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A Constitutive Enhancer in the Bovine Papillomavirus Upstream Regulatory Region Shares Genetic Elements with the Viral P1 Promoter†

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The bovine papillomavirus upstream regulatory region represents a common element in the regulation of transcription from the five early viral promoters. We have determined the sequences required for transcription from the viral P1 promoter, which is located at the 5′ end of the upstream regulatory region. In vitro transcription from P1 requires a 123-bp fragment (nucleotides 7153 to 7275; −33 to +90) consisting of an upstream TATA-like sequence as well as an unidentified protein which binds to sequences immediately downstream of the initiation site. In vivo, this promoter requires additional downstream sequences (to position +160; nucleotide 7345) for maximal activity but does not require any additional DNA sequence upstream of a putative TATA box. Four regions within the downstream sequence from +9 to +160 are protected from DNase I digestion by proteins present in a HeLa cell extract. The presence of these sites correlates with the level of P1 activity. A constitutive enhancer maps to this same region, and mutations in this enhancer have been shown to affect downstream promoters. Deletion analysis indicates that the same sequences are required by both the P1 promoter and the constitutive enhancer, suggesting that the same proteins function in both activities.

Bovine papillomavirus type 1 (BPV-1) causes a benign proliferation of epithelial and fibroblastic cells resulting in the formation of a fibropapilloma. Both the dermal and epithelial components of the wart maintain the viral DNA as a replicating, extrachromosomal plasmid (16). Vegetative replication of the proviral DNA is induced by terminal differentiation of the basal cells of the epithelium and is likely a viral response to a cellular environment specific to these more highly differentiated cells. Changes in the activities of the viral promoters accompany this process (2) and are undoubtedly important factors in the switch from latent to productive infection. It is therefore crucial to our full understanding of the viral life cycle that we identify the elements which regulate the activities of the viral promoters.

Six promoters have been identified in the BPV-1 genome (2, 6, 29); one of them (P_L) is active only in the more fully differentiated keratinocytes and presumably corresponds to the late-gene promoter (2). The five early promoters, P1 through P5, are each regulated by virally encoded E2 proteins. P2 through P5 are activated by the full-length E2 protein through a common E2-dependent, *cis*-acting enhancer (E2RE1; nucleotides 7611 to 7806) located in the upstream regulatory region (URR) (11, 13, 17, 21, 26, 31, 32). This stimulation is modulated by truncated forms of the E2 protein which can repress E2-dependent activation (6, 15). The P1 promoter, on the other hand, is negatively regulated by all forms of E2 through a single E2 binding motif which overlaps elements of the P1 promoter (3, 28, 29). Additional elements which regulate expression of the viral proteins, including the proximal transcription elements which define each promoter, are just beginning to be identified (17, 27, 28, 34).

We have focused our attention on identifying the sequences required for expression from the P1 promoter. P1 is located at position 7186 in the URR, upstream of the sequences known to regulate expression of the other early promoters. We have previously shown that the P1 promoter is unusual in that it requires transcriptional elements located downstream of the site of transcription initiation (28, 29). In this paper, we extend that analysis and demonstrate that multiple P1 promoter elements exist downstream of the cap site and that these elements overlap sequences exhibiting a constitutive enhancer activity which can activate transcription from the other early-gene promoters (31, 34, 35).

MATERIALS AND METHODS

Plasmid constructions. The constructs pUC237 (wild type [wt]), Ψwt, D234, D28, and D20 have been described previously (29). pUCH210ΔXB was created by cutting the plasmid H210 (18) with *Xba*I and *Bam*HI and recircularizing the vector by blunt-end ligating the restriction sites.

TATA sequence deletions were generated by exonuclease III digestion. Plasmid pUCH210ΔXB (10 μg) was linearized with *Hin*dIII. Exonuclease III was added to 3 U of DNA per μg, and aliquots were taken after 70, 75, 80, 85, and 90 s of incubation at 37°C; added to 0.5 volume of 250 mM EDTA; and quick frozen in dry ice-ethanol. The extent of exonuclease III digestion was determined by filling in the recessed ends of 0.25 μg of exonuclease III-treated DNA with Klenow fragment, [^{32}P]dATP, and dTTP at room temperature for 30 min. The products were digested with *Mlu*I and sized on a 5% sequencing gel. Samples which contained the most deleted material in the TATA region were treated with 6 U of S1 nuclease per μg in 100 mM NaCl–50 mM Tris–10 mM $MgCl_2$–1 mM dithiothreitol at room temperature for 15 min. The DNA from each time point was recircularized in the

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presence of phosphorylated *Bam*HI linkers. The extent of each deletion was determined by dideoxy sequencing.

All chloramphenicol acetyltransferase (CAT) constructs in Fig. 3 are inserted into the CAT vector RVCAT, which was derived from SP65CAT (32) by inserting an 8-bp *Eco*RV linker into the filled-in *Xba*I in the SP65 polylinker. 234CAT, D28CAT, D20CAT, and ΔHIICAT were constructed by treating the respective *Nar*I-cut parent DNAs with T4 DNA polymerase in the presence of only dATP and then removing the single-stranded DNA end with S1 nuclease. The resulting deletions to position 7273, which remove the ATG, were digested at the 5′ position with *Hin*dIII at nucleotide 6959 except for DHII (derived from pUCH210ΔXB), in which the *Hin*dIII site is from the pUC18 polylinker. The fragments were inserted into RVCAT at the *Hin*dIII and *Eco*RV sites. NarCAT, MluCAT, and ClaCAT were constructed by inserting into RVCAT a fragment from *Hin*dIII to the *Nar*I, *Mlu*I, or *Cla*I site after first filling in the downstream site. The promoter fragments were derived from P1ΔATG1-4, in which the four BPV ATGs located between the P1 promoter and the downstream *Cla*I site have been mutated. P1ΔATG1-4 was created by oligonucleotide-directed point mutagenesis (14). Each ATG was converted to CTG by using a 17-bp oligonucleotide. A gapped, hybrid M13 duplex was created by hybridizing a wt M13 double-stranded DNA cut at the polylinker sites *Hin*dIII and *Eco*RI to a single-stranded M13 amber mutant containing BPV sequence between *Hin*dIII and *Sma*I. The phosphorylated mutant oligonucleotide was hybridized to the gapped duplex, and the gap was filled in with Klenow fragment and ligated, resulting in incorporation of the mutation into the wt viral strand. This mutant was transformed into BMH 71-18 cells, which are *mutS* cells containing an amber supressor. After incubation overnight at 37°C, the supernatant was used to infect MK30 cells, which lack the amber supressor. These cells grow only the wt virus, which contains the mutated BPV sequence. Individual plaques were picked and screened for the correct mutation as described by Choe et al. (6).

