The EMBO Journal vol.7 no.9 pp.2815-2822, 1988

The bovine papillomavirus P_{2443} promoter is E2 *trans*-responsive: evidence for E2 autoregulation

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Communicated by R.Kamen

The bovine papillomavirus type 1 (BPV-1) P_{2443} promoter is located just upstream of the E2, E3, E4 and E5 open reading frames (ORFs) and is active in both transformed rodent cells and in productively infected warts. Analysis of viral RNA structures suggests that transcripts from this promoter encode the E2 transactivator as well as the E5 oncoprotein. To study expression of P_{2443}, the chloramphenicol acetyltransferase (CAT) reporter gene was placed downstream of this promoter, deleting the E2 and E5 ORFs, in a plasmid which contained all BPV-1 upstream sequences including the long control region (LCR). By itself, this plasmid demonstrated a low level of activity in transient assays and could be transactivated to a high level by the full-length E2 product. Transactivation of P_{2443} expression by E2 required the LCR *in cis* in an orientation- and position-independent manner, suggesting that this transactivation was mediated through the E2 responsive enhancer elements (E2RE) located within the LCR. Primer extension analysis of the 5′ ends of the viral transcripts from pooled cells expressing these P_{2443}/CAT plasmids confirmed that E2 transactivation results in an increase in the steady-state levels of RNA initiated from the P_{2443} promoter. Furthermore, E2 transactivation of the P_{2443} promoter could be inhibited by the *trans*-repressor encoded by the 3′ portion of the E2 ORF. Thus, expression of the E2 transactivator and the E5 oncoprotein is directly regulated by transcriptional factors encoded by the E2 ORF.

Key words: transcription/autoregulation/E2 transactivation

Introduction

Bovine papillomavirus type 1 (BPV-1) causes fibropapillomas in cattle and can latently infect and transform rodent fibroblasts in tissue culture where the viral DNA is maintained as a stable extrachromosomal plasmid. Only one strand of the BPV-1 genome is transcribed in infected cells (Amtmann and Sauer, 1982; Heilman *et al.*, 1982; Engel *et al.*, 1983) and the transcriptional organization is complex (Baker and Howley, 1987). Multiple promoters and complicated patterns of splicing have been revealed by cDNA and RNA analyses of both productively infected warts and latently infected tissue culture cells (Stenlund *et al.*, 1985; Yang *et al.*, 1985a; Baker and Howley, 1987). Splice donor sites at nt 304, 864, 1235, 2505, 3764 and 7385 and splice acceptor sites at nt 528, 3225, 3605 and 5609 have been identified (Baker, 1987). Five active promoters have thus far been identified in BPV-1-transformed cells and in infected dermal fibroblasts of a BPV-1-induced fibropapilloma (Baker and Howley, 1987). At the 5′ end of the early region is a 1-kb region which contains no large open reading frames (ORFs) and which has been referred to as the long control region (LCR) and sometimes as the upstream regulatory region. The LCR contains the origin of DNA replication (Waldeck *et al.*, 1984), sequences required for plasmid replication (Lusky and Botchan, 1984), DNase hypersensitive sites Rösl *et al.*, 1983) as well as viral transcriptional promoters, which include P_{7185}, P_{7940} and P_{89}. The P_{89} promoter, which is located just upstream of the E6 ORF, appears to regulate expression of the 5′ ORFs E6 and E7 (Stenlund *et al.*, 1985; Yang *et al.*, 1985a). This promoter, along with P_{7940}, is transactivated by the full-length E2 gene product (Haugen *et al.*, 1987; Spalholz *et al.*, 1987). E2 transactivation of these promoters occurs through an interaction of the full-length E2 gene product with the $ACCN_6GGT$ motifs in $E2RE_1$ which is located between nt 7611 and 7806 (Spalholz *et al.*, 1987, 1988). The $ACCN_6GGT$ motifs have been shown to be the sites to which the E2 gene products bind (Androphy *et al.*, 1987; Moskaluk *et al.*, 1987; McBride *et al.*, 1988). The 3′ portion of the E2 ORF encodes a factor expressed from a downstream promoter P_{3080} which can specifically repress E2 transactivation mediated through $E2RE_1$ (Lambert *et al.*, 1987).

Another major BPV-1 promoter, P_{2443}, has been identified just upstream of the complete E2 ORF as well as E3, E4 and E5 (Yang *et al.*, 1985a; Ahola *et al.*, 1987; Baker and Howley, 1987). Analysis of RNA structures from BPV-1-transformed cells suggests that this promoter directs expression of two important products: E2 and E5. Mutants in E2 are defective in stable plasmid replication and in transformation of contact-inhibited C127 cells (Sarver *et al.*, 1984; DiMaio, 1986; Groff and Lancaster, 1986; Rabson *et al.*, 1986b). These defects in DNA replication and transformation are probably due to indirect effects resulting from the inability to transactivate the E2 dependent enhancers of the LCR. The E5 gene product is directly involved in transformation and may also play a role in stable plasmid replication (Yang *et al.*, 1985b; Groff and Lancaster, 1986; Rabson *et al.*, 1986b; Schiller *et al.*, 1986; Schlegel *et al.*, 1986). The 3′ half of the E2 gene and the E5 gene, however, may also be expressed from upstream promoters through the splice acceptor site at nt 3225 (Yang *et al.*, 1985a; Baker and Howley, 1987; Hermonat and Howley, 1987). To date, two species of transcripts which initiate from the P_{2443} promoter have been identified. One RNA is unspliced and encodes the E2 gene product. The other, which accounts for 90% of the RNAs from this promoter, is spliced from nt 2505 to 3225 (Yang *et al.*, 1985a; Baker and Howley, 1987) and it has been postulated that this RNA is the major E5 RNA (Baker, 1987). It has been demonstrated that a cDNA representative of this spliced message can transform

