JOURNAL OF VIROLOGY, Oct. 1988, p. 3608–3613
0022-538X/88/103608-06$02.00/0

Vol. 62, No. 10

Bovine Papillomavirus E2 Gene Regulates Expression of the Viral E5 Transforming Gene

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Received 8 March 1988/Accepted 14 June 1988

We have performed transient-expression experiments with CV1 monkey kidney cells to investigate the role of the bovine papillomavirus type 1 (BPV1) E2 gene in the regulation of the E5 transforming gene. Direct analysis of the 7-kilodalton open reading frame (ORF) E5 protein and measurements of the expression of an E5-chloramphenicol acetyltransferase fusion protein indicate that the efficient expression of ORF E5 requires the full-length E2 gene, which can be supplied in *trans*. The viral long control region is required in *cis* for this response to ORF E2, and it acts in a position- and orientation-independent fashion characteristic of a transcriptional enhancer. Deletion analysis suggests that the P_{2443} promoter is required for efficient expression of the E5 gene. The E2 repressor activity encoded in the 3′ end of the E2 gene inhibits the expression of ORF E5. These effects define a major BPV1 regulatory circuit and appear to explain the transformation behavior of a variety of BPV1 mutants.

Bovine papillomavirus type 1 (BPV1) and BPV1 DNA readily induce tumorigenic transformation of cultured mouse C127 cells (10, 18). To identify and characterize the viral genes required for transformation, researchers in several laboratories have examined the biological activities of constructed viral mutants and subgenomic fragments of viral DNA. These experiments have identified two viral genes, open reading frame (ORF) E5 and ORF E6, each of which is sufficient to induce focus formation in mouse C127 cells in the absence of other BPV1 genes (24–26, 31). In the context of the full-length 7,945-base-pair viral genome, mutations that disrupt the E5 gene cause severe reductions in the efficiency of focus formation (6, 13, 16, 21). A third viral gene, ORF E2, has no detectable focus-forming activity when it is expressed in the absence of other BPV1 genes (25, 31), but mutations in the 5′ portion of this reading frame cause a substantial reduction in the ability of the viral DNA to induce foci and to replicate as a plasmid in transformed cells (5, 8, 13, 15, 19, 21). However, similar ORF E2 mutations have minimal effects on transformation efficiency when fragments of the BPV1 genome containing ORFs E2 and E5 are expressed from heterologous promoters (25, 31). The transformation and replication defects of these mutants can be complemented in *trans* by the full-length E2 gene, indicating that ORF E2 expresses a *trans*-acting factor required for these activities, and analysis of an ORF E2 temperature-sensitive mutant indicates that E2 activity is required for initiation and maintenance of transformation and normal viral DNA replication (8, 15, 21). The inability of the E2 gene to transform on its own and the pleiotropic effects of the E2 mutations suggest that ORF E2 may play an indirect role in transformation. Expression of the E2 gene affects transcription from promoters linked to the BPV1 long control region (LCR) (14, 17, 27, 28). The full-length E2 protein (encoded by nucleotides [nt] 2608 to 3837) is a transcriptional activator, whereas independent E2 proteins lacking the amino-terminal domain appear to be transcriptional repressors. These effects of ORF E2 evidently are mediated by direct binding of the E2 protein(s) to specific DNA sequences that are located in the LCR and elsewhere in the BPV1 genome (2, 19a). Although it has been suggested that the E2 gene may influence the transformation efficiency of BPV1 by controlling the activity of viral transforming genes, it has not been determined which BPV1 genes are affected by E2 activity (5, 14, 17, 19, 21, 27, 28). We demonstrate here in a series of transient-expression experiments that expression of the E5 gene is regulated by the E2 gene.

MATERIALS AND METHODS

Plasmid constructions. pBPV-E5-XL2 contains a frameshift mutation that inactivates ORF E5 (6). c59-X9 was derived from the ORF E2-expressing cDNA clone c59 (30) by insertion of the E5-XL2 frameshift mutation into ORF E5, and c59-F9 was generated by insertion of the ORF E2 E2-fs-1 mutation (20) into c59.