The constructs depicted in Fig. 5A will be described elsewhere (32). The plasmid DHIII contains a URR deletion from *Hin*dIII to *Mlu*I within the plasmid BPV_{100}.

DNase I protection and in vitro transcription. DNase I protection, HeLa whole-cell-extract preparation, and in vitro transcription assays were carried out as described previously (29). A test DNA and a Ψwt template (500 ng each) were mixed and added to the transcription extract. RNA was purified, and specific transcription was assayed by primer extension (29) with a primer binding to BPV nucleotides 7241 to 7264. Extension products were fractionated on a 5% denaturing polyacrylamide gel. Transcription levels were quantitated with a Hoefer GS300 densitometer.

Cell culture and CAT assays. HeLa, CV-1, and WHZ10 cells were maintained in Dulbecco's modified Eagle medium with 10% fetal bovine serum. Transfections were carried out by using standard calcium phosphate techniques (36). In CV-1 and WHZ10 cells, 10 to 20 μg of reporter plasmid, 1 μg of internal standard pON260 (25), and sonicated salmon sperm DNA to a total quantity of 25 μg were added to a 100-mm plate. For HeLa cells, 2 to 5 μg of reporter and 1 μg of internal standard were brought to 10 μg with carrier and added to 60-mm plates. Approximately 12 h after the addition of precipitate, the cells were treated with 15% glycerol for 2 min. Cells were harvested 48 h after glycerol shock and divided into two portions which were assayed for CAT and β-galactosidase activities as described previously (33). Briefly, 50 to 150 μg of total cell protein was incubated overnight at 37°C with [^{14}C]chloramphenicol and acetyl-coenzyme A. The products were separated by thin-layer chromatography and quantitated in a scintillation counter. The values were normalized to the β-galactosidase activity for the corresponding extract. For focus assays, 1 μg of each test DNA was brought to 10 μg with sonicated salmon sperm DNA and transfected into ~10^6 C127 cells on a 60-mm plate by calcium phosphate coprecipitation (36). Cells were split and put into a 100-mm dish the following day, and 3 to 4 weeks later, foci were stained with methylene blue and counted.

RESULTS

Multiple proteins bind downstream of the P1 promoter. We described in a previous report the identification of a DNA sequence immediately downstream of the P1 promoter (P_{7185}) that is essential for the activity of this promoter in vitro (29). Furthermore, we showed by DNase I protection that this sequence was bound by a protein(s) present in a HeLa cell extract (29). A more-extensive analysis of the protein-DNA interactions at the 5′ end of the URR reveals four regions of viral DNA which are protected from DNase I digestion by HeLa cell proteins (Fig. 1A). Site I (sequence from +9 to +30; nucleotides 7194 to 7215) corresponds to the previously identified cellular protein which demonstrates transcriptional activity in vitro. In addition, we detected protection of three other regions of DNA: site II, from +65 to +89 (nucleotides 7250 to 7274); site III, from +104 to +125 (nucleotides 7289 to 7310); and site IV, from +128 to +160 (nucleotides 7314 to 7345). The 5′ ends of the P1 RNAs and the direction of transcription are indicated in Fig. 1A by an arrow, illustrating that all four sites are located downstream of the site of P1 transcription initiation. We detect no additional protection of sequences to position +414 (nucleotide 7599; data not shown). Figure 1B summarizes the positions of the protected regions and specifies their locations within the BPV genome. These four sites are upstream of E2RE1 (positions 7611 to 7806), a critical component of the E2-dependent transcriptional enhancer (26), but overlap the E2RE2 enhancer (positions 7200 to 7336; 26). For convenience, we illustrate each binding site with a single component; however, we have no reason to believe that only a single protein is bound within each protected region.

P1 promoter responds to a −30 element in vitro. Since the sequence within binding site I had previously been shown to coincide with sequences required for P1 promoter activity (29), we were interested in determining whether sites II through IV also contributed to P1 activity. Initially, we analyzed deletions of these sequences in an in vitro transcription assay identical to that used to identify site I. Deletions which affect only binding sites II through IV, including a deletion of all sequences downstream of position 7275, have no effect on the levels of transcription (data not shown), indicating that of these downstream binding sites, site I alone is required for in vitro activity.

An A-T-rich sequence precedes the site of P1 transcription initiation by 31 nucleotides (Fig. 2A). It has been postulated that this sequence serves as the late polyadenylation signal (2, 8), but its position near the P1 promoter suggests that it could also serve as a binding site for the TATA binding protein component of the TFIID fraction. Deletions which remove all upstream BPV sequence to within 2 nucleotides of this A-T-rich region (position 7153) have no effect on in vitro transcription levels from the P1 promoter (Fig. 2, 75S.35 and H210ΔXB). Plasmid 80S.2, which disrupts this

