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Importance of the Bovine Papillomavirus P_{2443} Promoter in the Regulation of E2 and E5 Expression

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The full-length bovine papillomavirus E2 gene product (E2TA), which has a direct role in DNA replication and functions as a transcriptional activator, can be expressed from an unspliced mRNA transcribed from the P_{2443} promoter or from spliced mRNAs transcribed from other upstream promoters. The regulation of E2 expression from these promoters is still in question. In the background of wild-type protein coding sequences, this study identified the P_{2443} promoter as the major source of E2TA as well as E5 expression in C127 cells.

The bovine papillomavirus type 1 (BPV-1) E2 proteins are important regulators of viral transcription (for a review, see reference 24). In BPV-1, a 48-kDa protein (E2TA) is produced from translation of the full-length E2 open reading frame (ORF). This protein acts as a transcriptional activator when bound to tandem repeats of its cognate binding site, $ACCN_6GGT$ (2). Two smaller E2 proteins, each containing the DNA binding domain of the protein, act as repressors of E2-transactivated transcription (10, 20, 21). E2-dependent enhancers located in the long control region of the viral genome can activate transcription from the viral promoters P_{89} and P_{2443} and possibly also from P_{890} and P_{3080} (15, 17, 23, 27, 36, 37, 41).

Controlling elements in the regulation of E2 transactivator expression are still under investigation. Three species of mRNA from transformed cells which could encode the full-length E2 ORF have been described: an unspliced species transcribed from the P_{2443} promoter and two spliced species transcribed from P_{89} and P_{890} (10, 40, 46). The species transcribed from the P_{89} promoter is spliced from nucleotide (nt) 304 to 2558, and the species transcribed from the P_{890} promoter is spliced from nt 1235 to 2558. Since the first in-frame E2 AUG is found at nt 2608 in all three species, each encodes the same E2 protein. In addition, cDNA copies of each transcript are capable of expressing the transactivator function when cloned into heterologous expression vectors, although at different efficiencies (39, 43). The spliced E2 transcript derived from P_{890} is not believed to be a major component since it is a very rare species (10); however, studies differ on the relative abundance of the other two species. A study by Vaillancourt et al. (43) concluded that both the P_{89} and P_{2443} promoters contribute equally to the production of E2. This conclusion was based largely on experiments in which mutations in either the P_{2443} promoter or the nt 2558 splice acceptor site led to similar levels of the E2 transactivator expression. However, the P_{2443} promoter mutation examined in that study, *dl*2440 (27), also generates a large mutation in the E1 ORF, complicating interpretation of the data. Mouse C127 cells transformed by E1 ORF mutants have altered transcriptional profiles, characterized by a 20- to 30-fold increase in the level of P_{89} promoter activity (19, 33). Therefore, it is possible that the level of E2 expressed from P_{89} in the *dl*2440 mutant in the study by Vaillancourt et al. (43) was higher than seen in the wild-type genome. In addition, the reduction in E2 activity from the nt 2558 splice acceptor mutant was actually quite small compared with that from the *dl*2440 mutant when a relatively small amount of either plasmid was used in the transfection. Only when large amounts of the plasmids were transfected did E2 activity reach approximately 50% of the wild-type level for both mutants. A reexamination of the control of E2 gene expression was therefore carried out by using P_{2443} mutations silent in the overlapping E1 ORF.

Mouse C127 cells were cotransfected with BPV-1 DNA and an expression vector for the neomycin resistance gene (pdMMTneo) as previously described (31). Poly(A) RNA (9, 28) was extracted from G418-resistant cells transformed by wild-type (p142-6) BPV-1 DNA and from normal C127 cells. The RNA was annealed to the ^{32}P-5′-end-labeled, single-stranded DNA probes diagrammed in Fig. 1. S1 endonuclease protection analysis was performed as previously described (38). The protected fragments were separated on a 6% polyacrylamide denaturing gel and autoradiographed. In the experiment shown in Fig. 1A, RNA from wild-type BPV-1-transfected cells protected a 143-nt fragment of an E2-specific probe, corresponding to unspliced mRNA species from P_{2443}, and fragments 26 to 29 nt in length, corresponding to species spliced at nt 2558. A similar analysis was performed with an end-labeled probe from nt 2673 to 2333 (5′ to 3′) which protected 115 and 230 nt of the spliced and unspliced E2 species, respectively (data not shown). The intensities of the protected fragments corresponding to the spliced and unspliced species in both analyses indicate that the P_{2443} promoter is the predominant source of E2 mRNA in the cells. These results are consistent with previous results examining transient E2CAT mRNA in CV-1 cells (38).

