Bovine Papillomavirus E2 Repressor Mutant Displays a High-Copy-Number Phenotype and Enhanced Transforming Activity

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The methionine codon at bovine papillomavirus type 1 nucleotide 3091 was mutated to determine whether it may serve as an initiation codon for an E2 transcriptional repressor protein and to determine the role of the repressor in the biological activities of the virus. A series of transient expression experiments with CV1 cells documented that the mutation reduced expression of repressor activity from the viral genome and resulted in increased expression of the E5 transforming gene. Viral genomes containing the mutation displayed enhanced transforming activity in several assays in mouse C127 cells, including focus formation, colony formation in agarose, and tumorigenicity. In transformed cells, the mutant viral DNA was maintained as a plasmid with approximately 500 genomes per cell, whereas the wild-type copy number was approximately 75. These results indicate that the wild-type bovine papillomavirus type 1 genome encodes an E2 repressor protein that moderates the viral transforming activity and allows maintenance of the viral DNA at a relatively low copy number.

Papillomavirus transcription appears to be controlled by both viral and cellular factors. Numerous regulatory elements and protein binding sites have been identified in the viral genome, particularly in the long control region (LCR) 5' to the early genes (2, 11). In bovine papillomavirus type 1 (BPV-1), viral open reading frame (ORF) E2 encodes proteins that bind to the DNA sequence ACCNNNNNNGGT and its variants that occur in the LCR and elsewhere in the genome (1, 20, 24). The full-length BPV-1 E2 protein appears to bind these regulatory sequences as a homodimer, and when bound it can stimulate expression of linked promoters (9, 12, 13, 22, 35, 36). This activity is referred to as transactivation. Expression of the carboxyl-terminal portion of the E2 protein can inhibit transactivation by the full-length E2 protein (6, 19). This inhibitory activity is referred to as repression. The common carboxyl-terminal portion of the transactivator and repressor proteins contains sequences that mediate DNA binding and dimerization, and repression is thought to occur either via subunit mixing or via direct competition between the transactivator and repressor for DNA binding sites (9, 22, 23).

Lambert et al. (18, 19) and Choe et al. (6) have described subgenomic BPV-1 plasmids that express repressor activity under the control of heterologous promoters. On the basis of these studies, they have suggested that E2 repressor proteins can be synthesized by translation initiation at an internal methionine codon at nucleotide (nt) 3091 or by translation of a spliced ORF E8/E2 mRNA. In addition to the 48-kilodalton full-length E2 transactivator, BPV-1-transformed cells contain major 31- and minor 28-kilodalton proteins that are recognized by E2-specific antisera and contain carboxyl-terminal E2 sequences (14). These smaller proteins are not present in cells expressing mutant viral genomes unable to utilize the nt 3091 methionine codon or the E8/E2 splicing

pattern, suggesting that they are repressor species encoded by the wild-type genome (17). To confirm that BPV-1 expresses repressor activity and to determine its function, we have removed the putative initiation codon at nt 3091 and determined the effect of this mutation on expression of E2 repressor activity and on the other biological activities of BPV-1 DNA.

Mutant construction. We used standard oligonucleotide-directed mutagenesis of the BPV-1/M13 clone MR7 to convert the putative initiator ATG codon at BPV-1 nt 3091 to 3093 into an ACG codon (Fig. 1) (7, 16, 40). As well as presumably preventing translation initiation from this codon, this mutation results in the substitution of a threonine for a methionine in E2 proteins initiated upstream of this position. The base change also inactivates an *Nco*I cleavage site. The presence of the ATG→ACG substitution was confirmed by DNA sequencing, and the small *Bst*EII-to-*Bst*XI fragment (BPV-1 nt 2405 to 3881) containing the mutation was subcloned into the full-length BPV-1 genome (pBPV-142-6 [30]) to generate pBPV-RMC.

Transactivation and repression assays. A number of transient expression experiments were performed in monkey CV1 cells to determine whether the RMC mutation resulted in decreased repressor activity. Because the wild-type viral genome has the potential to express both positive and negative E2 regulatory activities, changes in either transactivation or repression or both might alter the net level of transactivation. Net transactivation was assessed by determining the expression of a bacterial chloramphenicol acetyltransferase (CAT) gene linked to BPV regulatory signals. Wild-type and mutant E2 proteins were expressed under the control of their endogenous regulatory signals in the full-length viral genome. The structures of the reporter plasmids and the viral genomes expressing ORF E2 used in these experiments are shown schematically in Fig. 2 above representative results of each type of assay.

p407-1 contains the CAT gene linked to the simian virus 40 early promoter and the entire BPV LCR such that CAT expression is dependent on E2-mediated transactivation

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FIG. 1. Schematic diagram of E2 genes studied in this report. The open bars represent ORF E2, with the 5'-most ATG codon (at left) and the one at nt 3091 to 3093 (at right) indicated. The lines below each E2 gene represent the active E2 proteins predicted to be present in cells if the internal methionine codon in wild-type DNA serves as an initiation codon for a repressor protein. A, Full-length E2 transactivator; R, internally initiated repressor. Both proteins are present in cells containing wild-type viral DNA, whereas only a mutant transactivator is present in cells containing the repressor mutant RMC. E2fsAK and RMC-E2fsAK contain a frameshift mutation in the 5' portion of ORF E2 that is followed shortly by a new in-frame stop codon, thereby preventing synthesis of the transactivator. For simplicity, forms of the repressor encoded by spliced mRNA have been omitted.