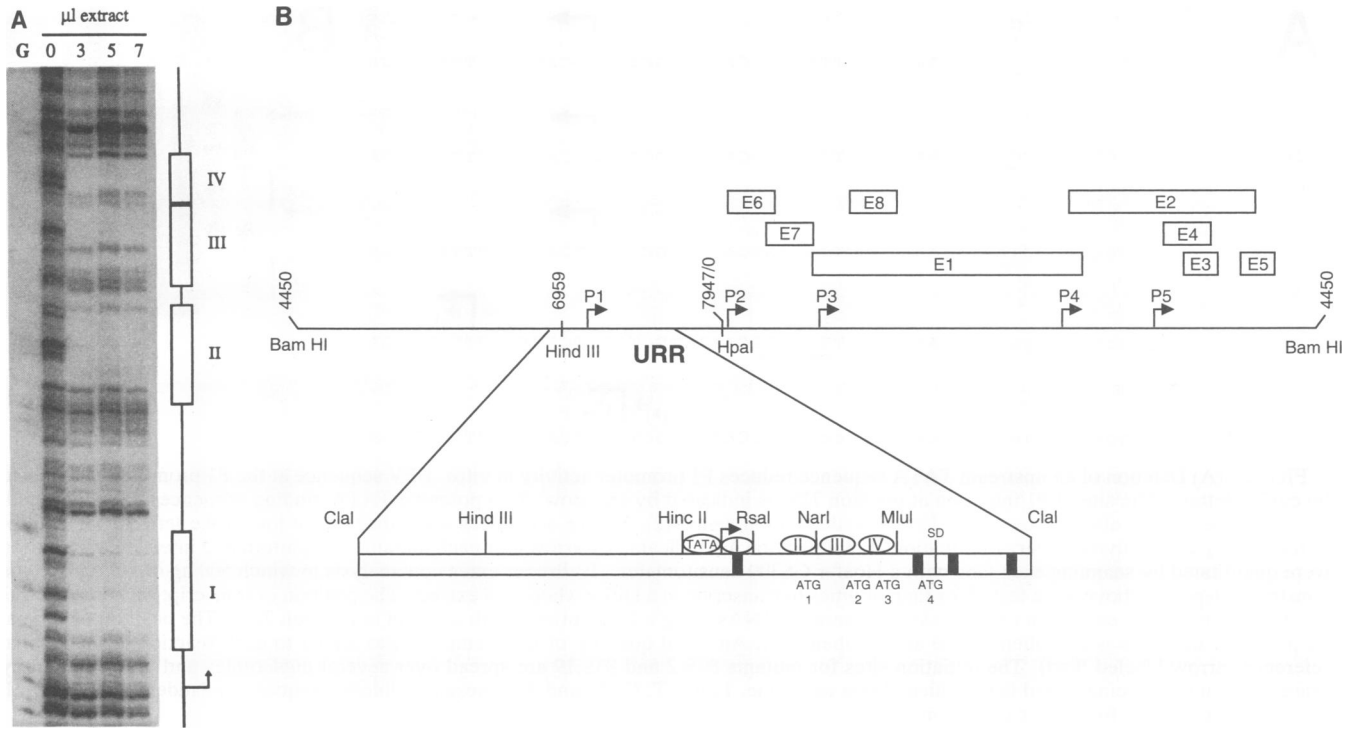


FIG. 1. (A) Footprint of the region downstream of the P1 promoter. A DNA fragment containing the P1 promoter region was 5′ labeled at nucleotide 7028, mixed with a HeLa extract fractionated on heparin agarose, and subjected to limited digestion with DNase I. In lane 0, no extract was added; in lanes 3, 5, and 7, extract (3, 5, or 7 μl, respectively) was added. Lane G is a sequence marker generated by Maxam and Gilbert sequencing (19) of the same fragment that was used for the protection assay. To the right of the gel, the extents of the protected regions are shown by open bars. The site of P1 transcriptional initiation is indicated by an arrow. Protected regions cover site I (positions 7194 to 7215), site II (positions 7250 to 7274), site III (positions 7289 to 7310), and site IV (positions 7314 to 7345). (B) Summary of the protected regions within the BPV genome and protein binding sites in the P1 promoter region. The top of the figure shows the entire BPV genome, with the known promoters and early-region open reading frames indicated. The URR spans the ~1-kb region between the end of the late coding region at nucleotide 7093 and the P2 promoter at nucleotide 89 and contains sequences which regulate both transcription and replication. Expanded below is a portion of the URR which identifies the P1 promoter and several nearby restriction sites used in this analysis. Each site of DNase I protection is indicated by an ellipse, which is not meant to indicate that only a single protein interacts with this site. The positions of the ATG codons in this region (we refer to these as ATG-1 through ATG-4, starting with the ATG nearest the site of P1 initiation) as well as a splice donor sequence (SD) are indicated. E2 binding sites 1, 2, 3, and 4 are identified on the URR fragment by black boxes. The nucleotide positions of the indicated restriction enzyme sites are 6835 and 7477 for *Cla*I, 6959 for *Hin*dIII, 7143 for *Hin*cII, 7213 for *Rsa*I, 7275 for *Nar*I, and 7352 for *Mlu*I.

A-T-rich domain, generates a heterogeneous initiation pattern with a twofold overall reduction in the level of transcription (Fig. 2), indicating that this sequence influences use of the P1 start site. The central band of this new group of cap sites is positioned 9 nucleotides downstream of the original initiation site. Interestingly, this new position for RNA 5′ ends is situated 26 bp downstream of a second A-T-rich sequence (underlined in Fig. 2A). Plasmid 85S.19 extends the deletion of BPV sequences to position 7171, disrupting this second A-T-rich domain. An initiation pattern identical to that of 80S.2 occurs with this mutation (down fivefold in overall transcriptional activity from that of wt), but the group of cap sites is now positioned back over the region of the original P1 start. It is unlikely that vector sequences brought into proximity with the promoter are affecting the position of initiation, since the same flanking sequence is introduced in both 80S.2 and 85S.19. More likely, cryptic TATA binding protein sequences are uncovered when the genuine sequence is deleted, as has been demonstrated with a model yeast system in which multiple DNA sequences can substitute for the prototype TATA binding protein recognition sequence (23).

