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Genetics

Translation of open reading frame E5 of bovine papillomavirus is required for its transforming activity

(viral oncogenes/oncogenic transformation/frameshift mutations)

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ABSTRACT **A series of mutations in open reading frame (ORF) E5 of bovine papillomavirus type 1 has been constructed to determine whether this putative gene is required for *in vitro* oncogenic transformation by viral DNA. Frameshift mutations at either of two different positions located exclusively in ORF E5 cause a substantial reduction in the ability of the cloned viral DNA to induce the appearance of transformed foci of mouse C127 cells. A genetic mapping experiment with one of the mutants indicates that this characteristic transformation defect is actually due to the constructed mutation in ORF E5. Analysis of 10 different mutants with sequence changes at a single position in the ORF showed that there is an exact correspondence between transformation-competence and the ability of the 3′ half of ORF E5 to be correctly translated. The transformation defect of an ORF E5 frameshift mutant can be suppressed by a second mutation that restores the correct reading frame to most of the ORF, but not by one that restores the reading frame near the 3′ end of the ORF. These results constitute strong genetic evidence that translation of ORF E5 is required for efficient transformation of mouse C127 cells by bovine papillomavirus DNA. Wild-type ORF E5 has the potential to encode a short hydrophobic protein or polypeptide domain.**

The papillomaviruses cause benign epithelial-cell tumors in infected animals and humans, and these lesions occasionally progress to malignancy (1, 2). Infection by a subset of the papillomaviruses, the fibropapillomaviruses, induces fibroblastic as well as epithelial proliferation (1). This property of the fibropapillomaviruses is reflected in their potent transforming activity toward fibroblasts in culture. The study of *in vitro* transformation by these viruses may shed light on their biological activity during natural infection as well as on the mechanism of oncogenic transformation.

The best-studied fibropapillomavirus is bovine papillomavirus type 1 (BPV), which efficiently induces the appearance of foci of morphologically transformed mouse cells *in vitro* (3, 4). The 7945-base-pair (bp) BPV genome contains a number of translational open reading frames (ORFs) that are thought to encode viral proteins (5, 6) (Fig. 1*a*). A 5437-bp subgenomic transforming fragment of viral DNA contains eight ORFs which have been designated E1 through E8 (5–7). All the ORFs are located on the same strand of the viral DNA and each ORF overlaps with at least one other. This complex genetic organization has prevented the precise definition of the papillomavirus transforming genes. Moreover, the assignment of specific proteins to individual ORFs has been hampered by the difficulty of identifying nonstructural viral proteins in infected or transformed cells, but recently a likely candidate for a protein product of ORF E6 has been detected in BPV-transformed mouse C127 cells (8). It has not been possible to isolate temperature-sensitive viral mutants because the papillomaviruses do not propagate in tissue culture.

Viral mutants have been constructed *in vitro* and assayed for their transforming activity. Mutations in apparent regulatory regions and in ORFs E2, E5, and E6 have been reported to inhibit *in vitro* transformation of C127 and NIH 3T3 cells (9–17). It has not been proven that the transformation defect caused by the mutations in any of the BPV ORFs is due to the absence of a protein encoded by the mutant ORF. Because there are multiple, overlapping ORFs in the transforming segment, many of the mutations affect more than a single ORF. Moreover, it is possible that some mutations lie within regulatory signals or interfere with RNA processing or stability, thus affecting expression of a distal ORF.

One of the BPV ORFs implicated in fibroblast transformation is ORF E5, a 300-bp ORF located at the 3′ end of the transforming segment. The 5′ half of ORF E5 overlaps with the 3′ end of ORF E2 (Fig. 1*b*), and ORF E5 contains two ATG triplets, both of which are located downstream from the termination codon of ORF E2. Some mutants with sequence changes at the *Bst*XI restriction site just downstream of the first ATG in ORF E5 display a profound defect in focus formation in mouse cells (15–17), whereas deletions of portions of the ORF upstream of the ATG did not result in significantly reduced transformation efficiency (13, 15). To examine in detail the requirement for ORF E5 in transformation, we have constructed an extensive set of mutations in this ORF and determined the ability of the cloned mutant viral DNAs to transform mouse C127 cells. The results of this analysis strongly suggest that ORF E5 is translated into a protein required for efficient focus formation by BPV DNA in these cells.