(36). Cotransfected BPV-RMC DNA stimulated severalfold more CAT expression from p407-1 than did wild-type viral DNA (in several different experiments, the mutant was $4.7\text{-fold} \pm 1.6\text{-fold}$ more active than the wild type) (Fig. 2A). Thus, in the standard assay, the RMC mutation results in increased net transactivation of the BPV-1 LCR.

To examine the expression of the E5 transforming gene under the control of its endogenous regulatory signals, we have used the plasmid pBPV-E5CAT, in which the CAT coding region is fused in frame to the ORF E5 initiation codon (26). We have previously shown that E5-CAT expression from this plasmid is stimulated by the E2 transactivator and inhibited by the E2 repressor. The RMC mutation was inserted into pBPV-E5CAT to generate pBPV-E5CAT-RM. Cells transfected with pBPV-E5CAT-RM expressed about fivefold (mean, 4.8 ± 1.3) more CAT activity than did cells transfected with the wild-type plasmid (Fig. 2B). To confirm that the RMC mutation was able to increase ORF E5 expression *in trans*, we tested the ability of viral genomes expressing E2 proteins to affect expression of the CAT gene from pBPV-E5CATΔB, a derivative of pBPV-E5CAT that contains a 101-base-pair deletion in the 3' end of ORF E2 that inactivates both the transactivator and repressor (Daniel DiMaio, data not shown). In the absence of a cotransfected E2 gene, CAT expression from pBPV-E5CATΔB is undetectable (Fig. 2D, lane N). In several independent transfection experiments, pBPV-RMC was severalfold more effective than wild-type DNA at stimulating E5-CAT expression from pBPV-E5CATΔB (Fig. 2C). The enhanced stimulatory effect of pBPV-RMC compared with wild-type DNA was more pronounced when the plasmids supplying the E2 proteins were present at low DNA concentrations (Fig. 2C). Thus, the increased net transactivation caused by the RMC mutation was observed repeatedly, both when the mutation and the reporter gene were present on the same plasmid and when they were on two different combinations of separate plasmids.

The increased net transactivation observed in the above experiments could be due to decreased repressor activity, as predicted from the nature of the introduced mutation. However, the methionine-to-threonine mutation also affected the structure of the full-length transactivator, and it is possible that this substitution results in a more efficient or more stable transactivator. To demonstrate that the RMC mutant expressed less repressor than did the wild type, repressor activity was measured directly from derivatives of pBPV-RMC and BPV DNA unable to express the transactivator. A frameshift mutation was introduced into the 5' end of the E2 gene in both the wild-type and RMC viral genomes by inserting four base pairs into the *AvrII* recognition site at nt 2766 to generate pBPV-E2fsAK and pBPV-RMC-E2fsAK, respectively (Fig. 1). The frameshift mutation eliminated transactivation from the resulting mutants (data not shown) but is not predicted to interfere with synthesis of repressor proteins. These frameshift mutants were then tested for their ability to inhibit transactivation of pBPV-E5CATΔB by cotransfected c59, a plasmid that expresses E2 transactivator (38). If the RMC mutation results in decreased repressor synthesis, pBPV-RMC-E2fsAK should express reduced repressor activity compared with pBPV-E2fsAK, despite the inability of these two plasmids to transactivate. Two independent clones of viral DNA containing both the 5' frameshift mutation and the RMC mutation inhibited transactivation less than did two clones of the viral DNA with the frameshift mutation only (Fig. 2D), indicating that the RMC mutation inhibits repressor synthesis. Moreover, cotransfection of RMC DNA plus wild-type DNA resulted in an intermediate rather than an additive level of transactivation (Fig. 2C). This result suggests that the wild-type genome is expressing an inhibiting factor that is not expressed by the repressor mutant.

Transformation assays. RMC and wild-type viral DNAs were separated from the bacterial plasmid vector and tested in C127 cell focus-forming assays (7). Several preparations of transfected BPV-RMC DNA reproducibly induced between two and three times more dense foci in monolayer culture than did wild-type DNA. In general, the foci induced by the mutant appeared earlier. Thus, the increased level of E5 expression detected in the transient expression experiments is accompanied by increased focus-forming activity.