Downstream elements are required for P1 transcription in vivo. With the information given above in hand, we turned our attention to the characterization of the P1 promoter in vivo. The difficulty in detecting RNA from the weak P1 promoter (2, 12, 29), especially in transient assays, prompted us to measure the effects of these downstream sites by using the CAT assay. When plasmids that contained additional sequences downstream of the *Nar* site were constructed, CAT activity was undetectable (data not shown). We reasoned that additional AUG codons added to the 5′ leader sequence of the CAT mRNA might lead to inefficient translation initiation at the authentic CAT AUG (3). We therefore used point mutagenesis to remove all BPV AUGs in the leader. The four BPV ATGs upstream of the *Cla*I site at position 7477 (Fig. 3A) were individually mutated (ATG → CTG) and then recombined into a single plasmid. The structure of this recombinant was confirmed by sequencing.

DNA fragments containing the P1 promoter and increasing amounts of downstream sequence were cloned upstream of a promoterless CAT gene; the 5′ end of the promoter fragment was the *Hin*dIII site at 6959 unless indicated otherwise in Fig. 3. CAT conversion levels in all cases were normalized

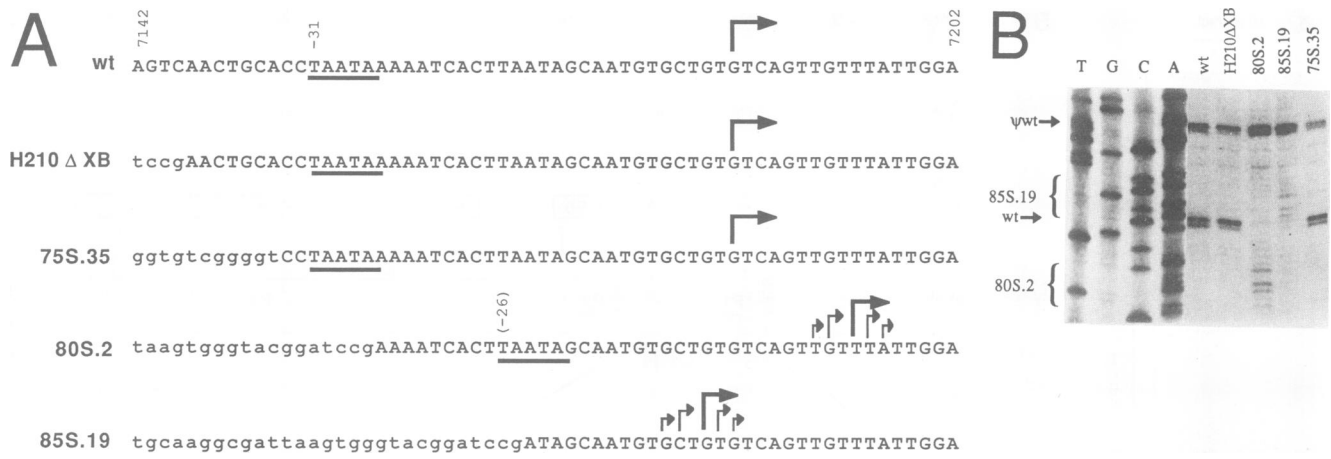


FIG. 2. (A) Deletion of an upstream TATA sequence reduces P1 promoter activity in vitro. BPV sequence at the P1 promoter is indicated by capital letters. The site of P1 initiation at position 7186 is indicated by an arrow. Two potential TATA binding sequences are underlined; the sequence at −31 also serves as the late polyadenylation signal (2). Vector sequences are indicated by lowercase letters. The site of initiation and the relative intensity of initiation at that position are indicated by arrows for each mutant. The pattern and intensity of initiation were quantitated by scanning each lane with a Hoefer GS300 densitometer. (B) Primer extension analysis in which 500 ng of DNA from the constructs depicted above were tested for their abilities to transcribe in a HeLa whole-cell extract. The position of transcription initiation was determined by primer extension on the in vitro-generated RNAs using a 24-bp primer with a 5′ end at position 7264. The position of normal P1 promoter initiation is identified by the arrow labeled wt. An equal quantity of Ψwt template was added to each reaction as an internal reference (arrow labeled Ψwt). The initiation sites for mutants 80S.2 and 85S.19 are spread over several nucleotides and are indicated by braces. The mutant being tested is identified above each lane. Lanes T, G, C, and A represent a dideoxy sequencing ladder generated with the same primer used for primer extension.

to levels of β-galactosidase produced from the cotransfected pON260 vector (25). For purposes of comparison, the activity of NarCAT was arbitrarily set to 1.0 (CAT conversion levels for this construct were from 2 to 7% in both CV-1 and WHZ10 cells).

The activities of NarCAT, MluCAT, and ClaCAT were tested in CV-1 monkey kidney cells and WHZ10 cells, a BPV-infected C127 cell line. As shown in Fig. 3, the presence of binding sites III and IV in MluCAT (3′ end point at position 7352) results in an 85-fold increase in activity relative to that of NarCAT (3′ end point at position 7275) when assayed in CV-1 cells. While the increase is a more modest sixfold in WHZ10 cells, the results indicate that these sequences contribute to P1 promoter activity. Addition of sequence to the *Cla*I site (position 7477; ClaCAT) gives only a small (less than twofold) additional increase in activity over that of MluCAT, suggesting that the requirements for transcriptional activity are limited to those regions which are bound by cell proteins.

To determine whether binding sites I and II were also active in vivo, we made the constructs 234CAT, D28CAT, and D20CAT (Fig. 3A). 234CAT deletes site I and a nonfootprinted region between sites I and II (deleted nucleotides 7187 to 7234). D28CAT deletes only site I (nucleotides 7187 to 7214), while D20CAT removes only the nonfootprinted region (nucleotides 7214 to 7234). In CV-1 cells, loss of activity correlates solely with loss of binding site I; both 234CAT and D28CAT show reduced activity, while D20CAT is unaffected. Site II is not specifically deleted in any of these constructions, making it difficult to define a role for this site, but since a CAT construct containing sequence from *Hin*dIII to *Rsa*I (nucleotide 7214; site I only) has no measurable CAT activity (data not shown) while NarCAT (nucleotide 7275; sites I and II) is active, binding site II may also be important for P1 transcription.

