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Mutational Analysis of Open Reading Frame E4 of Bovine Papillomavirus Type 1

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Open reading frame (ORF) E4 is a 353-base-pair ORF of bovine papillomavirus type 1. To determine the biological activities of this ORF in mouse C127 cells, we analyzed the effects of two constructed mutations which are predicted to prevent synthesis of ORF E4 proteins while leaving the amino acid sequence encoded by the overlapping ORF E2 unchanged. Neither mutation interfered with the abilities of the mutants to efficiently induce focus formation, induce growth in soft agarose, or transactivate an inducible bovine papillomavirus type 1 enhancer. Also, neither mutation prevented establishment of the viral DNA as an extrachromosomal plasmid in transformed cells. These results suggest that ORF E4 proteins are not required for these biological activities, and they are consistent with the observation of others (J. Doorbar, D. Campbell, R. J. A. Grand, and P. H. Gallimore, EMBO J. 5:355–362, 1986) that the ORF E4 protein of a human papillomavirus is associated with late gene expression during papilloma formation.

Bovine papillomavirus type 1 (BPV) induces the formation of fibroepithelial tumors in animals and oncogenically transforms certain established lines of rodent cells in vitro (15, 24). Mouse C127 cells transformed by BPV form foci on a monolayer of normal cells, display anchorage independence, and are tumorigenic (8, 17). In the nuclei of these transformed cells, the viral DNA is maintained as a circular plasmid (14, 16). A 5,500-base-pair fragment of the viral genome contains eight open reading frames (ORFs), designated ORFs E1 to E8, which are thought to encode proteins responsible for the biological activities of the virus (2, 17, 29). Mutational analysis of the viral genome indicates that ORFs E1, E2, E7, and E6-7 play a role in maintenance of the viral DNA as an autonomous plasmid (1, 3, 10, 18, 19) and that ORFs E2, E5, and E6 are involved in inducing the appearance of transformed foci (3–5, 10, 22, 26–28, 33). ORF E2 is also required for transactivation of an enhancer located in the BPV long control region (31, 35). As part of our evaluation of the biological activity of each of the viral ORFs, we constructed base substitution mutations that should prevent synthesis of the putative protein product(s) of ORF E4, and we analyzed the effects of these mutations.

ORF E4 is a 353-base-pair ORF (nucleotide [nt] 3173 to nt 3526) that totally overlaps with ORF E2 in a different reading phase (Fig. 1A) (2). BPV-transformed cells and bovine fibropapillomas contain mRNAs that are unspliced in the ORF E4 region, as well as RNAs with a splice junction at nt 3224 (Fig. 1B) (32, 34). If an ORF E4 gene product is encoded by an unspliced mRNA, translation presumably initiates at the first and only methionine codon in ORF E4, located at nt 3191. The E4mc1 mutation changes bases at positions 3189 and 3192 to convert this codon to a threonine codon (Fig. 1B and C). If a spliced mRNA is used, ORF E4 would be spliced in frame to either ORF E6 or ORF E1, depending upon which upstream splice donor is used (32, 34). The E4am1 mutation is a single-base substitution at position 3249 which creates an amber termination codon in ORF E4 25 bases 3′ to the mRNA splice acceptor site at nt 3224 (Fig. 1B and C). This mutation would affect the synthesis of E4 proteins that initiate at the E4 methionine, as well as proteins translated from spliced E4 messages. Neither of these mutations alters the amino acid sequence of the protein product encoded by the overlapping ORF E2 (Fig. 1C). Both mutations were constructed by oligonucleotide-directed mutagenesis of a segment of BPV DNA; they were subsequently cloned separately into the full-length viral genome (13, 21, 36).