*Hin*dIII-linearized pSV2CAT (12) was resected with T4 DNA polymerase followed by nuclease S1, and *Xba*I linkers (pCTCTAGAG) were inserted. This generated pSV2 CAT5X, which contains a single *Xba*I linker abutted to the sequence AAAAAA, with the resulting deletion of the chloramphenicol acetyltransferase (CAT) translation initiation codon (see Fig. 2A, bottom). Similar techniques were used to insert an *Xba*I linker at the *Bst*XI site immediately downstream of the ORF E5 start codon in pHBK9, a transformation-competent BPV1 mutant that contains a new *Eco*RV cleavage site immediately 3′ to the ORF E2 stop codon (9; D. DiMaio, unpublished results). The resulting plasmid, pHBK9X, contains the *Xba*I linker inserted 4 base pairs downstream of the E5 ATG (see Fig. 2A, top). The 747-base-pair *Xba*I-to-*Dpn*I fragment from pSV2CAT5X (containing the entire CAT coding region except for the initiation codon) was inserted at the *Xba*I site of pHBK9X, placing the CAT coding region in frame with the E5 initiation codon, as documented by DNA sequencing (see Fig. 2A, middle). By means of a series of subcloning steps, the in-frame E5CAT fusion gene was inserted in the cloned BPV1 genome to construct pBPV-E5CAT (see Fig. 2B). This plasmid is identical to pBPV-142-6, except for the presence

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of the in-frame CAT coding region inserted at BPV nt 3881 and the 2-base mutation generating the *Eco*RV site.

pHBK9X was digested with *Xba*I, filled in with Klenow DNA polymerase, and religated to generate pHBK9-XFS3, which has a 4-base-pair insertion immediately downstream of the E5 initiation codon. This frameshift mutation was inserted into pBPV-E5CAT to generate pBPV-E5CAT-XFS3. An *Xho*I linker (pCCCTCGAGGG) was inserted into the *Eco*RV site in pBPV-E5CAT to introduce a 10-base-pair insertion 30 base pairs upstream of the E5 initiation codon, thus generating pBPV-E5CAT-RVX3. The ORF E2 NIL deletion (nt 2878 to 3089) and fs-1 frameshift (at nt 3023) mutations (20) were inserted into pBPV-E5CAT to generate pBPV-E5CAT-E2N and pBPV-E5CAT-E2F, respectively.

pLCRD-E5CAT was generated from pBPV-E5CAT by deletion of the *Hin*dIII-to-*Hpa*I small fragment (nt 6958 to 3). After addition of *Sal*I linkers to its ends, the *Hin*dIII-to-*Hpa*I LCR fragment was then inserted into the *Sal*I site of pLCRD-E5CAT. The resulting plasmid, pLD5C-SL5, contains two tandem copies of the LCR fragment in the opposite orientation to that in wild-type viral DNA. The ORF E2 frameshift mutation in pBPV-E2fs1 was inserted into pLD5C-SL5 to generate pLD5C-SL5-E2F.

The mutation at P_{89} was generated by deleting the DNA segment between the *Hpa*I site (nt 3) and the *Xho*I linker (nt 108) in plasmid pXH731 (24). The E5 gene in this plasmid was replaced with the E5CAT gene from pBPV-E5CAT to generate pd1P_{89}-E5CAT. To construct the mutation at P_{2443}, *Bst*EII-linearized pBPV-142-6 was treated with T4 DNA polymerase in the absence of deoxynucleoside triphosphates at 11°C. After limited digestion with mung bean nuclease, *Xho*I linkers were added to the ends of the molecules, and the *Xho*I (formerly *Bst*EII)-to-*Kpn*I small fragment was ligated to the *Kpn*I-to-*Xho*I large fragment of a pBPV-142-6 derivative containing an *Xho*I linker at the *Nsi*I site at nt 2329. The E5 gene in this plasmid was replaced with the E5CAT gene from pBPV-E5CAT to generate pd1P_{2440}-E5CAT.

Detection of the E5 protein. CV1 cells in 100-mm plates were transfected with the indicated DNA plus 10 μg of calf thymus DNA by using calcium phosphate. DNA was removed after 6 h, a 15% glycerol shock was performed, and cells were incubated in medium plus sodium butyrate (11). At 48 h after transfection, the medium was changed to methionine- and cysteine-free medium supplemented with 0.25 mCi each of [^{35}S]methionine and [^{35}S]cysteine per ml. After an additional 5-h incubation, the cells were rinsed and harvested as described (26). E5 protein was detected by subjecting extracts of labeled cells to immunoprecipitation with the anti-E5 peptide antiserum generated by Schlegel et al. (26). Proteins were fractionated on a sodium dodecyl sulfate–15% polyacrylamide gel and visualized by treatment with En^{3}Hance (Du Pont, NEN Research Products) and fluorography.