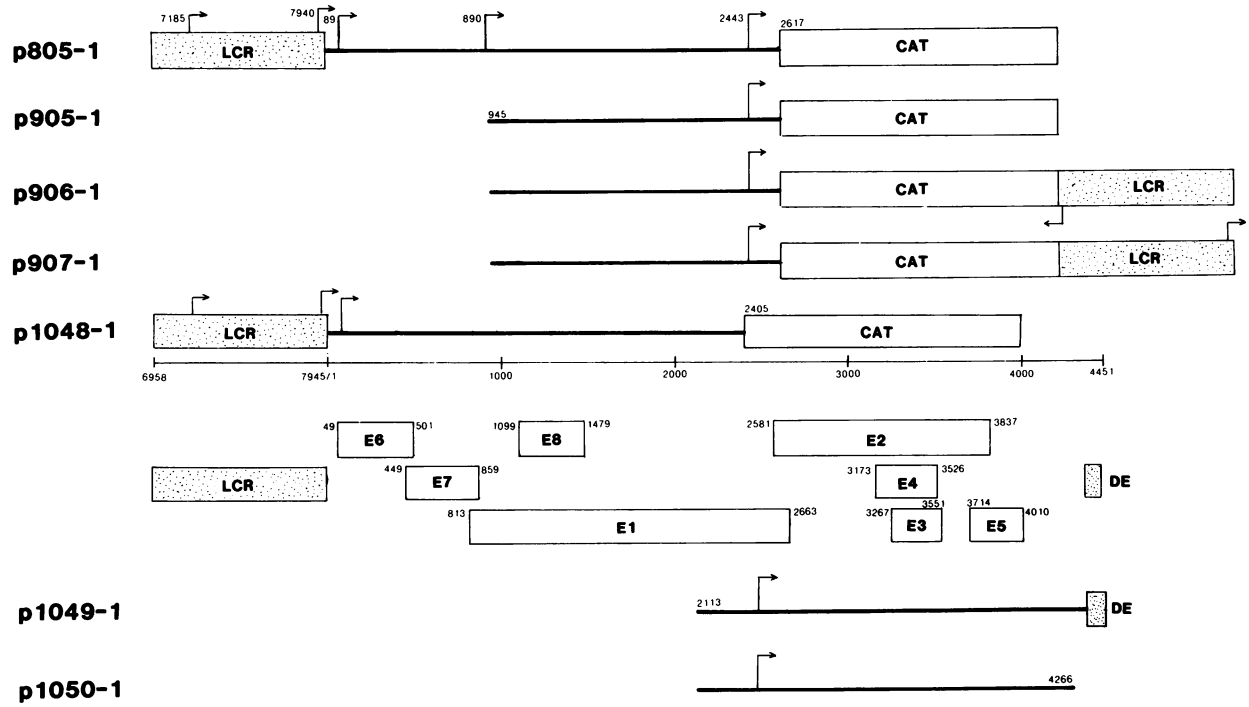

Fig. 1. Structure of BPV-1 recombinant plasmids. The genomic organization of BPV-1 is shown in the middle of the figure for reference. The left column names the plasmids whose structures are shown on the right. The nucleotide positions in the BPV-1 genome are indicated. Solid lines indicate BPV-1 sequences, open boxes represent the CAT gene or the ORFs, and stippled boxes represent the indicated *cis* regulatory regions. Various promoters are shown as bent arrows (Baker and Howley, 1987; M.R.Botchan, personal communication). Abbreviations are as indicated in the text. CAT refers to the sequences derived from pSV2-CAT (Materials and methods) and includes the prokaryotic CAT gene, the SV40 small T splice sequences, and the early polyadenylation sequences. DE indicates the distal enhancer (Lusky and Botchan, 1983).

C127 cells at low levels when expressed from the SV40 early enhancer/promoter (Hermonat and Howley, 1987), although the unspliced cDNA from the P_{2443} in the same SV40 based vector is much more efficient at inducing transformation (Yang *et al.*, 1985a). A mutation in this unspliced cDNA or in the full length BPV-1 genomic background, eliminating the 3′ splice junction at nt 3225, drastically reduces transformation efficiency suggesting that the nt 2505−3225 splice is required to generate stable E5 transcripts (Hermonat and Howley, 1987). It therefore appears that P_{2443} plays an important role in stable plasmid maintenance as well as in transformation. In this study we have analyzed the regulation of the P_{2443} promoter to determine its *cis* and *trans* requirements for expression and to discern its role in overall BPV-1 gene regulation. We have found that it is responsive to E2 *trans*-regulation through *cis*-acting sequences in the LCR.

Results

E2 transactivation of P_{2443}/CAT requires the LCR in cis

To study the expression and regulation of the P_{2443} promoter, the prokaryotic CAT reporter gene was placed downstream of the promoter at the *Sph*I site (nt 2617) as described in Materials and methods. The resulting plasmid, p805-1 (shown in Figure 1) contains BPV-1 sequences upstream from nt 2617 through the LCR to the *Hin*dIII site at nt 6958. Activity from the P_{2443} promoter was assayed by measuring levels of CAT activity from cell extracts after transfection of p805-1 into CV1 cells or the C127 cell subclone (PH116). In the initial experiments only very low levels of CAT activity could be observed using p805-1 by itself. High-level expression was noted, however, when p805-1 was co-transfected into CV1 cells with C59 or C59-3881 which express the full-length E2 transactivator product from the SV40 early promoter (Yang *et al.*, 1985a; Spalholz *et al.*, 1985; Yang *et al.*, 1985b). Similarly, with C127 cells, CAT expression from p805-1 required co-transfection of a full length BPV-1 construct such as p142-6 with an intact E2 ORF (data not shown). This suggested that the P_{2443} promoter of p805-1 could be transactivated by E2.