Since the spliced E2 mRNA species could originate from more than one promoter, the contribution from P_{89} was determined by annealing the same preparation of RNA to a probe specific for the E2 mRNA spliced from nt 304 to 2558. S1 endonuclease protection was carried out as before except that the gel was autoradiographed for 2 weeks instead of 2 days. Detection of protected fragments corresponding to transcripts derived from the P_{89} promoter were clearly seen, and the intensity of the fragment corresponding to the spliced P_{89} species is consistent with the intensity of the fragments corresponding to the spliced species detected in Fig. 1A. The band in Fig. 1B should represent only species

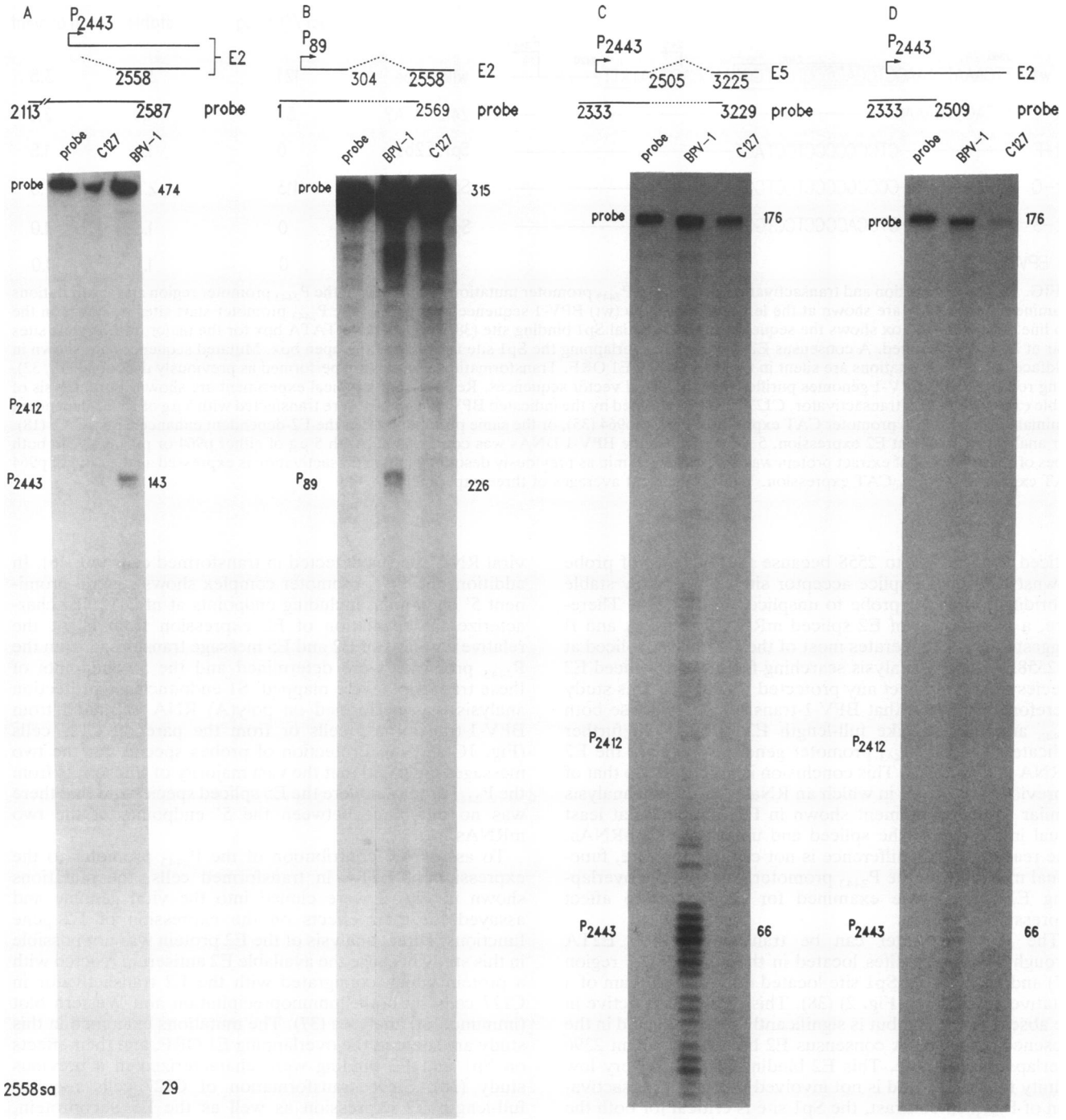
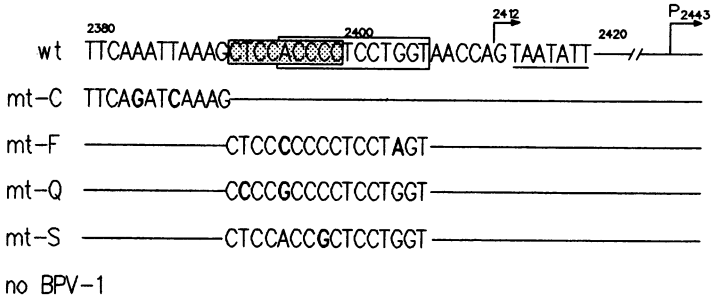