MATERIALS AND METHODS

General Methods. The starting plasmids for these experiments were pBPV-142-6, which consists of the full-length BPV genome inserted into pML2d (18), and pBPV-H11, which consists of the 5437-bp *Hin*dIII–*Bam*HI transforming segment of BPV DNA inserted into pBRd (19). Restriction endonucleases and other DNA-modifying enzymes were purchased from commercial sources and used according to the suppliers' instructions. After the *in vitro* manipulations outlined below, bacterial transformations were carried out in *Escherichia coli* strain DH-1 according to Hanahan (20), and ampicillin-resistant bacterial colonies were screened for mutant DNA by restriction analysis of small-scale DNA preparations (21).

***Bst*XI Mutations.** One microgram of *Bst*XI-digested pBPV-142-6 DNA was treated with 60 units of nuclease S1 for 10 min at 20°C, ligated at low DNA concentration with T4 DNA

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Abbreviations: BPV, bovine papillomavirus; ORF, open reading frame; bp, base pair(s).

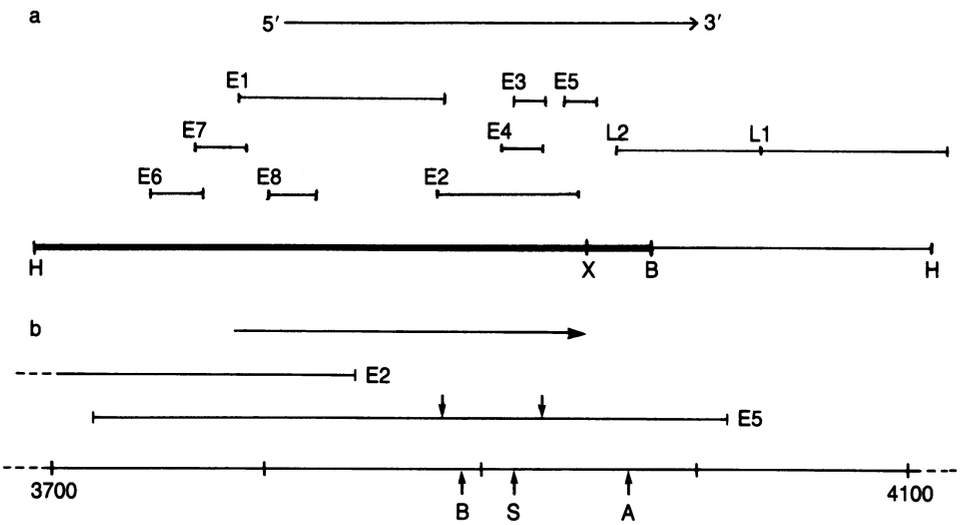

FIG. 1. (*a*) Genetic organization of BPV. The line at the bottom represents the 7945-bp genome of BPV, linearized by cleavage at the unique *Hin*dIII (H) site. Cleavage sites for *Bst*XI (X), and *Bam*HI (B) are indicated. The subgenomic transforming segment of BPV DNA (69% of the genome) is represented by the thick line. The lines above represent the translational open reading frames (5). The arrow at the top indicates direction of transcription. (*b*) Map of the ORF E5 region of BPV DNA. The line at the bottom represents a portion of the BPV genome. Crossmarks have been placed every 100 bp and are numbered according to convention (5, 6). The locations of cleavage sites for *Bst*XI (B, cleaves at nucleotide 3889), *Spe* I (S, 3911), and *Acc* I (A, 3967) are indicated. The positions of ORF E5 and 3' end of ORF E2 are shown, and the direction of transcription is indicated by the arrow at the top. The downward-pointing arrows indicate the positions of the two ATG triplets in ORF E5.

ligase, and digested with *Bst*XI. After bacterial transformation, mutants pE5DB-4, -6, and -13 were isolated.