To test the transforming activity of these mutants, we separated the viral DNA from its bacterial plasmid vector and assayed it for its ability to induce foci on C127 cells (8). In several independent experiments, transfection was performed by the calcium phosphate precipitation method, as previously described (4, 9), with 100 ng of *Bam*HI-digested plasmid DNA for each plate, and foci were stained and counted 14 days after transfection. Both of the ORF E4 mutants (pE4am1 and pE4mc1) induced C127 cell focus formation (1,378 and 1767 foci per μg of viral DNA, respectively) as efficiently as did wild-type BPV DNA (pBPV-142-6; 1,444 foci per μg). Foci induced by the E4 mutants appeared at the same time and were the same size as foci induced by wild-type BPV DNA. We have previously reported that both ORFs E2 and E5 are necessary for efficient focus formation by full-length BPV but that neither ORF E2 nor ORF E5 mutants are totally transformation defective (3, 4). To determine whether the residual focus-forming ability of these mutants was due to weak transforming activity of the ORF E4 protein, we tested the activity of double mutants in which each of the ORF E4 mutations was combined with either an E2 or an E5 mutation. BPV DNA containing a 211-base-pair deletion in ORF E2 transformed at approximately 2% of the level of wild-type BPV, and double mutants containing the ORF E2 deletion plus either of the ORF E4 mutations transformed at this level as well (Table 1). BPV DNA containing a frameshift mutation in ORF E5 induces about 0.5% as many foci as does wild-type BPV DNA (4). Double mutants containing the ORF E5 mutation plus either of the ORF E4 mutations were no more defective than is the ORF E5 single mutant (Table 1). Therefore, ORF E4 is not a transforming gene as assayed by focus formation in C127 cells.

We established cell lines from transformed foci induced by the ORF E4 mutants. In semisolid medium, these cells

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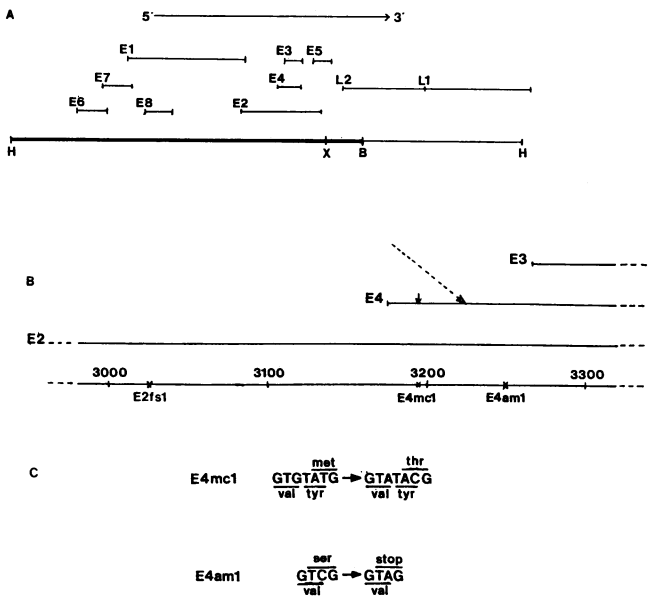

FIG. 1. (A) Genetic organization of BPV. The line at the bottom represents the 7,945-base-pair genome of BPV, linearized by cleavage at the unique *Hin*dIII (H) site. (X, *Bst*XI; B, *Bam*HI.) The lines above represent the translational ORFs, and the arrow indicates the direction of transcription (reproduced from reference 4). (B) Mutations in ORF E4. The figure represents a portion of the viral genome around the 5' end of ORF E4, and the locations of ORFs E2, E3, and E4 are indicated. The ×'s indicate the positions of constructed mutations in ORF E4 and ORF E2. E2fs1 is a frameshift mutant containing an insertion of 16 base pairs at the *Fsp*I site (nt 3023). The downward arrow indicates the position of the ORF E4 methionine triplet; the slanted arrow indicates the position of the splice acceptor site. (C) Base substitution mutations. Mutations in ORF E4 were constructed by oligonucleotide-directed mutagenesis (36) by using as a template M13-R4 (3, 21), an M13-BPV recombinant grown in the *Escherichia coli dut ung* strain (13). The sequences in the figure to the left of the arrows are the relevant wild-type BPV sequences, and those to the right are the mutant sequences. Phage DNAs containing these mutations were identified by *Acc*I digestion (mc1) or hybridization to the mutagenic oligonucleotide (am1). After being subcloned into the viral genome, the mutations were confirmed by DNA sequencing (20). Amino acids above the triplets correspond to the wild-type and mutant ORF E4 protein sequences, and those below correspond to the ORF E2 protein sequence (2).

efficiently formed colonies indistinguishable from colonies formed by cells transformed by wild-type viral DNA (Fig. 2). In addition, a cell line established from a single pE4am1-induced focus was plated in agarose, and cloned cell lines were established from four individual, well-separated colonies. During approximately 25 subsequent cell generations in continuous passage, each of these cell lines exhibited a uniform, piled-up appearance with no obvious evidence of an unstable transformed phenotype (data not shown).