FIG. 1. S1 endonuclease protection analysis of E2 and E5 RNAs from G418^r C127 cells stably expressing full-length wild-type BPV-1 DNA. Complementary-strand DNA probes were generated by asymmetric polymerase chain reaction (25) using 20-base synthetic oligonucleotide primers. The 5′ endpoints of the primers are indicated on the diagrams of the probes. The dotted lines indicate intron sequences which are not present in the probes. Each probe is specific for the RNA(s) shown above it. Probes were 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase after purification through 2% Nusieve agarose (FMC) gels. Specific activities of all probes were similar. Hybridization, S1 endonuclease digestion, and gel electrophoresis were performed as previously described (38) except that S1 digestion was performed at 42°C for 45 min. (A and B) Four micrograms of poly(A) total RNA (9, 28) extracted from either normal C127 cells or C127 cells transformed by wild-type BPV-1 DNA was annealed to 10,000 cpm of the indicated probes. The gels were autoradiographed with intensifying screens for 2 days (A) and 2 weeks (B). (C and D) One microgram of poly(A) RNA was annealed to 20,000 cpm of the indicated probes. Gels were autoradiographed for 2 days with intensifying screens. Probe lanes contain approximately 100 cpm of undigested probe. The sizes (in nucleotides) of fragments are shown at the right and were determined by comparison with sequencing reactions run on each gel.



Mutation	Transformation Foci/0.5 ug	E2 Transactivation stable	E2 Transactivation transient
wild-type	121	3.7	3.5
2412 TATA$^-$	57	1.9	2.0
Sp1$^-$, E2bs$^-$	0	1.1	1.5
Sp1 consensus	113	2.9	7.3
Sp1$^-$	0	1.3	1.0
	0	1.1	1.0

FIG. 2. Transformation and transactivation potential of P_{2443} promoter mutations. Diagrams of the P_{2443} promoter region and the mutations examined in this study are shown at the left. The wild-type (wt) BPV-1 sequence upstream of the P_{2443} promoter start sites is shown on the top line. The stippled box shows the sequence of an essential Sp1 binding site (38). The putative TATA box for the major mRNA start sites near nt 2443 is underlined. A consensus E2 binding site overlapping the Sp1 site is shown in the open box. Mutated sequences are shown in boldface. All of the mutations are silent in the overlapping E1 ORF. Transformation assays were performed as previously described (13, 32), using recircularized BPV-1 genomes purified from bacterial vector sequences. Results from a typical experiment are shown. For analysis of stable expression of E2 transactivator, C127 cells transformed by the indicated BPV-1 genomes were transfected with 5 μg of an E2-dependent minimal simian virus 40 promoter CAT expression vector, p964 (35), or the same plasmid without the E2-dependent enhancer, $pA_{10}CAT$ (18). For analysis of transient E2 expression, 5 μg of each of the BPV-1 DNAs was cotransfected with 5 μg of either p964 or $pA_{10}CAT$. In both types of assays, 30 μg of extract protein was assayed for 60 min as previously described (14). Transactivation is expressed as the ratio of p964 CAT expression to $pA_{10}CAT$ expression. Values represent averages of three experiments.