Stably transformed cell lines were established from pooled foci induced by BPV-RMC DNA and by wild-type viral DNA and tested for their transformed phenotype in two additional assays. First, the cells were plated in soft agarose to determine their anchorage independence. Cells transformed by the repressor mutant gave rise to substantially more large colonies than did cells transformed by wild-type viral DNA (Table 1). Second, these cell lines were tested for their tumorigenicity in nude mice. All mice injected with cells transformed by the mutant developed tumors, whereas tumors developed in only a fraction of mice injected with wild-type-transformed cells. Moreover, the tumors arising from cells transformed with the RMC mutant were larger than their wild-type counterparts. These effects on anchorage-independent growth and tumorigenicity were observed in several independently derived cell lines. Thus, by several standard criteria, the RMC mutation resulted in enhanced transformation.

Analysis of viral DNA in transformed cell lines. To assess the state and copy number of viral DNA, total DNA was isolated from transformed cell lines and analyzed by Southern blotting (34). The DNA of both the wild-type BPV and the RMC mutant was maintained largely as a monomeric

FIG. 2. Analysis of E2 regulatory activity in transient expression assays. The maps in each of the panels represent the DNAs used for transfection into CV1 cells. The top line in each set represents the full-length BPV-1 genome either with or without the E2 RMC mutation. AK (in panel D) indicates the position of the frameshift mutation that inactivates the transactivator. The bacterial CAT gene (■) is linked to the BPV-1 LCR and the simian virus 40 early promoter (P) in p407-1 and is fused in frame to the E5 translation start codon in the BPV-E5CAT plasmids. The triangles in the maps in panels C and D represent the 101-base-pair deletion of a *Bcl*I fragment that inactivates both the repressor and transactivator. c59 in panel D expresses the transactivator from a full-length E2 cDNA. CV1 cells were transfected and assayed for CAT activity, as previously described (26). The bottom portions of panels A, B, and D show autoradiograms of the reaction products of CAT assays separated by thin-layer chromatography; panel C shows quantitation of the proportion of conversion to product in a similar assay.

TABLE 1. Properties of transformed cells

Input plasmid	Colony formation (%) ^a	Tumor	
		Incidence ^b	Size (cm)
None	0	0/6	
pBPV-142-6	0.2	5/8	0.3-1
pBPV-RMC	2.6	8/8	1-2

^a Stably transformed cells were plated in agarose as previously described (25), and the percentage of cells giving rise to colonies >0.6 mm in diameter was determined.

^b Tumorigenicity of stably transformed cell lines in nude mice was determined as previously described (25).

unrearranged plasmid (Fig. 3A). In cell lines derived both from single foci and from pooled foci, the mutant viral DNA was maintained at a copy number severalfold higher than that of wild-type viral DNA. From a comparison of standards corresponding to known copy numbers, we estimate that there are about 75 copies of the viral genome per cell transformed with wild-type viral DNA, whereas there are approximately 500 copies in cells transformed by the repressor mutant.

To document that the mutation was present in the viral DNA in the appropriate cell lines, we tested DNA isolated from transformed cells for the loss of the *Nco*I site at the position of the methionine codon mutation. In the wild-type viral genome, digestion with *Nco*I plus *Bam*HI generates a 1,300-base-pair fragment containing the 3' end of the viral early region. In the mutant, an *Nco*I site is eliminated and the corresponding *Bam*HI-to-*Nco*I fragment extends for an additional 211 base pairs to the adjacent *Nco*I site. As shown in Fig. 3B, the fragment present in cells transformed by RMC DNA comigrated with the band derived from the mutant input DNA. Therefore, the mutation was retained in the viral DNA in cells transformed by this mutant.

Discussion. The reduction of repressor activity by elimination of the methionine codon at nt 3091 demonstrates that the intact viral DNA expresses an E2 repressor protein. Our results and the specific absence of the abundant 31-kilodalton E2 repressor protein in C127 cells stably transformed by this mutant (17) strongly suggest that this methionine codon serves as the initiation codon for the major form of the E2 repressor. These conclusions were also reached by Lambert et al. (18), who have independently constructed and analyzed an identical mutant.