CAT assays. CV1 cells in 60-mm plates were transfected as described above with 4 μg of a BPV-E5CAT plasmid plus 5 μg of calf thymus DNA. Some plates also received 2 μg of a cDNA expression plasmid, as indicated. After 48 to 50 h, the cells were harvested and CAT activity was measured by using standard protocols (12) with equivalent amounts of extract as determined by using the Bio-Rad protein assay.

To determine the effect of E2 repressor activity, we cotransfected CV1 cells with 4 μg of pBPV-E5CAT and the indicated amount of either pSB3R6, which contains the *Fsp*I (nt 3023)-to-*Bam*HI (nt 4450) fragment of BPV1 DNA under the control of the simian virus 40 early promoter, or pSB3RB, which is identical to pSB3R6 other than containing a deletion of BPV nt 3737 to 3838 (J. Settleman, D. J. Riese, and D. DiMaio, unpublished results). A plasmid consisting of the BPV LCR cloned into pUC18 was added to the transfection mixtures, containing less than 3 μg of repressor plasmid to maintain the amount of the LCR and the total amount of added DNA constant. After 48 h, cells were harvested and in vitro assays of CAT were performed on extracts after normalizing for protein content.

RESULTS

To test whether an ORF E2 mutation affects production of the E5 transforming protein, the amount of the 7-kilodalton E5 polypeptide (26) was determined transiently after cells were transfected with either wild-type or mutant BPV1 DNA. pBPV-142-6 contains wild-type BPV1 DNA (23), and pE2-NIL DNA contains a 211-base-pair deletion in the 5′ half of ORF E2 (20). This E2 mutation eliminates transactivation and causes a substantial C127 cell focus-forming defect that can be complemented in *trans* by the wild-type E2 gene (20, 21). Each of these intact plasmids was transfected into CV1 cells that were subsequently metabolically labeled with [^{35}S]cysteine and [^{35}S]methionine. The anti-E5 antiserum described by Schlegel et al. (26) was used to immunoprecipitate the E5 protein from extracts of these cells, and the immunoprecipitated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. The 7-kilodalton E5 transforming protein is readily detectable in cells transfected with wild-type viral DNA (Fig. 1, lane 2). As expected, the protein is not detectable in cells transfected with an ORF E5 frameshift mutant or with carrier DNA alone (data not shown; see also Fig. 1, lane 5). The amount of the E5 protein is also markedly reduced in cells transfected with the E2 mutant (Fig. 1, lane 3), suggesting that ORF E5 expression is dependent on ORF E2 activity. To test whether the reduced levels of the E5 protein can be corrected by supplying the E2 gene in *trans*, we cotransfected CV1 cells with pE2-NIL and a cDNA clone that expresses ORF E2 transactivation and contains a frameshift mutation in ORF E5. Expression of the wild-type E2 gene allows the production of the E5 protein (Fig. 1, lane 4). Lane 5 shows that no E5 protein is produced in cells cotransfected with a BPV1 E5 frameshift mutant and the cDNA expression plasmid. These results demonstrate that efficient production of the E5 protein requires expression of the E2 transactivation function which can be supplied in *trans*.

To examine the role of ORF E2 in the expression of the E5 gene in more detail, we have established a sensitive and convenient assay for the transient expression of the E5 gene. As described in Materials and Methods, we constructed a fusion gene consisting of the *Escherichia coli* CAT coding region inserted in frame immediately downstream of the ORF E5 initiation codon (3). To force translation of the fusion gene to initiate at the E5 start codon, we deleted the endogenous initiation codon of the CAT gene. This gene encodes a CAT protein with a 3-amino-acid amino-terminal extension (Fig. 2A). The fusion gene was inserted into the full-length BPV1 genome at the position of the E5 gene to construct pBPV-E5CAT, in which all the BPV1 genes (other than ORF E5) and regulatory signals are intact (Fig. 2B). CAT activity is readily detectable in extracts of CV1 cells transfected with pBPV-E5CAT (Fig. 3A, lane 1). To confirm that translation initiation is occurring at the E5 start codon and not from ATG codons upstream of the authentic initia-