spliced from nt 304 to 2558 because the 10 bases of probe downstream of the splice acceptor site do not allow stable hybridization of the probe to unspliced P_{89} mRNA. Therefore, a comparison of E2 spliced mRNA in Fig. 1A and B suggests that P_{89} generates most of the E2 mRNA spliced at nt 2558. A similar analysis searching for the P_{890} spliced E2 species failed to detect any protected fragments. This study therefore confirmed that BPV-1-transformed cells use both P_{2443} and P_{89} to make full-length E2 mRNA and further indicates that the P_{2443} promoter generates most of the E2 mRNA in C127 cells. This conclusion is in contrast to that of a previous study (43) in which an RNase protection analysis similar to the experiment shown in Fig. 1A found at least equal intensities of the spliced and unspliced E2 mRNAs. The reason for this difference is not clear. Therefore, functional mutations in the P_{2443} promoter, silent in the overlapping E1 ORF, were examined for the ability to affect expression of E2TA.

The P_{2443} promoter can be transactivated by E2TA through E2 binding sites located in the long control region (17) and an essential Sp1 site located directly upstream of a putative TATA box (Fig. 2) (38). This promoter is active in the absence of E2TA but is significantly transactivated in the presence of E2TA. A consensus E2 binding site at nt 2396 overlaps the Sp1 site. This E2 binding site has a very low affinity for E2 (22) and is not involved in the E2 transactivation of P_{2443}. In contrast, the Sp1 site is critical for both the basal and E2-transactivated expression of this promoter, and point mutations in this site can eliminate P_{2443} promoter activity in transient expression assays (23, 38).

Analysis of E2 transcription from P_{2443} is complicated by the fact that this promoter also transcribes a spliced message capable of encoding the E5 transforming gene (1, 12, 16, 34, 40, 46, 47). In addition, expression of E5 is responsive to the E2 transactivator (27), and morphologic transformation of C127 cells (13), which requires an intact E5 ORF, also requires E2 expression (11, 29, 32). These results are consistent with the E2 transactivation of the P_{2443} promoter. The E5 message is spliced from nt 2505 to 3225 and is the major viral RNA species detected in transformed cells (40, 46). In addition, the P_{2443} promoter complex shows several prominent 5′ endpoints, including endpoints at nt 2412. To characterize the regulation of E2 expression from P_{2443}, the relative amounts of E2 and E5 message transcribed from the P_{2443} promoter were determined and the 5′ endpoints of these transcripts were mapped. S1 endonuclease protection analysis was performed on poly(A) RNA extracted from BPV-1-transformed cells or from the parental C127 cells (Fig. 1C and D). Protection of probes specific for the two messages indicated that the vast majority of transcripts from the P_{2443} promoter were the E5 spliced species and that there was no difference between the 5′ endpoints of the two mRNAs.

To assess the contribution of the P_{2443} promoter to the expression of E2TA in transformed cells, the mutations shown in Fig. 2 were cloned into the viral genome and assayed for their effects on the expression of E2 gene functions. Direct analysis of the E2 protein was not possible in this study because the available E2 antiserum reacted with a protein which comigrated with the E2 transactivator in C127 cells by both immunoprecipitation and Western blot (immunoblot) analyses (37). The mutations examined in this study are silent in the overlapping E1 ORF, and their effects on Sp1 and E2 binding were characterized in a previous study (38). Since transformation of C127 cells requires full-length E2 expression as well as the E5 oncoprotein, full-length BPV-1 genomes containing the mutations shown in Fig. 2 were assayed for focus formation on mouse C127 cells. Mutations which destroyed the Sp1 site (mt-F and mt-S) resulted in a loss of focus formation, whereas mutation of the Sp1 site to a higher-affinity consensus binding site sequence (mt-Q) had little effect on focus formation. Interestingly, mutation of a putative TATA region for the nt 2412 mRNA start sites (mt-C) also resulted in a twofold reduction of focus-forming ability. Since expression of E5 has been shown to be dependent on E2 expression, these results could not distinguish whether E5 expression was directly or indirectly affected. E2 was therefore cotransfected with the

transformation-defective mutants to determine whether E2 expression from a heterologous plasmid could rescue transformation in these mutations. Under transfection conditions which did result in E2 complementation in transient DNA replication assays (see below), E2 could not rescue focus formation even after 3 weeks (data not shown), indicating that the C, F, and S mutations directly interfered with the expression of E5. This result suggests that P_{2443} activity is essential for E5 expression early after transfection and underscored the need for alternative assays to assess E2 levels in cells transformed by genomes mutated in the P_{2443} promoter. A similar conclusion can be inferred from the results of Prakash et al. (27).