In WHZ10 cells, both 234CAT and D28CAT show reduced activity, which is also consistent with a role for site I in P1 transcription; however, D20CAT is also transcriptionaly impaired. While at odds with the result in CV-1 cells, this result is consistent with the results of a transcriptional analysis of the P1 promoter in HeLa cells, in which a similar deletion was also inactive (28). Like WHZ10 cells, HeLa cells express papillomavirus proteins (20). It would have been interesting to compare the activities of these deletions in WHZ10 cells and the parent cell line C127 to determine the importance of these viral proteins to P1 activity. However, the low levels of CAT activity in this cell line made quantitation unreliable (data not shown).

Surprisingly, the P1 promoter demonstrates little requirement for sequences upstream of the −30 region. As Fig. 3A shows, constructs XbaCAT, NarCAT, and DHIICAT all have similar activities in CV-1 cells (XbaCAT levels are somewhat diminished in WHZ10 cells). The plasmid DHIICAT contains only 40 nucleotides of BPV sequence upstream of the P1 cap site; thus, the promoter elements for P1 are located predominantly downstream of the initiation site. Several polymerase II promoters whose activities require factors distinct from classical enhancer elements, which bind downstream of the transcriptional initiation site (see reference 1 and references within), have now been identified. The simian virus 40 late promoter, in particular, has been studied in some detail (1). As for P1, a protein which binds to DNA and corresponds to sequences required for transcription has been identified. This protein has been purified and shown to act directly on the formation of preinitiation complexes (1).

It has been demonstrated previously that the P1 promoter is downregulated by both activator and repressor forms of the E2 protein (28), most likely by competition for binding between E2 and the factor(s) binding to site I (28, 34). Stenlund and Botchan (28) measured P1 repression in a CAT construct containing only a single E2 binding site (BS1) and sequence downstream to position 7275 (HB-1CAT; similar to