We next determined whether CAT expression from p805-1 required the LCR *in cis*. The LCR has previously been shown to contain E2-responsive elements (Spalholz *et al.*, 1985, 1987; Haugen *et al.*, 1987) which can activate transcription from heterologous promoters, and specifically from the BPV-1 promoters P_{7940} and P_{89}. To test whether the E2 transactivation of the P_{2443} promoter was also mediated through responsive elements in the LCR, a series of plasmids derived from p805-1 was constructed and is shown in Figure 1. The LCR and sequences from nt 1−945 containing the E6, E7 and a 5′ segment of the E1 ORF were deleted to generate the plasmid p905-1. The 1-kb (nt 6958−7945) LCR fragment was then reinserted 3′ of the CAT expression cassette at the *Bam*HI site in the anti-sense or sense orientation to generate the plasmids p906-1 and p907-1 respectively. These plasmids were then individually transfected into CV1 cells in the presence or absence of the E2 viral product and assayed for CAT activity. The results are shown in Figure 2. The plasmid p407-1, which contains the LCR upstream of the SV40 enhancer deleted early promoter and CAT gene, can be transactivated by C59 and served as a control (Spalholz *et al.*, 1985). Like p407-1, each

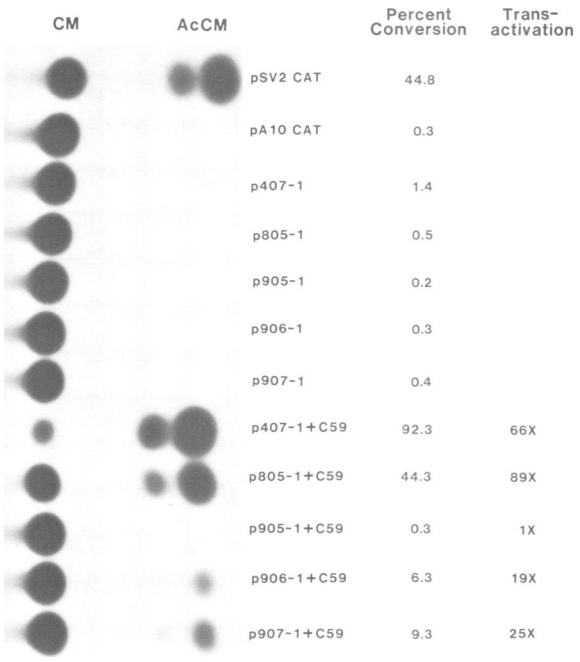

Fig. 2. Transactivation of p407-1, p805-1 (P_{2443}/CAT) and p805-1 derivatives. CV1 cells were transfected with 2 μg of the CAT-containing plasmid, 1 μg of C59 where indicated and 18 μg of high mol. wt salmon sperm DNA as carrier. The assay was carried out as described in Materials and methods with a reaction time of 1 h. The transactivation ratios were determined by dividing the percentage acetylated chloramphenicol obtained from cellular extracts transfected with the reporter plasmids plus C59 divided by the percentage acetylation obtained using the same plasmid transfected without C59. CM and AcCM are the non-acetylated and acetylated forms of chloramphenicol respectively.

of the four P_{2443}/CAT constructs being tested expressed only low levels of CAT activity in the absence of E2. When transfected with C59, however, p805-1, p906-1 and p907-1 were strongly transactivated, generating high levels of CAT (Figure 2), whereas p905-1 did not. Each of the constructs that was active in the presence of E2 contained the LCR in *cis* and p905-1 which does not contain the LCR was not. Similar results were obtained in the C127 cell subclone PH116 using the full length BPV-1 genome (p142-6) to provide E2 (data not shown). Thus, these data indicated that high-level expression of CAT from p805-1, p906-1 and p907-1 required the LCR *in cis* and the E2 product *in trans*. In p906-1 and in p907-1, in which the LCR is downstream of the CAT gene and of the SV40 small T splice and polyadenylation sequences, the only known promoter in the BPV-1 segment upstream of CAT is P_{2443}. This suggested that transactivation was mediated through the P_{2443} promoter.

Transactivation of P_{2443} is transcriptional

To examine whether the transactivation of the P_{2443}/CAT plasmids by E2 was due to an increase in the steady-state levels of CAT RNA from the P_{2443} promoter, we analyzed the CAT RNA expressed in cells transfected with the various reporter plasmids described above. Pooled lines of drug-resistant cells expressing these plasmids were established as described in Materials and methods by co-transfecting the P_{2443}/CAT plasmids into PH116 cells with or without the E2 expressing cDNA, C212 (Yang *et al.*, 1985a) and pSV2-Neo. Total RNA was isolated from these pooled cells