E2 strongly activates heterologous promoters linked to E2-dependent enhancers. A reporter plasmid containing the gene for chloramphenicol acetyltransferase (CAT) transcribed from the minimal simian virus 40 early promoter linked to an E2-dependent enhancer, p964 (35), was therefore transfected into cells to assess E2TA function. Two experimental strategies were used. Plasmid p964 was either transfected into C127 cells stably transformed by the BPV-1 P_{2443}-mutated genomes or cotransfected with the BPV-1 DNAs into normal C127 cells. The data in Fig. 2 indicated that the Sp1 mutations (F and S) made very little E2 transactivator and that the C mutation was somewhat impaired in its ability to provide E2 transactivation in both types of transfection. These data are not in agreement with the results of the study by Vaillancourt et al. (43), which showed that BPV-1 DNA containing a large deletion of the P_{2443} promoter region provided substantial E2 transactivation activity. Several explanations could account for this difference. It is possible that the C, F, and S mutations increase E2 repressor function; however, these mutations are outside the coding regions of the repressors, and primer extension analysis (see Fig. 4) suggests that there was no change in the transcription of the repressors. Another explanation is that the study by Vaillancourt et al. (43) measured transactivation in monkey CV-1 cells, and it is possible that the promoters may have different relative strengths than in mouse C127 cells. Finally, the P_{2443} deletion mutation examined in that study, *dl*2440, contains a large E1 mutation. C127 cells transformed by E1 mutants characteristically have elevated levels of P_{89} transcription (19, 33), probably as a result of loss of the transcriptional repressor properties of E1 (30). It is therefore possible that the level of spliced E2 messages from P_{89} was significantly elevated in that study. In the C127 cells used in the study presented here, E2 was expressed either stably or transiently in the background of wild-type E1; most of the E2 transactivation potential was removed when P_{2443} was impaired.

The full-length E2 protein also has a direct role in DNA replication. It is absolutely required in vivo for transient viral DNA replication (42, 45) and can form a complex with the E1 replication factor in vitro (6, 26). The ability of the P_{2443} mutations to replicate as episomes was therefore tested as another measure of E2TA expression. Wild-type BPV-1 DNA or DNA containing the P_{2443} mutations was electroporated into C127 cells (42). Low-molecular-weight DNA was harvested 48 and 72 h postelectroporation and digested with *Dpn*I and *Hin*dIII. *Dpn*I digests only unreplicated DNA, and *Hin*dIII linearizes the BPV-1 genome. The results shown in Fig. 3A are consistent with those shown in Fig. 2. The wild-type BPV-1 genome and the genome containing the mutation that generated a consensus Sp1 site in the P_{2443} promoter (mt-Q) replicated well. Genomes carrying mutations which impair the Sp1 site (mt-F and mt-S) replicated very poorly, while the genome with mutations upstream of the Sp1 site (mt-C) replicated at an intermediate level. Since no known *cis* replication elements were involved in the P_{2443} mutations, complementation with E2 was tested to determine whether loss of E2 was the sole block to replication of the mutated genomes. Complementation, under the same transfections conditions which could not rescue focus formation, clearly showed that these genomes were capable of replicating when E2 was supplied from an exogenous source. (In other experiments, mt-F replicated at higher levels in the presence of E2 than that seen in Fig. 3A.) Similar results were obtained when replication of the mutated genomes was examined after stable transformation of the C127 cells (Fig. 3B). Therefore, the only block to replication in these experiments was a loss of E2. This result further supports the conclusion that P_{2443} is critical in the expression of E2 and that P_{89} does not make sufficient levels of E2 for efficient replication under these conditions.