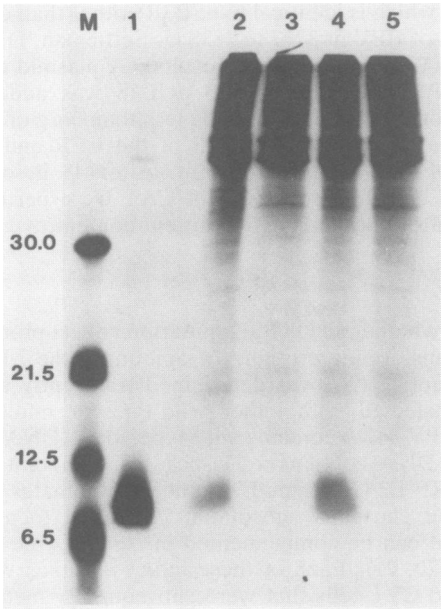

FIG. 1. Synthesis of the E5 protein in CV1 cells transfected with BPV1 DNA. E5 protein was immunoprecipitated (26) from cells transfected with the following DNAs: lane 2, 20 μg of pBPV-142-6 (23) plus 5 μg of pBRd; lane 3, 20 μg of pBPV-E2-NIL (20) plus 5 μg of pBRd; lane 4, 20 μg of pBPV-E2-NIL plus 5 μg of c59-X9; lane 5, 20 μg of pBPV-E5-XL2 plus 5 μg of c59-X9. Each of the plates also received 10 μg of calf thymus DNA. Lane M shows molecular mass standards with apparent sizes in kilodaltons indicated on the left. Lane 1 shows BPV1 E5 protein immunoprecipitated from cells engineered to overexpress it (J. Settleman and D. DiMaio, submitted for publication). Exposure was for 2 weeks.

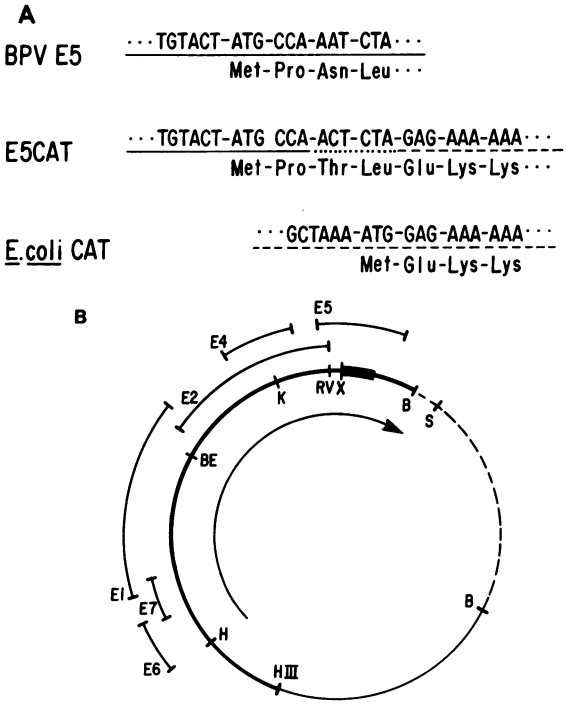

FIG. 2. (A) 5′ end of the E5CAT fusion gene and its encoded amino acid sequence. The nucleotide and predicted amino acid sequences surrounding the initiation codons of the indicated genes are shown. · · · , Sequences not contributed by either parent gene. (B) Structure of pBPV-E5CAT. Symbols: ▬, transforming segment of BPV1 DNA; ——, "late" region; -----, bacterial plasmid vector; ■, position of the inserted CAT gene. BPV1 transcription proceeds clockwise, and the positions of some of the BPV1 ORFs are indicated (4). Abbreviations for restriction sites: HIII, *Hin*dIII; H, *Hpa*I; BE, *Bst*EII; K, *Kpn*I; RV, *Eco*RV; X, *Xba*I; B, *Bam*HI; S, *Sal*I. The LCR is located in the small *Hin*dIII-to-*Hpa*I fragment.

