNA. EMBO J. **6**:2781–2784.
18. **Howley, P. M., and R. Schlegel.** 1987. Papillomavirus transformation, p. 141–163. *In* N. P. Salzman and P. M. Howley (ed.), The Papovaviridae. II. The papillomaviruses. Plenum Publishing Corp., New York.
19. **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 **79**:6453–6457.
20. **Lambert, P. F., B. A. Spalholz, and P. M. Howley.** 1987. A transcriptional repressor encoded by BPV-1 shares a common carboxy-terminal domain with the E2 transactivator. Cell **50**:69–78.
21. **Lambert, P. F., B. A. Spalholz, and P. M. Howley.** 1987. Evidence that bovine papillomavirus type 1 may encode a negative transcriptional regulatory factor, p. 15–22. *In* B. M. Steinberg, J. L. Brandsma, and L. B. Taichman (ed.), Cancer cells 5. Papillomaviruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Law, M.-F., D. R. Lowy, I. Dvoretzky, and P. M. Howley.** 1981. Mouse cells transformed by bovine papillomavirus contain only extrachromosomal viral DNA sequences. Proc. Natl. Acad. Sci. USA **78**:2727–2731.
23. **McBride, A. A., R. Schlegel, and P. M. Howley.** 1988. The carboxy-terminal domain shared by the bovine papillomavirus E2 transactivator and repressor proteins contains a specific DNA binding domain. EMBO J. **7**:533–539.
24. **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.
25. **Phelps, W. C., and P. M. Howley.** 1987. Transcriptional transactivation by the human papillomavirus type 16 E2 gene product. J. Virol. **62**:1630–1638.
26. **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.
27. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
28. **Spalholz, B. A., P. F. Lambert, C. L. Yee, and P. M. Howley.** 1987. Bovine papillomavirus transcriptional regulation: localization of the E2 responsive elements of the long control region. J. Virol. **61**:2128–2137.
29. **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.
30. **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.
31. **Thierry, F., and M. Yaniv.** 1988. The BPV1-E2 trans-acting protein can be either an activator or a repressor of the HPV18 regulatory region. EMBO J. **7**:3391–3397.
32. **Yang, Y.-C., H. Okayama, and P. M. Howley.** 1985. Bovine papillomavirus contains multiple transforming genes. Proc. Natl. Acad. Sci. USA **82**:1030–1034.