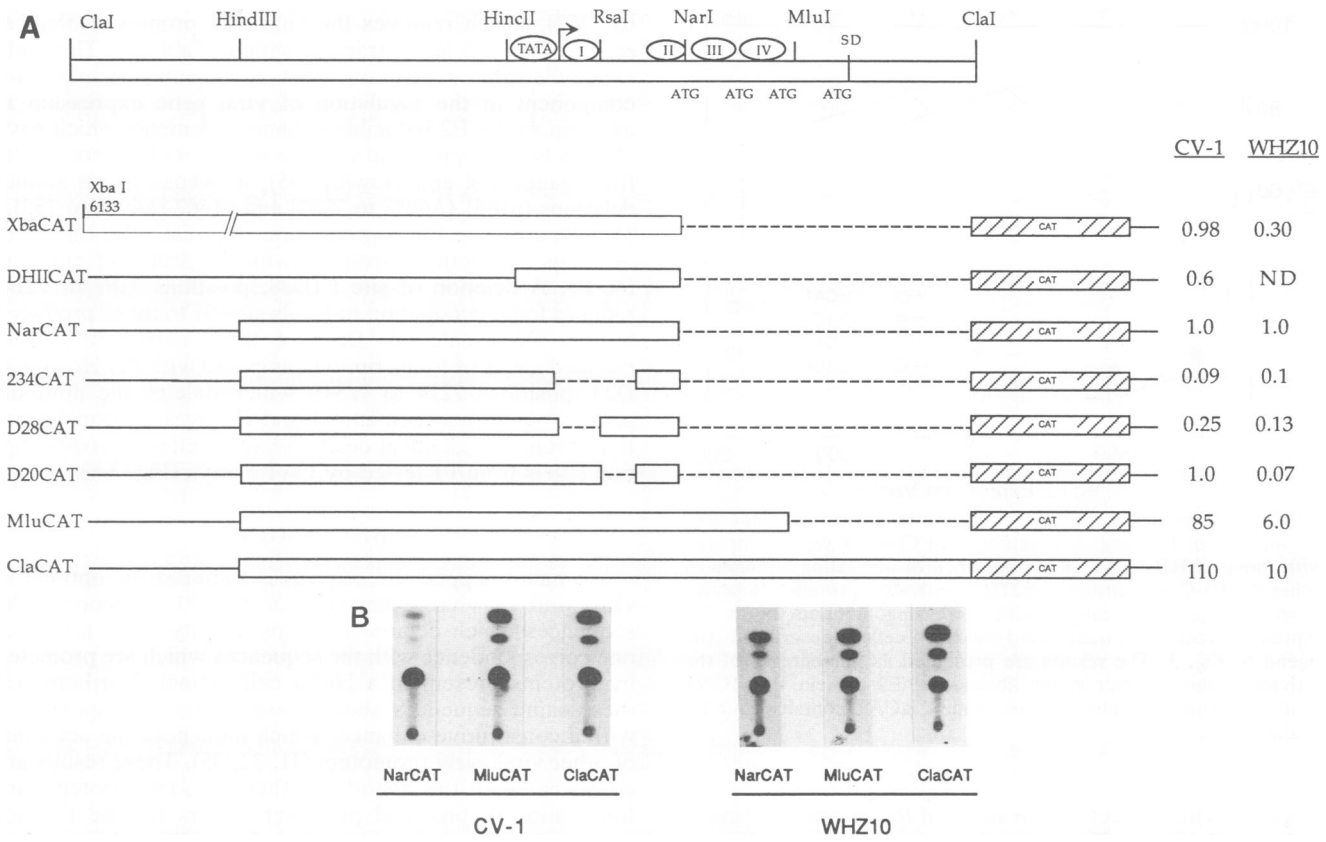


FIG. 3. (A) Binding sites I through IV are required for P1 promoter activity in vivo. The illustrated plasmids (10 to 20 μg each) were transfected into CV-1 or WHZ10 cells, and CAT extracts were made 48 h after glycerol shock. Reactions were carried out for 16 h with 75 to 100 μg of protein. CAT conversion levels were normalized to the β-galactosidase activity from a cotransfected pON260 vector. Corrected values of CAT conversion for each construct were normalized to that of the plasmid NarCAT (includes sites I and II) and are indicated to the right. Within an experiment, each template was tested in duplicate, and individual experiments were repeated at least three times. BPV sequences are indicated by open boxes. Nucleotide positions of the restriction enzyme sites are given in the legend to Fig. 1. D234 is a deletion of nucleotides 7187 to 7234 (loss of site I), D20 is a deletion of nucleotides 7214 to 7234 (site I still intact), and D28 is a deletion of nucleotides 7187 to 7214 (loss of site I). Dashed lines indicate the absence of DNA. ND, not determined; SD, splice donor sequence. (B) Autoradiogram of CAT assays prepared from extracts of CV-1 or WHZ10 cell lines which had been transfected with the indicated plasmids.

NarCAT in this study). Given the increase in P1 promoter activity with the addition of sequences downstream of the *Nar*I site, we were interested in the effect of E2 in this new transcriptional environment. The plasmid ClaCAT contains three additional E2 binding sites (BS2, BS3, and BS4), all located between the *Mlu*I and *Cla*I restriction sites. None of these sites overlap the cellular protein binding sites identified in this study, however. We compared the level of repression of ClaCAT with that of HB-1CAT after cotransfection of plasmids expressing the E2-C repressor protein or E2TR$^-$, which makes only the activator form of E2. With either form of E2, CAT activities from both HB-1CAT and ClaCAT are reduced to roughly the same levels (Fig. 4). This suggests that repression of P1 is specific to the competition between E2 and the cellular factor(s) which occurs at site I. It would be interesting to know the effect of having the E2ER1 enhancer also present on the P1 expression plasmid. However, the problem of introducing ATGs into the CAT mRNA leader sequence prevents this situation from being tested by a CAT assay. It is interesting to note the difference in the level of repression by E2-C and E2TR$^-$ (Fig. 4). This difference was not observed in the original description of E2 repression (28). In those experiments, the recipient cells were HeLa cells.

Downstream binding sites overlap constitutive enhancer. A constitutive enhancer has recently been mapped to the same region as the P1 promoter (31, 32, 34) and has been shown to activate transcription from the P2 through P5 promoters. To determine whether the sequences responsible for P1 promoter activity are also required for this enhancer activity, we transfected the constructs shown in Fig. 5A into CV-1, WHZ10, and HeLa cells. In these plasmids, the P3 promoter drives CAT gene expression; the BPV URR is positioned downstream in the opposite orientation, so that promoters within the URR, including P1, do not contribute to the expression of CAT message. The level of activation of P3 by the URR in this construct averaged about eightfold.

Deleting sequences downstream of position 7610 does not inhibit the stimulatory effect of the URR, indicating that elements in E2RE1 do not contribute to the constitutive enhancer activity we are measuring (Fig. 5, deletion B). Deletion C, which removes all of the sequences shown to affect the P1 promoter in vivo (3′ end point of deletion at position 7345), gives virtually undetectable levels of CAT activity in CV-1 cells, thus mapping at least part of the constitutive enhancer activity to this region. The effects of the individual protein binding domains on P3 activation were tested by using constructs D through J (Fig. 5). Only those

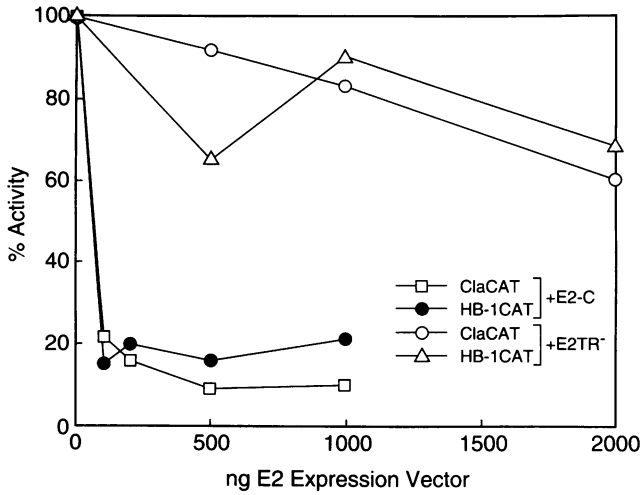


FIG. 4. Repression of the P1 promoter by E2 is independent of the number of E2 sites. The activities of ClaCAT were compared with those of HB-1CAT in the presence of increasing amounts of either the E2-C (repressor) or E2TR$^-$ (activator) proteins. Reporter plasmid (9 μg) was mixed with increasing amounts of an E2 expression vector and transfected into CV-1 cells as described in the legend to Fig. 3. The results are presented as percentages of the activity of the reporter in the absence of E2 protein. HB-1CAT contains a single E2 binding site, while ClaCAT contains four E2 binding sites.

deletions which affect one or more of the protected regions (−30, I, II, III, or IV region) have an effect on enhancer activity (Fig. 5, constructs E through H). Deletions outside the footprinted areas (D, I, and J) have either no effect or slightly elevated enhancer activities. Thus, the requirements for constitutive enhancer activity appear to overlap completely with the sequences required for P1 promoter activity.