tion codon or from internal ATG codons within the CAT coding region, we tested the effects of frameshift mutations bracketing this position. As expected, a 4-base-pair insertion between the E5 initiation codon and the CAT coding region abolishes CAT activity in transfected cells (Fig. 3A, lane 2), whereas a 10-base-pair insertion 30 base pairs upstream of the E5 start codon has minimal effect on CAT expression (lane 3). We have previously shown that frameshift mutations upstream of the methionine codon have no effect on ORF E5 expression as assessed by focus-forming efficiency (3). We also determined the effects of two mutations in the 5′ end of ORF E2, the E2-NIL deletion and the E2-fs1 frameshift insertion. Extracts of CV1 cells transfected with these mutant BPV-E5CAT plasmids exhibit a dramatically reduced level of CAT activity compared with cells transfected with the plasmid containing the wild-type E2 gene Fig. 3A, lanes 4 and 5). In the experiment shown, the mutants expressed less than 1% of wild-type levels, and similar results were obtained in multiple independent transfection experiments. We next tested whether this decrease in CAT expression could be corrected by supplying the E2 gene in *trans*. CV1 cells were cotransfected with E2 mutant BPV-E5CAT plasmids and either a cDNA clone that expresses E2 activity or a derivative cDNA clone containing the E2-fs1 frameshift mutation that inactivates the E2 gene. It is apparent that CAT expression is restored to the E2 mutant plasmids by the addition of a wild-type E2 gene but not a mutant one (Fig. 3B). Therefore, the results obtained with the E5CAT plasmids confirm those obtained when the level of the E5 protein was determined directly and document that the level of CAT activity in CV1 cells transfected with these plasmids is a valid measure of the expression of the E5 gene.

It has recently been found that overexpression of the 3′ portion of ORF E2 can block BPV1-induced transformation and transactivation of the viral enhancers (17). To test whether the E2 repressor negatively regulates the E5 gene, we determined the effect of cotransfecting the wild-type E5CAT plasmid and a plasmid expressing this repression activity. The expression of the E5CAT fusion gene is inhibited in a dose-dependent fashion when increasing amounts of a plasmid expressing this repressor are added in *trans* (Fig. 4, dashed line). This inhibitory effect is eliminated by a frameshift mutation in the portion of ORF E2 encoding the transcriptional repressor (solid line). Thus, expression of the E5 gene responds to both the positive and negative effects of the E2 gene.

To test whether the previously described E2-dependent enhancers are required for E5 expression, we tested the effect of deleting and repositioning the viral LCR which contains these elements (Fig. 5A). CAT activity is greatly reduced in CV1 cells transfected with an E5 CAT plasmid from which the LCR is deleted (Fig. 5B, lanes 3 and 4). This defect persists even if a wild-type E2 gene is supplied in *trans*, establishing that the defect is not due solely to the inability of the LCR deletion mutant to express ORF E2 (lane 3). Supplying the full-length BPV1 genome in *trans* does not correct the defect of the LCR deletion mutant,