Synthetic *Xho* I linkers were inserted into *Bst*XI-treated DNA as described (14) to generate pE5XL-1. pE5XL-2 was derived from pE5XL-1 DNA by complete digestion with *Xho* I and ligation at low DNA concentration. After *Xho* I-digested pE5XL-1 DNA was treated with nuclease S1, ligated, and redigested with *Xho* I, mutant pE5DX-10 was recovered. Mutant pE5DX-10 contains a deletion in the pML2d segment of the plasmid in addition to the mutation at the *Bst*XI site, but this second extraneous mutation was separated from the viral DNA sequences prior to assay for biological activity.

pE5DX-8 was constructed by treating *Xho* I-digested pE5XL-2 with nuclease S1, ligase, and *Xho* I. pE5DX-16 was constructed by digesting pE5XL-2 DNA with *Xho* I, filling in the ends of the DNA molecule by incubation with the Klenow fragment of DNA polymerase I plus all four deoxyribonucleoside triphosphates, ligation, and redigestion with *Xho* I. pE5DX-17 was constructed by treating *Xho* I-digested, polymerase-repaired pE5XL-2 DNA with nuclease S1, followed by ligation and redigestion with *Xho* I. pE5DX-18 was constructed by treating *Xho* I-digested pE5XL-2 DNA with the Klenow fragment of DNA polymerase I plus dCTP, dGTP, and dTTP, followed by digestion with nuclease S1, ligation, and *Xho* I digestion.

***Spe* I Mutations and Genetic Mapping.** pBPV-H11 was digested to completion with *Spe* I. The linear DNA was digested with nuclease S1, ligated, and redigested with *Spe* I. pE5HS-9 was isolated following bacterial transformation. pE5HS-9 and pBPV-142-6 were digested to completion with *Bst*XI and *Sal* I (the latter enzyme cleaves in the bacterial vector segment 275 bp from the *Bam*HI site at the junction with viral DNA). The two DNAs were ligated at equimolar concentration (total DNA concentration 30 μg/ml) and used to transform bacteria. A plasmid the size of pE5HS-9 containing the wild-type DNA segment downstream of the *Bst*XI site identified by the presence of the *Spe* I site and designated pE59MR-2.

Double Mutations. pXLBR-1 was constructed by inserting the *Hin*dIII–*Bam*HI segment of pE5XL-2 into pBRd. pE5XS-1 was constructed by sequentially treating pXLBR-1 with *Spe* I, nuclease S1, T4 DNA ligase, and *Spe* I. pXLBR-1 was also partially digested with *Acc* I in the presence of ethidium bromide at 5 μg/ml. Full-length linear partial digestion products were purified by agarose gel electrophoresis, treated with the Klenow fragment of DNA polymerase plus dATP and dTTP, and treated with T4 DNA ligase. pE5XA-1 was identified by restriction analysis.

Nucleotide Sequencing. The nucleotide sequences of all the mutations described in this report were determined by the method of Maxam and Gilbert (22) after the Klenow fragment of DNA polymerase I was used to end-label mutant plasmid DNA digested with *Xho* I, *Spe* I, or *Acc* I.

C127 Cell Transformation. Purified plasmid DNA was digested with *Bam*HI (*Bst*XI mutants) or with *Bam*HI plus *Hin*dIII (*Spe* I and double mutants); without further purification, 50–200 ng of the DNA plus calf thymus DNA carrier was added to mouse C127 cells as a calcium phosphate precipitate as described (23). After a 12-hr incubation with the DNA, the cells were subjected to a 20% (vol/vol) dimethyl sulfoxide shock and then incubated for 12 hr at 37°C in the presence of 5 mM sodium butyrate (24). The cells were then passaged 1:3 and incubated at 37°C with medium changes twice weekly. Foci were counted between 14 and 18 days after DNA transfer. Table 1 and Figs. 3 and 4 show the results of three separate transformation experiments, expressed as the number of transformed foci per μg of plasmid DNA. Each plasmid exhibited comparable activity in at least two additional experiments. No foci appeared on plates that did not receive viral DNA.

RESULTS

To generate mutations located exclusively in ORF E5, we used two restriction endonucleases that cut within E5 but at no other sites in the transforming region of BPV DNA. *Bst*XI cleaves 5 bp 3' of a possible initiator ATG triplet in the middle of ORF E5; *Spe* I cleaves 22 bp downstream (see Fig. 1).