To determine whether ORF E4 is required for extrachromosomal BPV replication, we analyzed the state of the BPV DNA in mouse C127 cells transformed by the ORF E4 mutants by the Southern blotting technique (30, 33) (Fig. 3). A number of cell lines were established from individual foci induced by wild-type viral DNA and by the two ORF E4 mutants. Viral DNA isolated from three of four cell lines established with wild-type DNA migrated at the position of full-length supercoiled circular BPV DNA molecules. Similarly, three of four cell lines induced by pE4am1 and two of four cell lines induced by pE4mc1 contained unit length, circular, extrachromosomal BPV DNA. Moreover, the cells transformed by wild-type DNA or the mutant BPV DNAs contained similar amounts of viral DNA. After digestion of DNAs from these cell lines with *Hin*dIII, which cuts once in the BPV genome, wild-type and mutant viral DNAs migrated at the position of full-length linear BPV molecules (data not shown). In addition, one cell line induced by wild-type DNA, as well as one cell line induced by each of the ORF E4 mutants, contained extrachromosomal viral DNA that had apparently suffered rearrangement, and the viral DNA in one cell line induced by E4mc1 had apparently integrated into cellular DNA. These results indicated that neither ORF E4 mutation prevents BPV DNA from becoming stably established as a plasmid in transformed mouse cells, but we have not ruled out more subtle effects of the mutations on replication.

The 5' noncoding region of BPV DNA contains a transcriptional enhancer which is only active in the presence of an intact ORF E2, but a role for ORF E4 in transactivation has not been ruled out (31, 35). To test this possibility, we used the assay described by Spalholz et al. (31), in which efficient expression of a bacterial chloramphenicol acetyltransferase gene in plasmid p407-1 requires the BPV transactivation function. We cloned each ORF E4 mutation into plasmid C59, which contains BPV ORFs E2, E3, E4, and E5 under the control of simian virus 40 regulatory sequences, and we tested the ability of the resulting plasmids (C59-E4mc1 and C59-E4am1) to transactivate chloramphenicol acetyltransferase gene expression from p407-1. Neither mutation affected transactivation, whereas a mutation that disrupts ORF E2 profoundly inhibited this activity (Fig. 4).

This paper reports the construction of base substitution mutations which should prevent translation of BPV ORF E4 but which are silent in the overlapping ORF E2. Neither of the mutations we tested caused defects in focus formation in C127 cells or in transactivation of an inducible BPV enhancer in CV1 cells. The viral DNA was maintained as a plasmid in many transformed cell lines established with the mutants, and the cells efficiently formed colonies in agarose. Our analysis thus indicated that ORF E4 is not essential for these in vitro biological activities. There are three possible explanations for these results. (i) ORF E4 is not a protein-coding sequence. (ii) the mutations do not prevent its expression, or (iii) it is required for an activity other than those we tested. The presence of an ORF in the same position as BPV ORF E4 in all sequenced papillomavirus genomes and the analysis of ORF E4 codon usage strongly suggest that it

TABLE 1. C127 cell transformation by double mutants[a]

Plasmid	Mutant ORF(s)	No. of foci/μg of viral DNA
pBPV-142-6	None	1,399
pE2-NIL	E2	22
pE2-4A	E2 and E4 (E4am1)	20
pE2-4M	E2 and E4 (E4mc1)	45
pE5-XL2	E5	4
pE5-4A	E5 and E4 (E4am1)	3
pE5-4M	E5 and E4 (E4mc1)	12

[a] The 211-base-pair *Nco*I fragment (nt 2878 to 3089) was deleted from p142-6 (6, 25) and from both ORF E4 mutants to generate the ORF E2 mutants. A frameshift mutation at the *Bst*XI site in BPV DNA has been previously described (4). A fragment containing this mutation was inserted into both ORF E4 mutants to generate the ORF E5 mutants. Transformation was performed by the calcium phosphate precipitation method, as previously described (4, 9), with 100 ng of *Bam*HI-digested plasmid DNA for each plate, and foci were stained and counted 14 days after transfection.