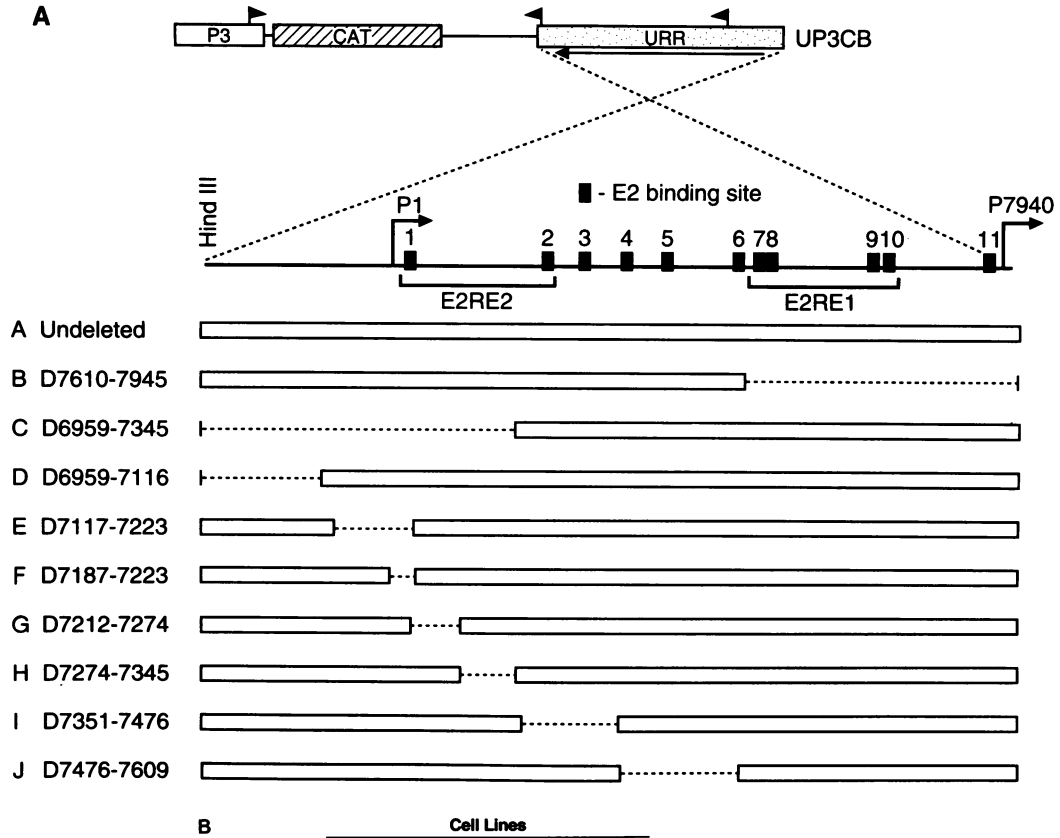
Similar results were obtained by using WHZ10 and HeLa cells (Fig. 5B). The measured effects of the various deletions on enhancer activity varied slightly between cell lines, but these differences correlate with the effects similar deletions have on P1 promoter activity. For instance, the level of construct H, which deletes binding sites III and IV, is down more than 30-fold in CV-1 cells, whereas in WHZ10 cells, there is only a mild effect due to deletion of these sites. For P1, deletion of these sites also has a more dramatic, 85-fold effect on promoter activity in CV-1 cells, whereas in WHZ10 cells, there is only a 6-fold effect, suggesting that these sequences have similar effects on P1 transcription and constitutive enhancer activity and may therefore represent the same proteins. It also suggests that the levels or activities of these proteins differ in different cell types. In WHZ10 cells, the most important factors are probably located upstream of the *Nar*I site, while in CV-1 cells, sequences downstream of *Nar*I have a more pronounced influence on transcription.

WHZ10 cells express the viral transactivator E2 protein. That we can measure an effect of deleting the constitutive enhancer, even in the presence of E2 stimulation, suggests that this enhancer may be an important component in the regulation of viral promoter activity. To determine whether removal of this region is deleterious to viral functions, we tested deletions of this region built back into the complete virus for their abilities to transform C127 cells. Deletion of site I (D234; positions 7187 to 7234) gave a small but reproducible reduction in number of foci, while a much larger deletion from *Hin*dIII to *Mlu*I (DHIII; positions 6959 to 7352), which removes the entire P1 promoter/enhancer region, resulted in no transformation (Table 1). This indicates that this region of DNA represents an important component in the regulation of viral gene expression in addition to the E2-inducible enhancer elements which have already been mapped and which correlate with recent results from vande Pol and Howley (35), in whose hands similar deletions (p1661 [Δ6958 to 7281] and p1662 [Δ6958 to 7351]; 35) also abrogate focus production. Interestingly, the effect on focus production correlates with the promoter mapping for P1. A deletion of site I (D28; positions 7187 to 7214) reduces focus production to levels similar to those produced by the D234 deletion. Both of these deletions showed reduced levels of transcription compared with P1. However, D20 (positions 7214 to 7234), which deletes the nonfootprinted region between sites I and II, gives wt transformation levels. An identical deletion had no effect on transcription levels from P1 tested by CAT assays (Fig. 3A).

DISCUSSION

We have mapped the sequences required for optimal in vitro and in vivo expression of the P1 promoter. The sequences which demonstrate this activity show a one-to-one correspondence with the sequences which are protected by proteins present in a HeLa cell extract. Furthermore, these same sequences show a one-to-one correspondence with a constitutive enhancer which influences the activities of other viral early promoters (31, 32, 35). These results are consistent with the hypothesis that the same proteins are functioning as proximal promoter factors for the P1 promoter as well as enhancer factors for additional promoters downstream. However, a more complete point mutational analysis will be required to definitively address this problem. An analogous situation has been described for the immunoglobulin heavy-chain enhancer (Ig-HCE; 30). The Ig-HCE has previously been shown to stimulate transcription from the variable region promoter. Su and Kadesch (30) have recently shown that, in addition, the Ig-HCE elements function as a promoter, directing transcription of the sterile transcript Iμ. The functional equivalence of enhancer and promoter factors in many situations has been recognized (7). For example, the flexibility in localization of a particular *cis*-acting site in a given promoter is probably more a consequence of its position within a transcription unit in relation to other factors than an intrinsic property of the factor itself (22, 24). Moreover, it is likely that any given factor stimulates transcription by the same pathway whether it contributes from a distal or a proximal position. Nevertheless, a transcription factor bound to a site is believed to act only on a single promoter. However, in those situations, such as that described here for BPV, in which a factor(s) binding to a single site can influence transcription from two or more promoters, it is not clear whether the factor involved can function in both capacities at the same time. For BPV, this functional duality of transcription factors may provide a mechanism for differential regulation of the activities of multiple viral promoters.