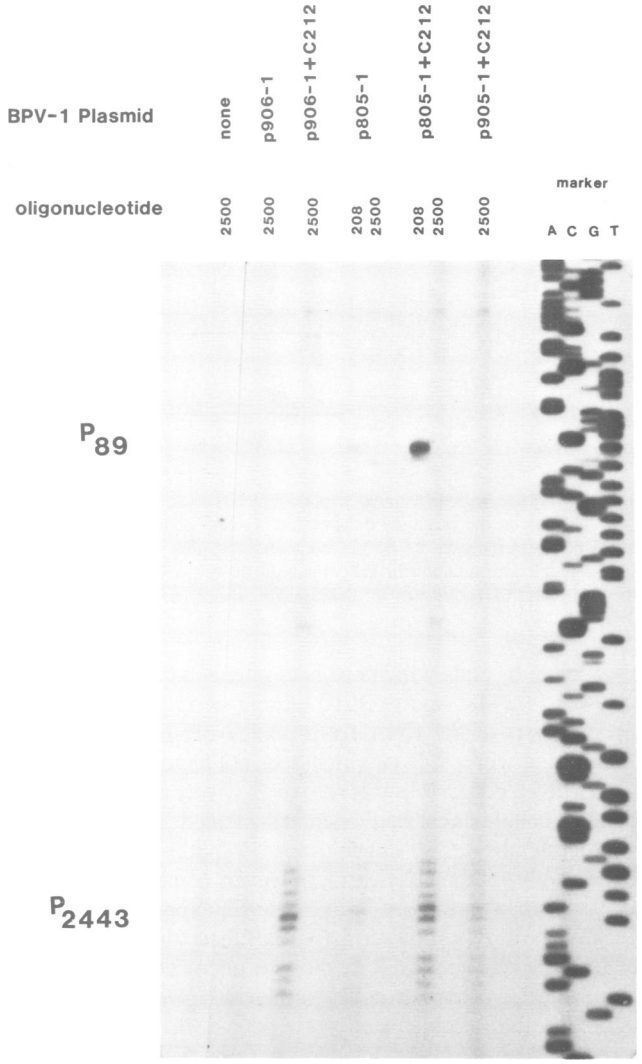

Fig. 3. Primer extension analysis of CAT RNA extracted from pooled cell cultures of the C127 cells transfected with the indicated BPV-1 plasmids and pSV2-Neo. The oligonucleotide primers used are as indicated and described in Materials and methods. The 5′ nt 2500 primer and the 5′ nt 208 primer were used to analyze the P_{2443} and P_{89} promoters respectively. The positions of the P_{89} and P_{2443} promoter initiation sites are indicated on the left of the autoradiograph. On the right is a sizing dideoxynucleotide sequencing ladder using the nt 2550 oligomer as a primer and a complementary M13mp7/BPV-1 DNA as template.

at ~4 weeks post-transfection and used as a template for reverse transcription primer extension analysis. A 20-base synthetic oligonucleotide complementary to the BPV-1 coding strand with a 5′ terminus at nt 2500 was end-labeled with ^{32}P and served as a primer to assess initiation from the P_{2443} promoter. This primer will only anneal to transcripts from the P_{2443}/CAT plasmids and not to those from C212 since the 5′ end of the BPV-1 sequences of C212 is at nt 2535 (Yang *et al.*, 1985a). Because the primer oligonucleotide is present in great molar excess, the products of the reverse transcription provide a quantitative measure of the levels of complementary RNA to which it can anneal. The reverse transcription products were separated on a sequencing gel alongside a marker dideoxy sequence reaction ladder created using the same primer, and ssDNA from a chimeric M13/BPV-1 recombinant as the template. The results are shown in Figure 3. The RNAs initiated from

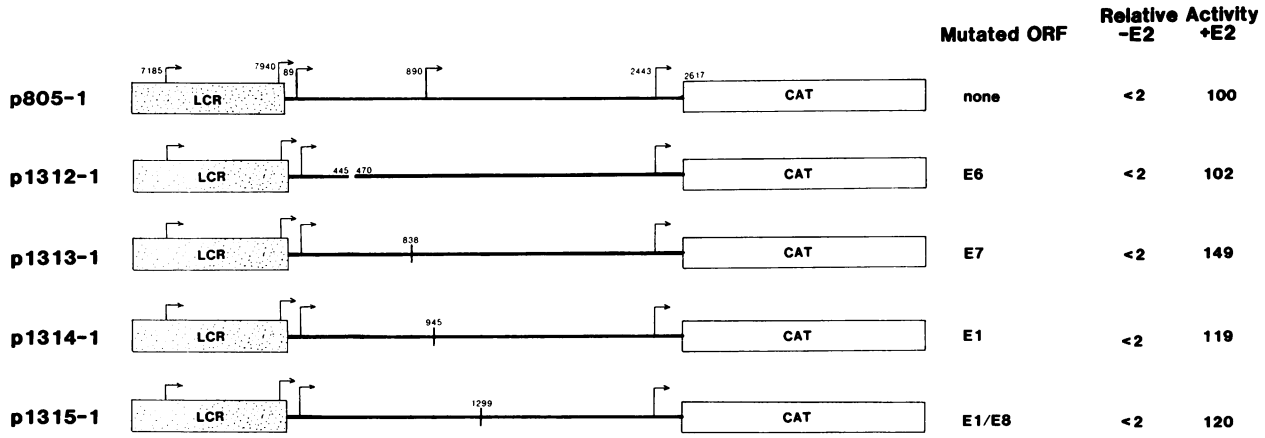

Fig. 4. Transactivation of mutated p805-1 derivatives. The names of the plasmids are indicated to the left and the structures to the right. The single vertical lines on the plasmids indicate the position of termination codon linker insertions and the broken line indicates the position of a deletion. The small numbers indicate the BPV-1 nucleotide position. The first column on the right indicates the mutated ORF of the plasmid structure. The p1312-1 deletion mutation in E6 extends to the E7 ORF but does not affect the initiation codon of the E7 ORF. The two columns on the right give the relative CAT activity of the indicated plasmids compared to the activity of p805-1 in the presence or absence of C59 using p805-1 plus C59 as 100%.

P_{2443} have 5′ ends which are heterogeneous and map from nt 2438 to 2448, and at 2410 (Ahola *et al.*, 1987; Figure 3). Analysis of the RNA from pooled drug-resistant cells transfected with p905-1 (which does not have the LCR) and C212 revealed very low levels of P_{2443} promoter activity. Pooled cells which had been transfected with plasmids containing the LCR, either 5′ or 3′ (p805-1 and p906-1 respectively) of the CAT gene, however, contained abundant P_{2443} products in the presence of the E2 expressing plasmid C212. The P_{2443} promoter activity of these two plasmids in cells in the absence of E2 was low or undetectable. Activity from the P_{89} promoter could similarly be observed in the pooled cells co-transfected with p805-1 using a complementary 20-base oligonucleotide with its 5′ terminus at nt 208 as a labeled primer. As shown in Figure 3, this promoter was also transactivated by E2 confirming previous results that P_{89} is E2 responsive (Haugen *et al.*, 1987; Spalholz *et al.*, 1987). These data indicated further that the transactivation of CAT observed with the P_{2443}/CAT plasmids was mediated by transcriptional activation of the P_{2443} promoter by E2 and that this transactivation requires the LCR *in cis*.