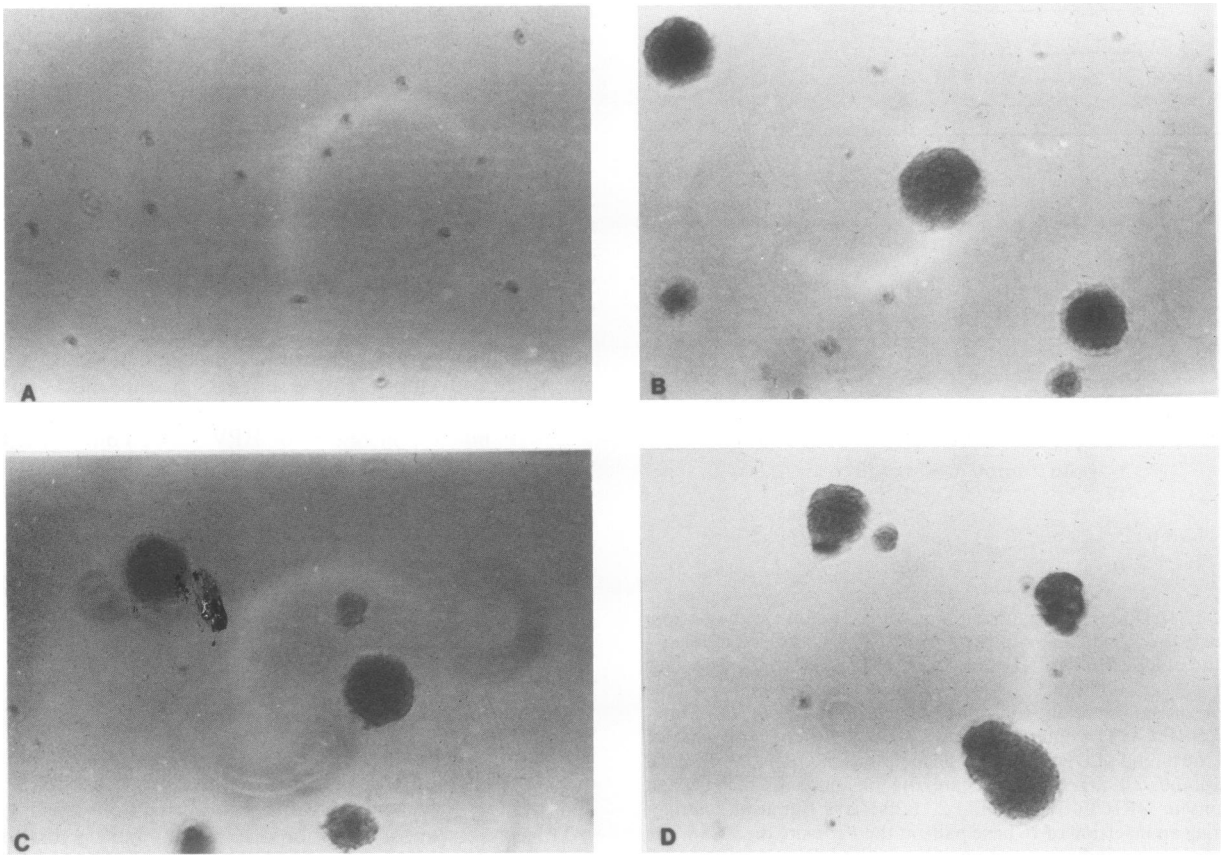

FIG. 2. Colony formation in semisolid medium. Transformed cell lines were established from a plate containing >50 foci induced by the indicated viral DNAs. Cells were then grown in 0.3% agarose in Dulbecco modified Eagle medium–10% fetal calf serum for 2 weeks with refeeding every 4 days. Colonies were photographed by phase-contrast microscopy. Panels: A, C127 cells; B, cells transformed with pBPV-142-6; C, cells transformed with pE4mc1; D, cells transformed with pE4am1.

is a protein-coding region (5, 23, 24a). Moreover, Doorbar et al. (7) have recently detected human papillomavirus 1a ORF E4 protein products in human papillomavirus 1a-induced human papillomas.

Translation of the vast majority of, and perhaps all, eucaryotic mRNAs initiates at AUG codons (12). Therefore, the E4mc1 mutation, which removes the sole methionine codon in ORF E4, is predicted to prevent translation of the intact ORF. The amber mutant E4am1 can express only eight codons of ORF E4 downstream of the splice acceptor site, whereas the wild-type spliced ORF has the potential to encode 101 amino acids. Although it is possible that the first eight amino acids downstream of the splice confer full biological activity to a protein product of the spliced RNA, it is difficult to reconcile this with the conserved length of the ORF in the sequenced viruses. It is also possible that a low level of initiation at the mutant ATG codon (or at some other, nonconventional initiation codon) or of readthrough past the amber codon would allow synthesis of enough E4 protein for biological activity.