It is not yet clear which cellular transcription factors bind within the constitutive enhancer. Binding site I contains a CCAAT homology as well as a simian virus 40 enhancer core homology, but neither CTF nor AP2-AP3 binds to these sequences (4). The sequence A/GTTG is repeated eight times within the 200 bp which make up the in vivo P1 promoter (positions 7143 to 7352). Seven of these eight elements are located within or immediately adjacent to



B

Construct	Cell Lines CV-1	WHZ10	HeLa	Footprint Affected
A	1.0	1.0	1.0	none
B	1.1	–	1.1	none
C	0.01	–	–	TATA, I, II, III, IV
D	1.9	–	0.95	none
E	0.24	–	0.23	TATA, I
F	0.18	0.25	0.31	I
G	0.69	0.17	0.41	II
H	0.03	0.39	0.33	III, IV
I	0.98	1.6	0.76	none
J	2.4	0.81	1.4	none

FIG. 5. (A) Plasmids tested for URR-specific enhancement of P3 promoter activity. Deleted sequences are indicated by dotted lines, and the position of each deletion is indicated by nucleotide numbers. Deletions B through J were built into the plasmid UP3CB, which has the URR containing the *Hin*dIII-to-*Hpa*I fragment cloned downstream in the orientation opposite to that of P3 transcription. The P3 promoter is contained on a *Pst*I-to-*Sma*I restriction fragment (positions 575 to 945). CAT activity was tested in CV-1, HeLa, and WHZ10 cells. Assays were performed as described in the legend to Fig. 3. (B) Summary of the activities of constructs depicted in Fig. 5A. CAT activity was tested in CV-1, HeLa, and WHZ10 cells. Assays were performed as described in the legend to Fig. 3. Values represent averages from at least two separate experiments in which each plasmid was tested in duplicate and are normalized to those of the wt URR contruct (construct A).

regions protected by cellular proteins. Multiple copies of a similar sequence, G/ATTG, are found in the URRs of many HPVs and are often protected from DNase I digestion by proteins present in extracts of HeLa and CV-1 cells and in other cell types and tissues (9, 10). A similar sequence has been implicated in the constitutive enhancer activity of the HPV-11 URR (5). A more detailed mutational analysis of the P1 promoter should indicate whether this sequence is an important component of the promoter/enhancer activities of this region.

Cell-type specificity of these factors may be an important element regulating the activity of the promoter and enhancer. Vande Pol and Howley (34) have already shown that the sequences from positions 7162 to 7275 (sites I and II in this study) are ~10-fold more active in primary bovine keratinocytes, cells which would serve as a natural host for the virus, than in several other cell lines, including C127 and CV-1. In our study, binding sites III and IV exhibited the highest activities in CV-1 cells, while site II was almost inactive in these cells. The situation was reversed in WHZ10

TABLE 1. Transforming abilities of templates with P1 promoter regions deleted

Template	No. of colonies/μg of DNA in expt[a]:			
	1	2	3	4
BPV_{100}	69	63	103	172
D234	18	18	40	75
D20	86	ND	ND	ND
D28	18	ND	ND	ND
DHIII	ND	0	0	0

[a] ND, not determined.

cells, in which site II had the greatest effect on enhancer activity, while sites III and IV had the least effect. Certain of these factors may be specifically associated with the virus, since activity of the region between sites I and II has been detected only in virus-containing cells. An overall high level of constitutive enhancer activity is apparently not required for stable viral replication, since the virus is capable of episomal maintenance in C127 cells, in which this enhancer has relatively low activity. This enhancer may influence the extent of replication, however (34), possibly by a direct effect on levels of replication proteins (35).

The effect of P1/enhancer deletions on the induction of foci provides evidence that this region is an important element for regulating viral gene expression. Preliminary results from our laboratory also suggest that the largest deletion (DHIII) inhibits the ability of the virus to replicate (3). Vande Pol and Howley have made similar observations and suggest that these effects are the direct result of decreased E2 levels (35); however, all promoters which utilize this enhancer region may be affected, and the phenotypes of the deletions may be suppressed by elevated E2 levels supplied in *trans*. The low activity of P1 in infected cells makes it likely that these mutant phenotypes result from a defect in the constitutive enhancer rather than a decrease in P1 activity.

The function of the P1 promoter in BPV gene expression is obscure. The facts that this region binds cellular factors and that transcription is solely dependent on these factors suggest that the P1 promoter could be an important regulator of transcription immediately after infection. Thus, expression from the P1 promoter is possible without E2 activation. Our analysis of enhancer activity in WHZ10 cells (Table 1) suggests also that the P1 promoter potentially remains active beyond the initial infection, even in the presence of concurrent E2-dependent stimulation. The low activity of P1 promoter in infected cells may reflect its repression after establishment of latency. E2 can repress the level of P1 transcription by up to 1 order of magnitude (28; this study). In the absence of E2, competition between the P1 start site and downstream promoters (such as P3) for the P1-associated enhancer factors may modulate the level of expression down at both sites. By binding to its site in this region (BS1), the E2 protein represses starts from the P1 promoter and may block the assembly of a preinitiation complex at P1. These activities may serve to liberate cellular factors which are bound to the downstream constitutive enhancer to activate transcription from other viral promoters. Along these lines, it is also interesting that the late polyadenylation signal overlaps with the P1 TATA box as defined here; competition between the transcription initiation complex and the end processing sequences could also have regulatory consequences in vegetative replication. Whether such switches are utilized in the natural life cycle of the virus awaits further elaboration.

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