A series of mutations has been constructed in the full-length viral genome at the *Bst*XI site to determine whether there is a correlation between the genetic integrity of ORF E5 and transformation competence. To construct the mutations, wild-type BPV DNA and mutant DNA with two tandemly arranged *Xho* I linkers at the *Bst*XI site (mutant pE5XL-1) were linearized with *Bst*XI and *Xho* I, respectively. The ends

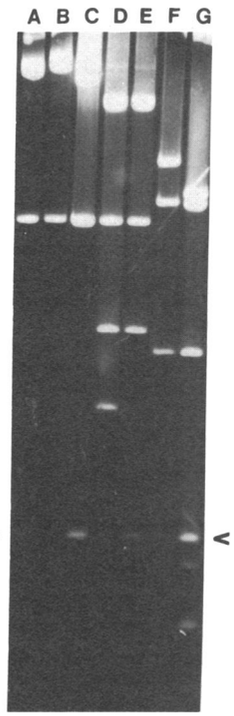

FIG. 2. Presence of new restriction endonuclease-cleavage sites in ORF E5. Wild-type or mutant plasmid DNA was digested with *Bam*HI plus an additional enzyme (indicated below in parentheses). Samples were electrophoresed in a 1.4% agarose gel and visualized with ethidium bromide. The arrowheads indicate the position of the 562-bp fragment of wild-type DNA extending from the *Bst*XI site in ORF E5 to the *Bam*HI site at the 3' end of the transforming region. Lanes: A, pBPV-142-6 (*Bst*XI); B, pBPV-142-6 (*Xho* I); C, pE5XL-2 (*Xho* I); D, pBPV-142-6 (*Stu* I); E, pE5XL-1 (*Stu* I); F, pBPV-142-6 (*Fnu*DII); G, pE5DX-18 (*Fnu*DII). The band in lane G that comigrates with the 562-bp wild-type fragment is a doublet.

of these molecules were then enzymatically modified as described in *Materials and Methods*; several different starting plasmids and enzymatic treatments were used to maximize the number of different mutations. From the known nucleotide sequence of wild-type BPV DNA and of mutant pE5XL-1, it was possible to predict that a number of the mutations would generate new restriction endonuclease-recognition sites. Several of the mutants that had acquired the predicted sites are shown in Fig. 2. For each of these mutants, the DNA fragment extending from the *Bam*HI recognition site at the 3' end of the transforming region to the new cleavage site comigrated with the 562-bp fragment extending from the *Bam*HI site to the *Bst*XI site in wild-type DNA. This result documents the existence of the expected mutations in ORF E5 in these mutants. We determined the nucleotide sequences of these mutations and of some others which did not result in the acquisition of a new cleavage site. Table 1 lists the sequence of each mutation and indicates whether ORF E5 is retained in-frame. Three different in-frame and seven out-of-frame mutants were isolated.

The transforming activity of each mutant was assayed by releasing the full-length BPV genome from its bacterial vector and adding the fragments as a calcium phosphate precipitate to C127 cells. Results of a representative transformation experiment are shown in Table 1. All seven mutants with out-of-frame mutations in ORF E5 exhibited a severe transformation defect. Two independent DNA preparations were tested for each defective mutant, and in several cases, multiple independent isolates containing the same mutations were tested and displayed identical defects. Moreover, an independent isolate of pE5XL-2 displayed a similar focus-forming defect when assayed by our standard procedure or when the viral DNA was not separated from bacterial plasmid sequences prior to DNA transfer and the dimethyl sulfoxide and sodium butyrate treatments were omitted (J.T.S., data not shown). In contrast, the three mutants with in-frame mutations (pE5XL-1, pE5DX-8, and pE5DX-18) induced transformed foci as efficiently as did the wild-type viral genome. The foci induced by wild-type viral DNA and these mutants became visible the same number of days after DNA transfer, they enlarged at the same rate, and there were no obvious differences visible by phase-contrast microscopy.

Mutations at the *Spe* I site in ORF E5 also caused a transformation defect. These mutations were constructed in pBPV-H11, a plasmid that contains the 5437-bp subgenomic transforming fragment of the viral genome. (This plasmid was used in these experiments to facilitate mutant construction because the nontransforming segment of the viral DNA contains a second *Spe* I site.) Two independent mutants were identified by the loss of the *Spe* I site, and sequence analysis showed that both contained the same 4-bp deletion at the *Spe* I site (see legend to Fig. 3). When wild-type and mutant viral DNA were separated from the prokaryotic vector sequences and added to mouse C127 cells, both independent mutants were far less efficient than wild-type at inducing the formation of foci (Fig. 3 shows the results for one of the isolates, pE5HS-9).