The known effects of the E2 regulatory proteins on viral promoters suggested that reduction of repressor activity would result in increased transcription (12, 36). We have documented in transient experiments that expression of the E5 gene is increased in cells transfected with the repressor mutant. This effect is not due to increased viral DNA copy number, because our expression assays were performed in CV1 cells in which BPV-1 DNA does not replicate (29). The biological activity of the repressor mutant in C127 cells implies that viral gene expression is increased in these cells also, a fact documented by the RNA mapping and immunoprecipitation experiments of Lambert et al. (18). In C127

FIG. 3. Analysis of viral DNA in cells transformed by the repressor mutant. (A) Total cellular DNA was purified from cell lines derived from individual foci induced by wild-type BPV-1 and BPV-RMC. After agarose gel electrophoresis without prior digestion with restriction endonuclease, samples were transferred to nitrocellulose and hybridized to BPV-1 probe (28, 33). Lane a, Representative cell line transformed by wild-type DNA; lanes b to e, cell lines transformed by BPV-RMC DNA. Arrow indicates the position of supercoiled viral DNA. (B) DNA digested with *Nco*I and *Bam*HI was analyzed as in panel A. Lane w, Marker of pBPV-142-6 (wild type); lane m, marker of pBPV-RMC; lanes d and e, DNA from two independent cell lines established from individual foci induced by pBPV-RMC. The caret indicates the band diagnostic of the introduced mutation.

cells, this increase is probably the effect of increased gene dosage and of a direct effect on transcription.

E2 transactivation is required for efficient transformation and normal viral DNA replication, so it is not surprising that the repressor mutation affects these biological activities (7, 8, 10, 15, 21, 27). The increased E5 gene expression and focus-forming activity of the repressor mutant strongly support our previous proposal that these activities are largely controlled by the net amount of E2-mediated transactivation (26). Lusky and Botchan (21) have described an ORF E1 mutant with markedly increased transforming activity, and it would be of interest to determine whether this mutation indirectly affected repressor synthesis. The enhanced transforming activity of the repressor mutant may be the consequence of overexpression of several viral transforming genes because the expression of either ORF E6 or ORF E5 can cause focus formation, anchorage-independent growth, and tumor induction and because ORF E7 has also been implicated in affecting anchorage-independent growth of BPV-1-transformed cells (5, 25, 31, 32, 38). The results reported

(A) Plates received 1 μ g of p407-1 plus no added BPV-1 DNA (0), 1 μ g of pBPV-RMC (R), or 1 μ g of the wild-type BPV-1 plasmid, pBPV-142-6 (W) (30). (B) Each plate received 4 μ g of pBPV-E5CAT (W) or pBPV-E5CAT-RM (R). (C) Each plate received 4 μ g of pBPV-E5CAT Δ B plus the indicated amount of wild-type DNA (▨) or pBPV-RMC DNA (□). ▨, CAT activity in cells cotransfected with pBPV-E5CAT Δ B plus 0.75 μ g of wild-type and 0.75 μ g of mutant DNAs. The numbers above the bars indicate the ratio of mutant to wild-type CAT activity. (D) Each plate received 4 μ g of pBPV-E5CAT Δ B, 0.5 μ g of c59, and either no additional BPV DNA (0) or 1 μ g of pBPV-RMC-E2fsAK (two different isolates) (R) or pBPV-E2fsAK (W) (two different isolates). Lane N received only pBPV-E5CAT Δ B.

here imply that the phenotype of cultured cells stably transformed by BPV-1 is not an all-or-nothing phenomenon but rather is correlated with the dose of viral gene products. This conclusion was previously suggested by the behavior of BPV-1-transformed hamster cells and C127 cells harboring a low-copy-number BPV-1 mutant (4, 39). However, a recent study of BPV-transformed C127 cell clones failed to reveal such a correlation (37). In C127 cells acutely transformed by the BPV-1 E5 protein, there is a clear correlation between the dose of the viral E5 gene and both the extent of morphologic transformation and the ability of the cells to escape growth arrest (33).

The control of viral DNA copy number in BPV-1-transformed cells is complex and incompletely understood. The number of viral genomes can initially be amplified from a low level yet be maintained at a constant copy number once transformation becomes established. We have shown here that the repressor mutant results in increased viral DNA copy number. In contrast, at least under some conditions, mutations in ORFs E6 and E7 result in a low copy number (1 to 5 genomes per cell), and mutations in the E1 M gene have been reported to result in increased initial replication that often culminates in cell death (3, 4, 21). The ability of *trans*-acting mutations to either increase or decrease the steady-state copy number in transformed cells implies that the viral DNA copy number is determined by the relative level of expression of virally encoded positive (and possibly negative) replication factors. If this is the case, it may be possible to increase the copy number even further by inactivation of alternative E2 repressors, by overexpression of the transactivator, or by other manipulations that increase the net expression of genes encoding positive replication factors. Such effects may increase the utility of cloning vectors based on BPV-1.

It is reasonable to speculate that a repressor is also expressed during natural infection by the papillomaviruses and that it contributes to the controlled expression of the viral genes. The increased transforming activity and replication potential we and Lambert et al. (18) have observed in a cultured-cell system may have counterparts in naturally arising lesions induced by the papillomaviruses. In these settings, inactivation of the E2 repressor by mutation, gene rearrangement, or other events may also result in increased oncogenicity or pathogenicity.

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