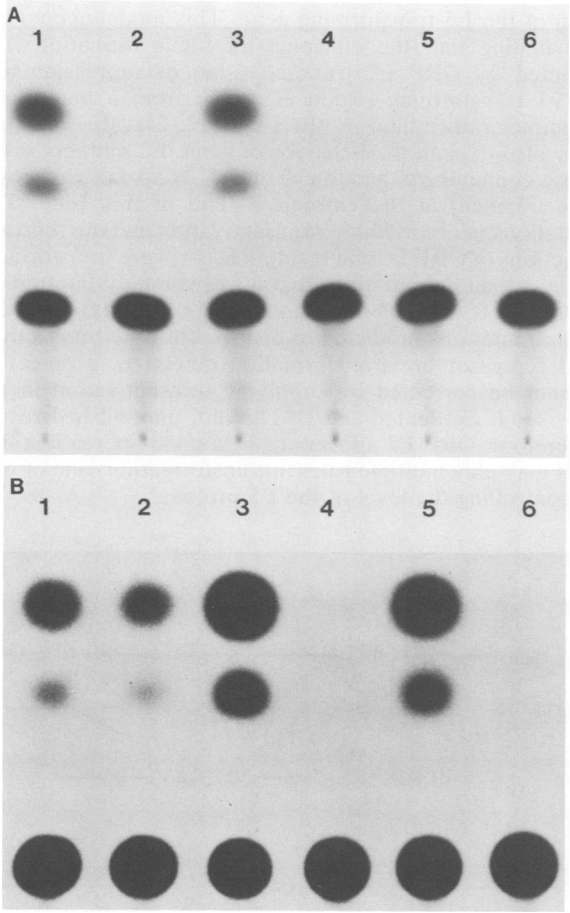

FIG. 3. CAT expression in CV1 cells transfected with the BPV-E5CAT plasmids. (A) We determined CAT activity in CV1 cells transfected with the following plasmids: lane 1, pBPV-E5CAT (wild type); lane 2, pBPV-E5CAT-XFS3 (frameshift mutation between the E5 initiation codon and the CAT coding region); lane 3, pBPV-E5CAT-RVX3 (frameshift mutation located between the 3′ end of ORF E2 and the E5 initiation codon); lane 4, pBPV-E5CAT-E2F (frameshift mutation in the 5′ end of ORF E2); lane 5, pBPV-E5CAT-E2N (deletion mutation in the 5′ end of ORF E2); lane 6, p451-NSI (BPV1 plasmid without an inserted CAT gene). (B) Complementation of CAT expression defect by cotransfected ORF E2. CV1 cells were transfected with the following plasmids: lane 1, pBPV-E5CAT plus c59 (expresses wild-type ORF E2) (3); lane 2, pBPV-E5CAT plus c59-F9 (expresses mutant ORF E2); lane 3, pBPV-E5CAT-E2N plus c59; lane 4, pBPV-E5CAT-E2N plus c59-F9; lane 5, pBPV-E5CAT-E2F plus c59; lane 6, pBPV-E5CAT-E2F plus c59-F9.

indicating that the LCR is required in *cis*, and the LCR mutant does not inhibit transactivation by a cotransfected E2 gene, indicating that the mutant does not overexpress E2 repressor activity (data not shown). If the LCR is inserted into the deleted plasmid in either orientation at the *Sal*I site downstream of the E5 gene, CAT expression is restored (Fig. 5B, lanes 5 and 6, show the results for a plasmid containing a tandemly arranged LCR dimer in the opposite orientation from that of the wild type). E5 expression from this construct is still E2 dependent, as shown by the elimination of CAT expression by the introduction of an ORF E2 mutation (lane 8) and its restoration by supplying ORF E2 in *trans* (lane 7). These results strongly suggest that a transcriptional enhancer located in the LCR is the crucial element

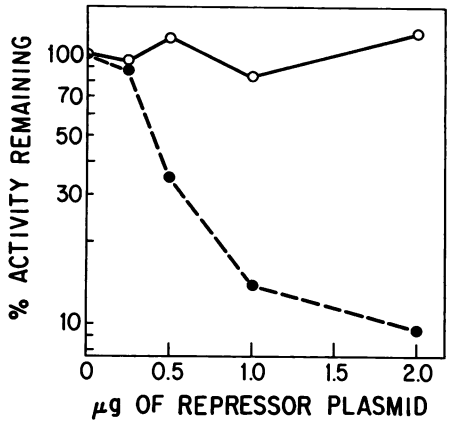

FIG. 4. Inhibition of CAT expression by the E2 repressor activity. Cells transfected with pBPV-E5CAT were cotransfected with increasing amounts of either pSB3R6 which expresses the E2 repressor activity (-----) or pSB3RB, a derivative plasmid containing a mutation inactivating the repressor (——). CAT activity in cell extracts was measured and is expressed as percentage of activity in the absence of added repressor plasmid.

required for E5 expression. We also found that E5 gene expression is not significantly impaired by a deletion (nt 1015 to 2329) that removes most of the E1 gene which is required for BPV1 DNA replication in cultured fibroblasts (data not shown).

The importance of the two major viral promoters active in BPV1-transformed C127 cells has also been assessed (Fig. 6). Deletion of the P_{89} promoter just 5′ to ORF E6 had no effect on E5CAT expression. In contrast, deletion of the P_{2443} promoter consensus sequences just 5′ to ORF E2 resulted in a severe reduction in E5CAT expression, even if E2 transactivation function is added in *trans*. These results are consistent with the P_{2443} promoter's being required for synthesis of the major mRNA(s) encoding the E5 transforming protein.