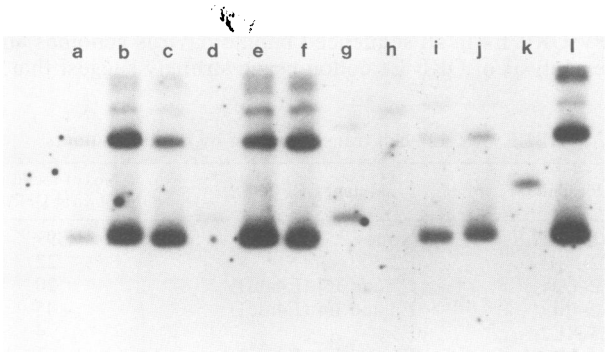

FIG. 3. State of viral DNA in transformed cells. Transformed cell lines were established from individual foci induced by p142-6, pE4mc1, or pE4am1. After approximately 20 cell generations, low-molecular-weight DNA was prepared from each cell line (11) and electrophoresed on a 1% agarose gel. After transfer to nitrocellulose, and hybridization to BPV DNA labeled with ^{32}P by nick translation, viral sequences were detected by autoradiography. DNAs from different clonal cell lines established with pBPV-142-6 (lanes a to d), pE4mc1 (e to h), or pE4am1 (i to l) were used.

We feel that it is most likely that ORF E4 is a gene involved in an activity we have not assayed. Different assays for transformation may reveal a requirement for BPV ORF E4. Detection of large quantities of human papillomavirus 1a ORF E4 proteins in cells expressing viral structural proteins raises another intriguing possibility, namely, that ORF E4 encodes proteins required for productive viral growth in animals (7). Moreover, the most abundant viral RNA species in the productively infected epithelial component of bovine fibropapillomas could potentially encode an ORF E4 protein product (C. Baker, personal communication). The results reported here are consistent with ORF E4 playing a role in productive infection, but we cannot directly test this hypo-

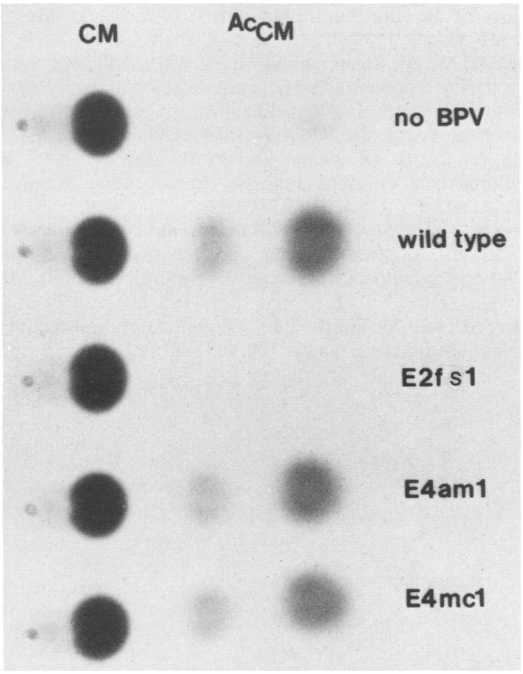

FIG. 4. Transactivation of the BPV enhancer. CV-1 cells were transfected with p407-1 alone or with p407-1 plus either C59 (containing wild-type DNA [31]) or a derivative of C59 containing a defined mutation in ORF E2 (E2fs1) or ORF E4 (E4am1, E4mc1). At 48 h after transfection, cellular extracts were prepared and chloramphenicol acetyltransferase activity was determined as previously described (31). The figure shows an autoradiogram of labeled reaction products separated by thin-layer chromatography. Abbreviations: CM, chloramphenicol; ^{Ac}CM, acetyl-chloramphenicol.

thesis because there is currently no in vitro system for the propagation of papillomaviruses. Regardless of the biological activities eventually attributed to it, our results indicate that neither efficient C127 cell focus formation nor transactivation requires BPV ORF E4, a gene totally embedded in the coding region of ORF E2, which is required for both activities.

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