E2 transactivation of P_{2443} is not dependent on other viral encoded factors

We next determined whether E2 transactivation of P_{2443} in p805-1 was only dependent on the presence of the LCR *in cis*, or whether it was the indirect result of the activation of another gene whose product acted directly on P_{2443}. Although p906-1 and p907-1, which could be transactivated by E2, had deletions of E6, E7 and the 5′ end of E1, the possibility existed that the mechanism by which these plasmids were transactivated might be different than p805-1 since the LCR in these plasmids had been positioned 3′ of the CAT gene transcriptional cassette. Mutations were therefore introduced independently into each of the BPV-1 early ORFs in the plasmid p805-1 as shown in Figure 4. This was accomplished as described in Materials and methods creating deletions within the p805-1 background in E6 (dl nt 445–470), E7 (termination linker insertion at nt 838), E1 (termination linker insertion at nt 945) and E1/E8 ORFs (termination linker insertion at nt 1299), generating p1312-1, p1313-1, p1314-1 and p1315-1 respectively. Although the deletion in p1312-1 extends to the 5′ end of the E7 ORF, it is upstream of the first E7 methionine codon at nt 479 and we therefore considered it to be an E6-specific mutation. Each of these mutated plasmids was assayed for CAT activity in the presence or absence of the E2 expression plasmid C59, and found to have approximately the same activity as p805-1 (Figure 4). These data indicated that no

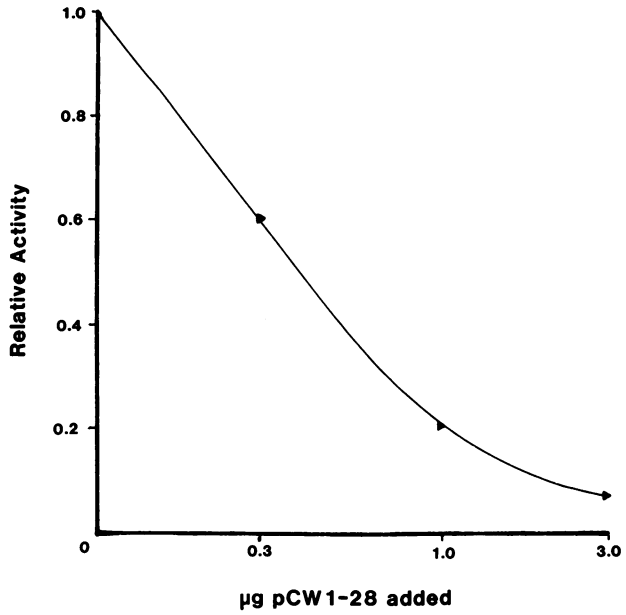

Fig. 5. Inhibition of P_{2443} transactivation by the 3′ E2 repressor. CV1 cells were transfected with 2 μg of p805-1, 0.3 μg of C59 and 18 μg of high mol. wt salmon sperm DNA as carrier, plus the indicated amount of pCW1-28 (E2-tr) or C48 (E7). The CAT assays were performed as described in Materials and methods. The data were normalized for each cDNA concentration, with the amount of conversion observed with C48 at each concentration defined as 100%. Increasing amounts of C48 resulted in a slight non-specific inhibition of CAT activity. The addition of 3.0 μg of C48 resulted in a 57% inhibition of CAT activity compared to p805-1 and C59 without C48.

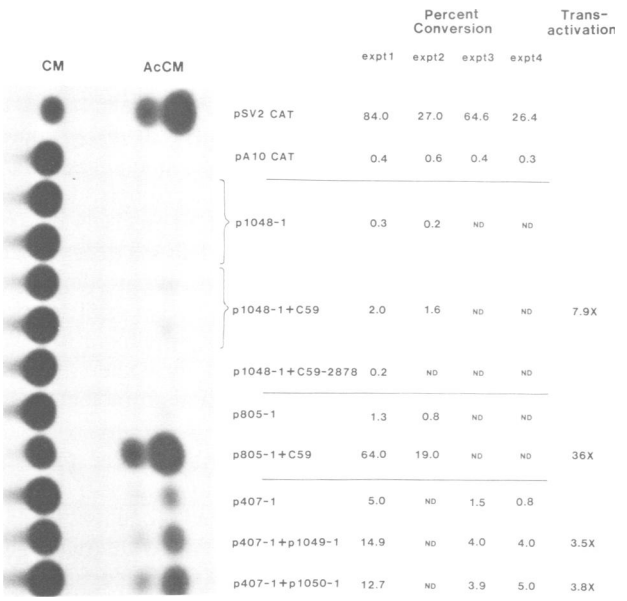

Fig. 6. Direct and indirect CAT analysis of E2-independent activity of the P_{2443} promoter. The indicated plasmids were transfected into CV1 cells as described in Materials and methods. The CAT reactions were allowed to proceed for 3 h. The columns on the right indicate four separate experiments and the transactivation numbers represent the percent conversion from extracts of transfections of the indicated plasmids with C59 divided by the conversion of the same plasmid alone. The brackets indicate assays of duplicate plates. **Lanes CM** and **AcCM** are the unacetylated and acetylated forms of chloramphenicol respectively.

BPV-1 encoded product other than the E2 transactivator was required for the transactivation of the P_{2443} promoter, and that the E2-mediated transactivation of this promoter through the LCR is direct.