DISCUSSION

Because the known E2-responsive enhancers and promoters are at the 5′ end of the transforming region, it has been speculated that ORF E2 controls the expression of the BPV1 genes located in this region, ORFs E6, E7, and E1 (27, 28). We have not examined these upstream genes, but our results show that the E2 gene acts in *trans* to control the expression of the E5 transforming gene which is located at the 3′ end of the transforming region. BPV1 DNA does not replicate in CV1 cells (22), and our results confirm that ORF E1-dependent BPV1 DNA replication is not required for this effect. Analysis of the deletion mutants establishes that nt 3 to 97, nt 1015 to 2329, and nt 2878 to 3089 do not contain *cis*-acting sequences required for E2-mediated induction of E5 expression. We have not ruled out a role for the E2 protein-binding site at nt 3088 to 3099, because this site is regenerated by the deletion. The response does require a *cis*-acting element in the viral LCR, which is located about 4,000 base pairs away from the E5 initiation codon. However, the LCR does not contain a promoter required for expression of the E5 gene, since deletion of the P_{89} promoter has no effect on E5 expression and the rest of the LCR can function in a position- and orientation-independent fashion.

Although we have not established that control of E5 gene expression is exerted at the level of transcription, this seems

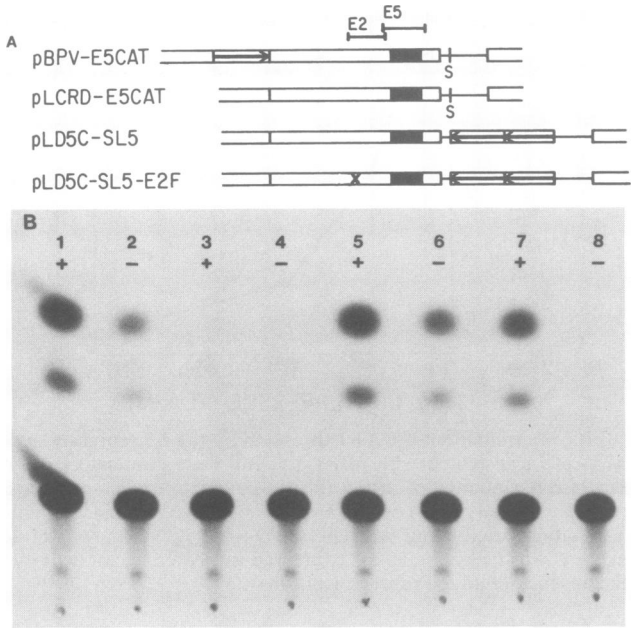

FIG. 5. (A) Structure of plasmids used to assess the requirement for the LCR in E5CAT expression. Symbols: ═, the BPV1 sequences; ——, bacterial vector; ▭, LCR; ▬, CAT coding region. The positions of ORF E2 and E5 are indicated. In pLD5C-SL5-E2F, the position of the frameshift mutation in ORF E2 is shown by the ×. The position of the *Sal*I site in the vector is indicated by the S. Transcription of BPV1 proceeds from left to right. (B) Effect of the LCR on E5CAT expression. CAT activity in extracts of CV1 cells transfected with the following plasmids: lanes 1 and 2, pBPV-E5CAT; lanes 3 and 4, pLCRD-E2CAT; lanes 5 and 6, pLD5C-SL5; lanes 7 and 8, pLD5C-SL5-E2F. Lanes labeled with (+) also received c59 (which expresses ORF E2); lanes labeled with (−) also received c59-F9 (which does not express ORF E2). Transfections and CAT assays were performed as described in the text.

a likely mechanism, because E2 gene products have been shown to induce a transcriptional enhancer in the LCR and to regulate the levels of viral mRNA transcribed from the viral promoters at nt 7940, nt 89, and nt 2443 (14, 17, 27, 28; P. L. Hermonat, B. A. Spalholz, and P. M. Howley, EMBO J., in press). Moreover, deletion of the promoter consensus sequences around nt 2400 greatly reduces expression of the E5CAT gene. These results are consistent with the conclusion that the E2 protein affects E5 gene expression by binding to the E2-responsive enhancer in the LCR, thereby influencing the activity of the P_{2443} promoter. This promoter directs the synthesis of a relatively abundant spliced viral RNA in which ORF E5 is not preceded by a long ORF following an in-frame ATG codon (30). Although this RNA is a plausible candidate for an E5 mRNA, all known viral RNAs in BPV1-transformed cells contain ORF E5 intact (1, 29, 30). The existence of these numerous mRNAs containing the E5 gene suggests that measurements of individual viral mRNA species may not accurately reflect the expression of the E5 gene.