As many of the BPV-1 promoters can be transactivated by E2 (15, 17, 23, 36, 41), the effects that the P_{2443} mutations have on viral transcription were examined. It should be noted that no substantial difference in total viral DNA copy number was observed between C127 cells transformed by wild-type and mutated genomes. Mutants which did not efficiently replicate BPV-1 DNA as an episome had equivalent copy numbers in an integrated form (data not shown). Figure 4 shows the results of primer extension analysis from poly(A) RNA extracted from $G418^r$ C127 cells expressing the P_{2443}-mutated BPV-1 genomes. As expected, mutations which disrupt the required Sp1 site, mt-F and mt-S, showed a loss of activity from P_{2443} and a reduction in signal from the nt 2412 start sites. mt-C also showed a reduction in the nt 2412 start sites but had retained the P_{2443} start sites. In most assays, a twofold reduction in the P_{2443} start sites was observed with the C mutation, which is consistent with the level of E2TA function observed. A more typical experiment showing this reduction is shown in the additional lanes in the second panel of Fig. 4.

Since the P_{89} promoter can be transactivated by E2TA (15, 36), mutation of the P_{2443} promoter might result in a reduction in activity from this promoter. mt-F and mt-S did show a reduction in activity from the P_{89} promoter, suggesting that this promoter is normally activated by E2 in transformed C127 cells. It is also possible that the loss of E2TA function in these mutations was due to the loss of both the spliced and unspliced E2 mRNA species. However, the C mutation consistently had no effect on P_{89} transcription. This result is important for two reasons. It suggests that the intermediate level of E2 transactivator expressed from mt-C was enough to fully activate the P_{89} promoter, and it indicates that significant amounts of E2 were not made from P_{89}. The reduction in E2TA function in mt-C is consistent with its reduction in P_{2443} activity.

Primer extension analysis for P_{3080} and P_{890} showed that P_{2443} mutations had little effect on these promoters. (In both cases, the reactions represented in lanes marked 142-6 contained one-half the amount of RNA used in the other reactions.) These promoters have been reported to be transactivated by E2 (23, 41); however, reduction of E2 consistently did not result in loss of P_{3080} or P_{890} activity. This result suggests that under the conditions of this study, the activity seen from P_{890} and P_{3080} did not result from E2 transactivation. It is possible that these promoters are primarily under the control of the constitutive enhancer (44).