Transactivation of P_{2443} can be inhibited by the 3′ E2 repressor

A previous study has mapped a transcriptional repressor (E2-tr) to the 3′ portion of the E2 ORF (Lambert *et al.*, 1987). This repressor shares a carboxy-terminal DNA binding domain with the full-length E2 gene product (McBride *et al.*, 1988) and is believed to inhibit E2 transactivation by directly competing for the E2 binding sites of the LCR. To determine if E2 transactivation of the P_{2443} promoter could also be repressed by E2-tr, p805-1 was co-transfected with C59 into CV1 cells with increasing amounts of a cDNA, pCW1-28, which expresses the repressor. This cDNA was originally cloned from RNA isolated from a bovine fibropapilloma (Baker and Howley, 1987). As a negative control, C48, a possible E7 cDNA which does not encode the repressor activity was also used (Yang *et al.*, 1985a). As shown in Figure 5, transfection of increasing amounts of pCW1-28 resulted in a strong repression of E2-mediated transactivation of p805-1. The data presented in Figure 5 show the level of CAT expression in the presence of pCW1-28 relative to that in the presence of C48. Co-transfection of 3 μg of pCW1-28 (a 10-fold excess over C59) resulted in a 93% repression of CAT activity. Thus, like the P_{89} promoter, the P_{2443} promoter is also subject to both positive and negative E2 transregulation. Similar experiments were also carried out with p906-1 which showed that it was also subject to repression by pCW1-28 (data not shown) indicating that the negative regulation of P_{2443} through repression of E2 occurred through the LCR in an orientation-independent manner.

The P_{2443} promoter exhibits an E2-independent activity which does not require the 'distal enhancer'

These data indicate that the P_{2443} promoter was subject to both positive and negative transregulation by the E2-encoded factors and that the full-length E2 gene product which is expressed from the P_{2443} positively autoregulated its own expression. This raised the question of what activates transcription from P_{2443} in newly infected cells. One potential mechanism by which expression of E2 might initiate is via an E2-independent activity of the P_{2443} promoter. Alternatively, since the E2 gene is located downstream of several other viral promoters (e.g. P_{89}), it is possible that E2 and E5 could also be expressed from an upstream promoter. To address this question, the plasmid p1048-1 was made from p805-1 by deletion of the BPV-1 sequences from nt 2405 to 2617 as described in Materials and methods initiation codon as described in Materials and methods (Figure 1). This deletion removes all the sequences corresponding to the known initiation sites of the P_{2443} promoter (Ahola *et al.*, 1987; Figure 3) as well as an A−T-rich region which may serve as the TATA box of this promoter. The plasmid p1048-1 and the parental plasmid p805-1 were then transfected into CV1 cells and assayed for CAT activity in the absence or presence of the E2 expression plasmid C59 (Figure 6). Both p1048-1 and p805-1 expressed very low levels of CAT without E2, although the levels observed with p805-1 were 4- to 5-fold higher activity than those seen with p1048-1. This difference suggested that some low level of expression from P_{2443} could occur in the absence of E2. Each of these two plasmids could also be transactivated by E2, although p805-1 could be transactivated to give much higher levels of CAT activity than p1048-1. As a control for potential recombination between p1048-1 and C59 (which would place the SV40 early enhancer/promoter directly upstream of the CAT gene) a plasmid derived from C59 with a mutated E2 gene (C59-2878; Yang *et al.*, 1985b) was transfected with p1048-1. In this co-transfection, no higher CAT activity was observed indicating that the CAT levels observed were not a consequence of plasmid recombination. It was therefore apparent that a low level of E2-responsive CAT activity continued to be expressed from p1048-1. The comparison of p1048-1 and p805-1 CAT activity in the absence of the E2 transactivator suggested that a low level of E2-independent activity mapped to the region of the P_{2443} promoter. The comparison of activities of these same two plasmids in the presence of E2 transactivator suggested that either the crippled P_{2443} promoter in p1048-1 is still responsive to E2 transactivation or that the CAT expression seen from p1048-1 is from an upstream E2-responsive promoter, or both.

We have further analyzed the E2-independent activity of the P_{2443} promoter using an indirect assay of the E2 protein itself. A plasmid containing the subgenomic region of the BPV-1 P_{2443} promoter directing expression of the E2 gene in the natural configuration would, if active, be able to transactivate an E2-responsive CAT plasmid such as p407-1 (LCR-CAT). The wild-type BPV-1 genome plasmids, p142-6, and a genomic plasmid with the distal enhancer p735-1 (Howley *et al.*, 1985) deleted, were digested with *Eco*RI and religated to generate the plasmids p1049-1 and

p1050-1 respectively (Figure 1). The plasmid p1049-1 includes the BPV-1 sequences from nt 2113 to 4450 containing the full-length E2 ORF and the P_{2443} promoter. The plasmid p1050-1 was similar, but was further deleted of sequences from nt 4266 to 4450 which removed the distal enhancer previously described by Lusky and Botchan (1983) (Howley *et al.*, 1985). To test if these plasmids expressed E2 and transactivated an E2 conditional enhancer each was co-transfected with p407-1 into CV1 cells, and assayed for CAT levels. As shown in Figure 6, p1049-1 and p1050-1 each could transactivate p407-1 to levels ~3.5- and 3.8-fold respectively. The isolated *Eco*RI to *Bam*HI fragment from p142-6, containing the same BPV-1 sequences as included in p1049-1, could also transactivate p407-1 at low levels (data not shown). These data, although indirect, indicated that there was a low level of E2-independent expression from the P_{2443} promoter in p1049-1 and p1050-1. Furthermore, the ability of p1050-1 to provide E2 transactivation at levels similar to that of p1049-1 suggested that the 'distal enhancer' was not required for this activity.