In C127 cell transformation assays, an intact E5 gene is required for efficient focus formation. Elimination of E2 transactivation activity, removal of the LCR, or increased E2 repressor synthesis causes dramatic decreases in transient expression of the E5 gene and severely inhibits focus formation. These results suggest that the efficiency of focus formation by intact BPV1 DNA reflects the net effect of E2-mediated transactivation and repression on the expression of the E5 transforming gene. This model accounts for the finding that the efficiency of focus formation is not affected by ORF E2 transactivation or repression if the BPV1 transforming region is driven from a heterologous promoter rather than by the LCR (17, 24, 30). This model may also explain the behavior of some E2 mutants such as those containing a deletion of the 101-base-pair *Bcl*I restriction fragment at the extreme 3′ end of this gene. These mutations inhibit both the transactivation and the repression functions of ORF E2 and result in less-severe transformation defects than do mutations that inactivate only the transactivator (7, 8, 21; DiMaio, unpublished results). However, other mutations predicted to disrupt both the transactivator and repressor are transformation defective, a defect that cannot be corrected by supplying transactivation in *trans* (15, 21; J. Settleman and D. DiMaio, unpublished results). Therefore, ORF E2 appears to be a complex reading frame that may play multiple roles in transformation, one of which is controlling the level of the E5 protein.

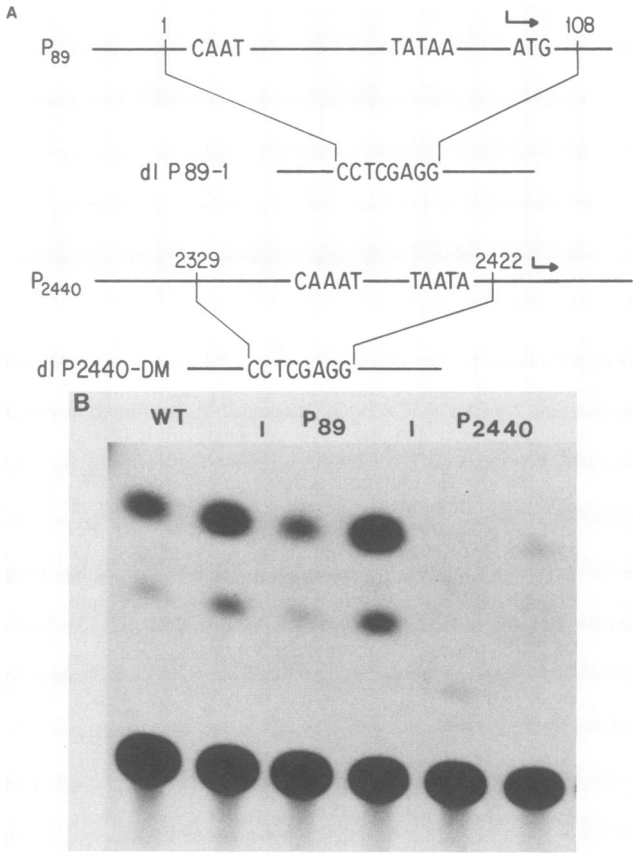

FIG. 6. (A) Structure of the promoter deletions. Promoter consensus elements are shown in the top line in each pair, with the 5′ end of the RNA indicated by the arrow. The numbers represent the limits of the wild-type sequence retained in the mutants, as determined by DNA sequencing. The bottom line in each pair shows the *Xho*I linker substituted for the consensus elements in pdlP_{89}-E5CAT (dlP_{89}-1) and in pd1P_{2440}-E5CAT (dlP_{2440}-DM). (B) Effects of the promoter deletions on E5CAT expression. CAT activity in extracts of CV1 cells transfected with pBPV-E5CAT (WT) pdlP_{89}-E5CAT (P_{89}), or pdlP_{2440}-E5CAT (P_{2440}), as indicated, is shown. In each pair, the sample on the left was cotransfected with c59-F9 and that on the right was cotransfected with c59.

ACKNOWLEDGMENTS

We thank D. J. Riese for help with the E2 repressor experiments, R. Schlegel for the gift of the anti-E5 antiserum, and D. Lowy and J. Schiller for mutant pXH731.

This work was supported by a Public Health Service grant from the National Cancer Institute. D.D. is the recipient of a Mallinckrodt Scholar Award.

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