The results of this study document the important role that

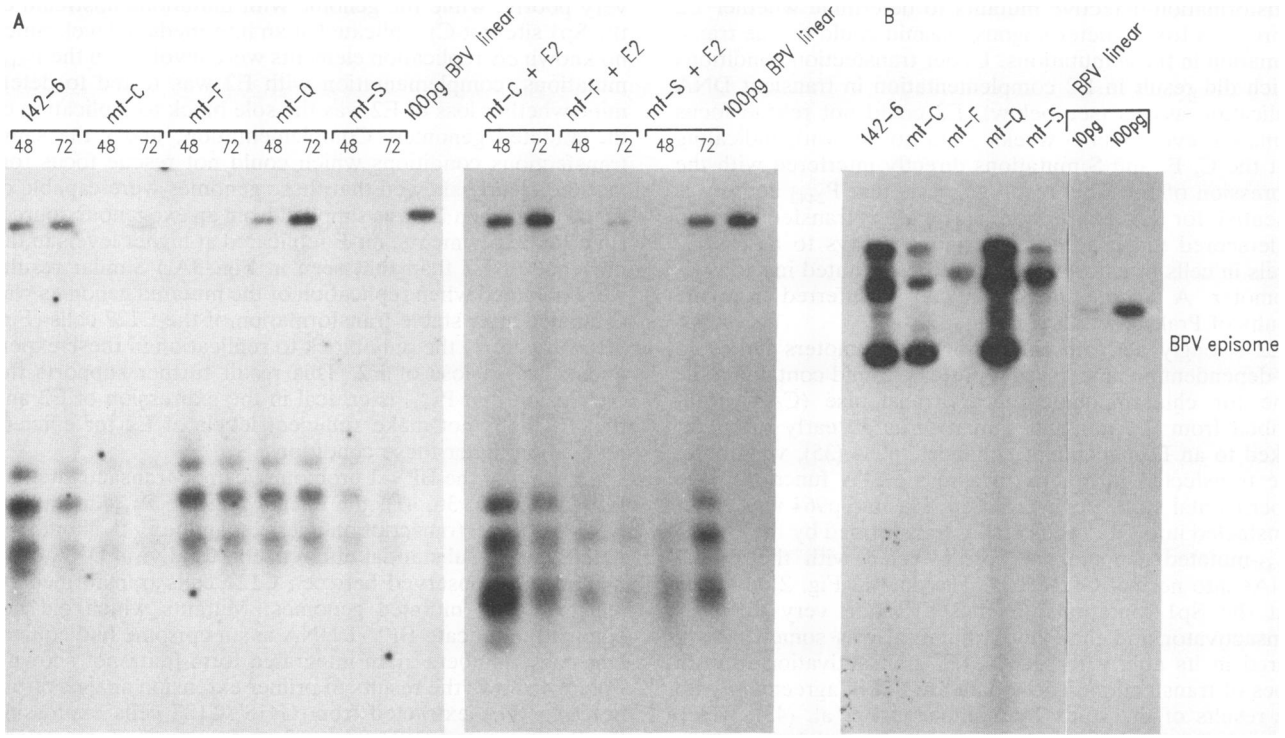


FIG. 3. Analysis of replication competence of BPV-1 DNA mutated in the P_{2443} promoter. (A) Transient replication analysis. A total of 5×10^6 C127 cells were electroporated (42) with 5 μg of BPV-1 DNA and 40 μg of salmon sperm DNA as carrier and with 5 μg of C59BstXI as a specific source of E2 (47) where indicated. After electroporation, cells were plated onto two 10-cm-diameter plates. Low-molecular-weight DNA was harvested 48 and 72 h postelectroporation and digested with *Dpn*I and *Hin*dIII. Replicated DNA was detected after electrophoresis through 1% agarose gels by Southern blot analysis using a nick-translated BPV-1-specific probe. (B) Stable replication. C127 cells were cotransfected with the BPV-1 DNAs and pdMMTneo (31). $G418^r$ colonies were pooled, and low-molecular-weight DNA was extracted from 10^6 cells. Undigested DNA was analyzed as in panel A.

the P_{2443} promoter has in the expression of both E2TA and E5 and show that mutation of this promoter has dramatic effects on the expression of viral functions. The E2 transactivator was made predominantly from P_{2443} in transformed mouse C127 cells, and although the spliced full-length E2 species from P_{89} was detected in these cells, it represented only approximately 10% of the E2 mRNA. C127 cells appear to be a good model system for the expression of many papillomavirus gene products; however, they do not completely mimic the natural host. In the fibropapilloma, E5 is expressed in the basal cells of the epithelium (7) and in the dermal cells apparently from P_{2443} transcripts (4). In contrast, high levels of E2 proteins are detected in the lower portion of the spinous layer (8). Although this latter study did not discriminate between the E2 repressors and E2TA, E2TA expression here would be consistent with the onset of vegetative viral DNA replication. E5 protein and the spliced E5 mRNA are not found in this region (4, 7). The C127 model system suggests that P_{2443} activation would lead to more E5 expression than E2 expression. The situation in the spinous layer could be explained by P_{2443} activation followed by suppression of E5 mRNA processing or by activation of other promoters which make E2 independently from E5. Transcripts spliced from nt 305 to 2558, possibly transcribed from P_{89}, can be detected in the spinous layer, which would be consistent with activation of E2 expression from P_{89} (5). It is possible, therefore, that P_{2443} is making both E2 and E5 in the basal layer of the epithelium, then in the spinous layer P_{2443} is shut down and P_{89} is activated to make the E2 required for viral DNA replication.

The study by Vaillancourt et al. (43) suggested that feedback mechanisms could control the level of E2 transactivator in the cells, so that when one E2 promoter was impaired, others could be activated to keep transactivator function constant. The current study, however, did not show significant E2 transactivator function when P_{2443} was disrupted in the absence of E1 mutations. Therefore, differential activation of P_{2443} and P_{89} in the fibropapilloma is likely to be determined by cellular factors specific to the differentiated state of the host. The ability to express E2 and E5 from different promoters and activation of the promoters in different cell types must provide some advantage to the virus. It is possible that the ability to transcribe E2 from different promoters may actually be a determining factor in the biology of this viral infection. To date, analogous internal promoters to make E2 have not been found in most of the human papillomaviruses which are associated with epithelial papillomas. Perhaps the activity of P_{2443} in the basal cells and dermal fibroblasts is instrumental in generating a fibropapilloma rather than a purely epithelial tumor. In addition, as E2TA plays a direct role in episomal DNA replication, it will be interesting to determine whether the ability to express E2TA from several differentially controlled promoters is related to the relatively benign outcome of a BPV-1 lesion compared with some human papillomavirus infections in