Discussion

This analysis of P_{2443} promoter regulation revealed an important additional regulatory pathway involved in BPV-1 gene expression. The P_{2443} promoter has a central role in BPV-1 gene regulation as it directs the synthesis of both the E2 and the E5 gene products (Yang *et al.*, 1985a,b). The full-length E2 product has been previously shown to be required for high levels of expression from the P_{89} and P_{7940} promoters through transactivation of $E2RE_1$ in the LCR (Spalholz *et al.*, 1987). Mutations in E2 result in BPV-1 genomes which are defective in both plasmid maintenance and transformation (DiMaio, 1986; Groff and Lancaster, 1986; Rabson *et al.*, 1986b). Approximately 10% of the transcripts from the P_{2443} promoter are unspliced and could code for E2 (Baker and Howley, 1987). The remainder are spliced from nt 2505 to 3225 and likely code for E5. A cDNA representative of this transcript (Yang *et al.*, 1985a) can transform PH116 cells but at lower levels than the unspliced P_{2443} cDNA suggesting that the splicing event is needed for efficient E5 expression (Hermonat and Howley, 1987). This spliced cDNA is also capable of complementing a transformation defective BPV-1 mutant to 10-fold higher levels than either plasmid alone (P.L.Hermonat and P.M.Howley, in preparaton).

The data presented in this study indicate that high levels of expression from the P_{2443} promoter require the LCR *in cis* and the E2 product *in trans*. The dependence of P_{2443} on E2 for expression provides a mechanistic explanation for the transformation defectiveness of E2 mutants due to their inability to express levels of E5 sufficient for transformation. This conclusion is supported by a recent study employing a reporter gene inserted in place of the E5 gene which demonstrates E2 regulation of E5 (Prakash *et al.*, 1988). Furthermore, P_{2443} transactivation can be repressed by the E2-tr encoded by the 3′ portion of the E2 ORF providing an explanation for the striking transformation inhibitory activity imparted by E2-tr.

These data implicate the involvement of the E2-responsive elements in the LCR, which are required for high levels of P_{89} promoter expression, as the same *cis* sequences needed for P_{2443} promoter expression. It should be noted that there is an E2 binding motif at nt 2397−2408 just upstream of P_{2443} which could potentially bind E2. Although one E2 binding site has no enhancer activity by itself (Hawley-Nelson *et al.*, 1988; Hirochika *et al.*, 1988; Spalholz *et al.*, 1988), E2 protein bound at the motif near P_{2443} could potentially interact with other E2 proteins bound to sites in the LCR to activate and regulate transcription at P_{2443}. Experiments are underway to define more precisely the *cis* elements involved in P_{2443} regulation. This *cis* dependence is somewhat surprising, however, because of the large distance between the P_{2443} and the LCR. No viral factors other than those encoded by the E2 ORF were found to have an effect on the P_{2443}. Thus, the P_{2443} promoter may be viewed as being similar to the LCR region promoters, such as P_{89}, but removed by 2.5 kb.

Our data indicate that the full-length E2 gene product autoregulates its expression in a positive manner and is subject to negative regulation by a second product of the E2 ORF, E2-tr, through P_{2443}. Perhaps this is a strategy to ensure balanced expression of the critical E2 and E5 genes from P_{2443}. Runaway expression from the P_{2443} promoter does not occur in warts or in latently infected tissue culture cells, indicating that like P_{89}, P_{2443} is maintained in a repressed mode (Yang *et al.*, 1985; Baker and Howley, 1987). We should point out that the full-length E2 protein could also be expressed from promoters upstream of P_{2443}. E2 transactivator expression from such promoters could arise possibly from polycistronic messenger RNAs or possibly through the use of a splice acceptor upstream of the E2 ORF (Stenlund *et al.*, 1985; Ahola *et al.*, 1987).

Surprisingly p1048-1, which has a crippled P_{2443} promoter, can still be transactivated by E2 (Figure 6). A cryptic promoter could be responsible for this activity and further experiments will be necessary to determine the origin of RNA initiation from this plasmid. These data also support the possibility that some level of expression of the E2 and E5 ORFs may be from upstream E2-responsive promoters. In the case of the E5 ORF this hypothesis is further supported by the putative E6-E4 cDNA, C119 (Yang *et al.*, 1985a), which is able to transform PH116 cells at very low levels (P.L.Hermonat and P.M.Howley, in preparation). A mutation in the E5 ORF of this cDNA eliminates this transformation activity indicating that E5 can be expressed to some level from this cDNA which derives from P_{89}.

The P_{2443} promoter also appears to have a low-level activity independent of E2 or the LCR. E2-independent activity of this promoter could be detected by direct RNA primer extension analysis of transfections with p805-1 (Figure 3). Two additional assays demonstrated this activity. In a direct analysis with the CAT gene located at nt 2617 (p805-1, Figure 6) there was a low level of CAT activity without co-transfection of an E2 expression plasmid (e.g. C59). Deletion of the sequences between nt 2405 and 2617 (p1048-1) in this plasmid background reduced E2-independent activity by 80% (Figure 6). Furthermore, in an indirect analysis of P_{2443} activity, both p1049-1 (nt 2113−4450) and p1050-1 (nt 2113−4250) were able to express E2 and transactivate expression from the E2-dependent CAT plasmid p407-1 (Figure 6). As the P_{2443} promoter is the only known BPV-1 promoter present 5′ of the E2 ORF in these constructs, these data suggest a low-level constitutive E2-independent activity for the promoter.

Materials and methods

Cells

Simian CV1 cells and a single cell subclone of murine C127 cells (subclone PH116) selected for efficient transformation were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (GIBCO Laboratories). Cells were fed every 3–4 days.