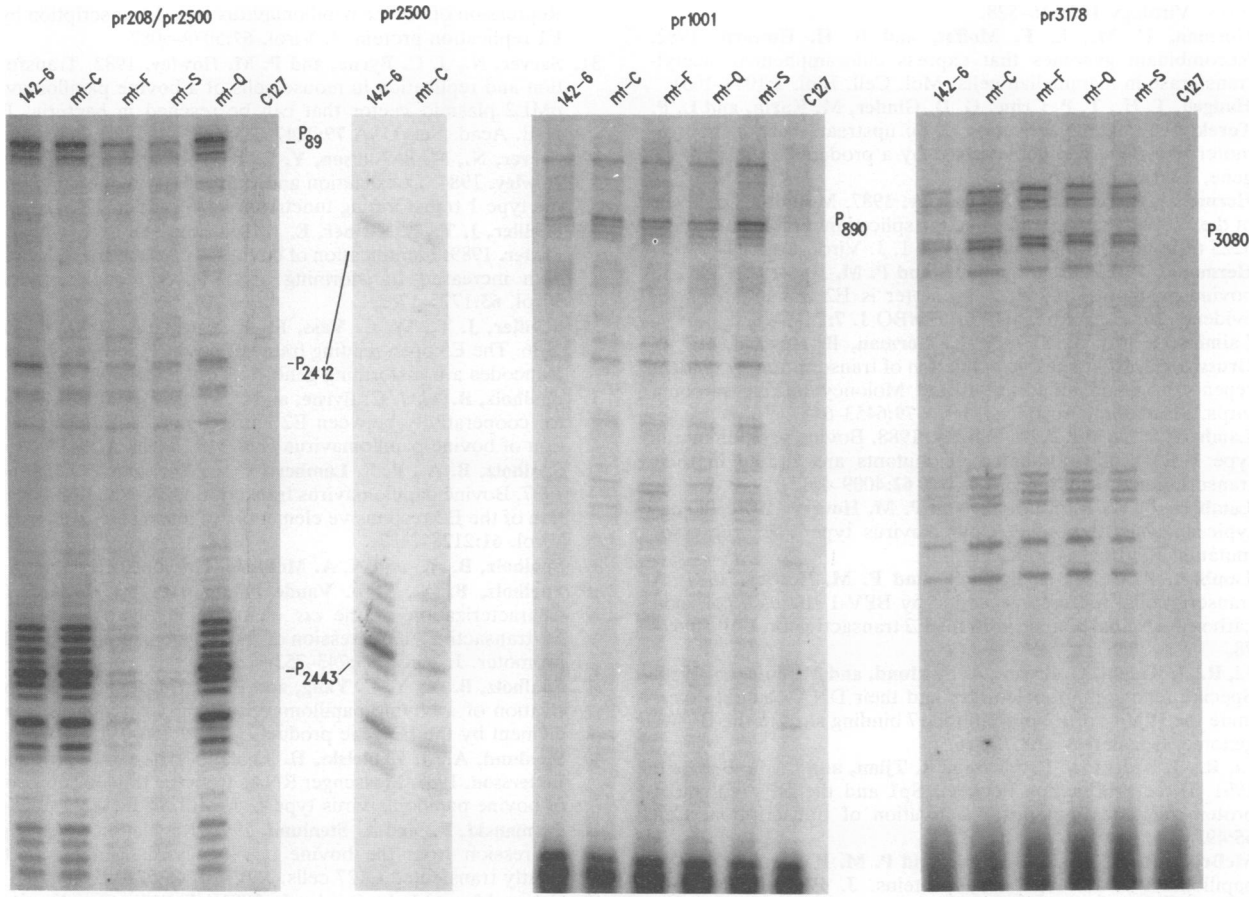


FIG. 4. Primer extension analysis of BPV-1 promoters in G418^r C127 cells transformed by wild-type and P_{2443}-mutated BPV-1 DNAs. Excess complementary-strand 20-base oligonucleotide primers, labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, were annealed to 2 μg of poly(A) RNA (prepared as in Fig. 1) from extracted C127 cell lines transformed by each of the indicated DNAs. (Only 1 μg of RNA was used in the P_{890} and P_{3080} analyses with 142-6.) The 5′ endpoints of the probes are indicated above the lanes. Primer extension analysis was performed as previously described (3). The positions of the promoters were determined by comparison with sequencing reactions run on each gel (not shown).

which viral DNA integration is associated with progression to carcinoma.

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