Recombinant plasmids

The recombinant plasmids p142-6, p407-1, C212, p51-1 and p735-1 have been described previously (Sarver *et al.*, 1982; Howley *et al.*, 1985; Spalholz *et al.*, 1985; Yang *et al.*, 1985a). A BPV-1 P_{2443}/CAT plasmid, p805-1, was constructed by ligating the 1.6-kb *StuI–BamHI* fragment of pSV2-CAT (Gorman *et al.*, 1982; Laimins *et al.*, 1982) to the *SphI* to *Bam*HI fragment from p51-1 (Sarver *et al.*, 1982) which contains the BPV-1 sequences clockwise from nt 6958 to 2617. The *StuI–Bam*HI fragment of pSV2 CAT includes 17 bp of SV40 DNA upstream of CAT, the CAT gene and the SV40 small T splice and polyadenylation sequences. The ligation with BPV-1 sequences at nt 2617 placed the start codon of the CAT gene downstream from the *2443* promoter. Although the first methionine initiation codon of the E2 ORF located at nt 2608 is present in the construct, it is followed by a translational termination codon prior to the CAT ATG. The plasmid p905-1 was derived from p805-1 by sequential restriction endonuclease digestion with *Cla*I and *Sma*I, overhang fill-in with Klenow fragment, circularization and ligation with an 8-bp *Xho*I linker. This plasmid, p905-1, contained the BPV-1 sequences from nt 945 to 2617 upstream of the CAT gene (Figure 1). The BPV-1 *HpaI–Hin*dIII fragment containing the LCR (nt 6958–7945) was isolated, modified with *Bam*HI linkers and ligated into the unique *Bam*HI site 3′ of the CAT gene and SV40 polyadenylation sequences in p905-1 in either orientation to yield p906-1 and p907-1 (Figure 1). The plasmid, p1048-1, was constructed by digestion of p805-1 with *Bst*EII (BPV-1 nt 2405) and *Avr*II (located 52 bp upstream of the CAT initiation codon), generation of blunt ends with the Klenow fragment and religation. This deleted the BPV-1 sequences from nt 2405 to 2617 (Figure 1). This plasmid does not contain the initiation sites for the P_{2443} promoter and deletes an A–T-rich region which likely functions as the promoter for transcription initiation. The plasmids p1312-1, p1313-1, p1314-1 and p1315-1 were derived by exchanging the *Mlu*I (nt 7351) to *Bst*E2 (nt 2405) fragments from previously characterized BPV-1 mutants for the analogous fragment of p805-1. The plasmid 775 (Schiller *et al.*, 1984; Lusky and Botchan, 1985) was used to generate a deletion mutant in E6 (p1312-1) and the plasmids p775-1, p745-1 and p743-23 (Rabson *et al.*, 1986a,b) were used to generate termination linker insertion mutations in E7 (p1313-1), E1 (p1314-1) and E1/E8 (p1315-1) respectively, as shown in Figure 4. The plasmids p1049-1 and p1050-1, which contain the E2 gene under the P_{2443} control, were constructed from the larger BPV-1 plasmids p142-6 and p735-1 respectively by digestion with *Eco*RI and religation. This results in a deletion of most of the BPV-1 sequences leaving from nt 2113 to 4450 in p1049-1 and nt 2113 to 4266 in p1050-1.

Pooled cell selection for drug resistance

The pooled cell cultures were generated by transfection of 2 μg of CAT-containing plasmid, 0.5 μg of pSV2-Neo (Southern and Berg, 1982), 1 μg of C212, a cDNA which expresses E2 from the SV40 early promoter (Yang *et al.*, 1985), plus 15 μg of salmon sperm carrier DNA onto C127 cells (subclone PH116) using calcium phosphate co-precipitation (Graham and van der Eb, 1973). Cells were selected with 600 μg/ml of G418.

CAT assays

CAT assays were performed as previously described (Gorman *et al.*, 1982) with the following modifications. Two micrograms of the CAT reporter plasmid plus 1 μg of an E2-expressing plasmid (where indicated) were transfected with 18 μg of high mol. wt salmon sperm DNA using the calcium phosphate procedure (Graham and van der Eb, 1973). When CV1 cells were transfected, the C59 cDNA (Yang *et al.*, 1985a) was used to provide the E2 transactivation product. When C127 cells (subclone PH116) were used, the E2 product was provided by various plasmids containing the full-length BPV-1 genome, e.g. p142-6. The cells were subjected to a 1-min shock with 8% glycerol in PBS and then treated with sodium butyrate (5 mM) in complete DMEM overnight for CV1 cells or 8 h for the C127 cell subclone. Extracts were prepared 48 h post-transfection for CV1 cells or 72 h post-transfection for the C127 cells. One-third of the cell extract from a 10-cm plate was used for each CAT reaction. Extracts were incubated in the presence of 0.2 μCi [^{14}C]chloramphenicol plus Acetyl CoA for 1–3 h when using CV1 cells or 12 h when using C127 cells.

RNA primer-extension analysis

Mapping of the 5′ ends of the RNAs from the pooled cell cultures was carried out by primer-extension analysis essentially as previously described (Baker and Howley, 1987; Spalholz *et al.*, 1987). The 20-base oligonucleotide primer complementary to the BPV–CAT hybrid RNA (nt 2500–2481) was used to assess transcription of the P_{2443} promoter and the 20-base oligonucleotide primer complementary to the BPV-1 RNA from nt 208 to 189 was used to analyze transcription from the P_{89} promoter. RNA was isolated from pooled G418 resistant cell cultures at ~4 weeks post-transfection. The ^{32}P-labeled primers were annealed to 15 μg of total cellular RNA isolated from the pooled cell cultures before addition of cloned MMLV reverse transcriptase (Bethesda Research Laboratories).

Acknowledgements

We are grateful to Drs Carl Baker and Alison McBride for critical readings of this manuscript. P.L.H. was supported by a National Research Service Award CF32 CA 07830-02S2 from the National Cancer Institute.

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Received on April 12, 1988; revised on May 25, 1988