A genetic mapping experiment was performed on the *Spe* I mutant pE5HS-9 to prove that the defect in transformation was caused by the constructed mutation in ORF E5. A DNA fragment, containing the 562-bp segment of wild-type viral DNA from the *Bst*XI site in ORF E5 to the 3' end of the transforming segment, was substituted for the corresponding mutant DNA segment. This substitution restored efficient focus-forming activity to the reconstructed genome (pE59MR-2), which now contained a wild-type E5 region (Fig. 3). The fragment that was substituted in this experiment contains ORF E5 but no other viral ORF expressed in transformed cells; thus, the transformation defect displayed by the viral DNA with the mutation in ORF E5 is not caused by an undetected mutation in another ORF.

These results suggested that ORF E5 has to be translated to allow efficient focus formation in our transformation assay. To examine this hypothesis in more detail, we con-

Table 1. Mutations at the *Bst*XI site

Plasmid	Sequence of ORF E5*	E5 in-frame†	Foci‡
pBPV-142-6	ATG-CCA-AAT-CTA-TGG	+	8300
pE5XL-1	ATG-CCA-ACC-TCG-AGG-CCT-CGA-GGA-TGG	+	6000
pE5DX-8	ATG-CCA-ACC-GGA-TGG	+	6000
pE5DX-18	ATG-CCA-ACC-TCG-CGA-GGA-TGG	+	9000
pE5XL-2	ATG-CCA-ACC-TCG-AGG-ATG-G	−	15
pE5DX-16	ATG-CCA-ACC-TCG-ATC-GAG-GAT-GG	−	<10
pE5DX-10	ATG-G	−	<5
pE5DX-17	ATG-CCA-ACC-TCG-ACG-AGG-ATG-G	−	10
pE5DB-4	ATG-CCA-ATG-G	−	17
pE5DB-6	ATG-CCA-AAT-GG	−	10
pE5DB-13	ATG-CCA-ATC-TAT-GG	−	10

*The sequence of ORF E5 is shown from the first ATG to the TGG originally located four triplets downstream.
†+ indicates that the 3' end of ORF E5 is in-frame relative to the ATG; − indicates that it is out-of-frame.
‡No. per μg of plasmid DNA.

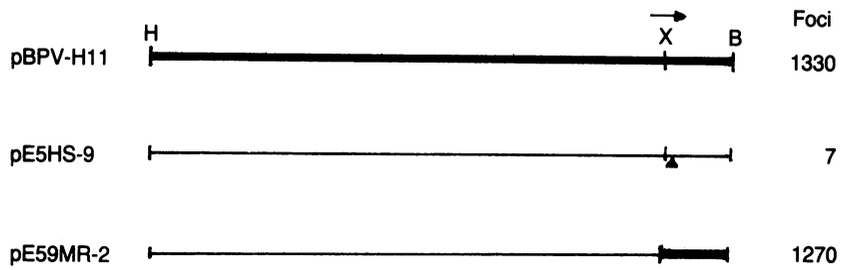

FIG. 3. Effect of mutation at the *Spe* I site in ORF E5. The *Hin*dIII–*Bam*HI subgenomic transforming segment of BPV DNA is represented by the horizontal lines, and the location of cleavage sites are indicated as in Fig. 1*a*. The small arrow at the top shows the location and size of ORF E5. The mutation at the *Spe* I site is represented by the solid triangle. The wild-type sequence at the *Spe* I site is ...GGACTAGTT...; the mutant sequence is ...GGATT... . The thick lines represent DNA derived from wild-type DNA (either pBPV-H11 or pBPV-142-6); the thin lines represent DNA derived from the mutant, pE5HS-9.

structed two double mutants, each of whch combined the 4-bp insertion of pE5XL-2 and a second mutation downstream in ORF E5. Sequence analysis demonstrated that the region between the two mutations is out-of-frame in both mutants (but it does not contain any stop codons); the correct reading frame is restored downstream of the second mutation (Fig. 4). For pE5XS-1, the second mutation is the same 4-bp deletion at the *Spe* I site as is present in pE5HS-9; thus three-fourths of ORF E5 downstream of the first ATG is in-frame. As shown in Fig. 4, pE5XS-1 with mutations at both the *Bst*XI and *Spe* I sites efficiently transforms cells even though either mutation by itself causes a severe transformation defect. For pE5XA-1, the downstream mutation that restores the correct reading frame is a 2-bp insertion at the *Acc* I site near the 3′ end of ORF E5; less than one-third of ORF E5 is in-frame. Several independent isolates of this mutant display a marked transformation defect. Efficient focus formation was restored when the *Xho* I–*Bam*HI fragment of pE5XA-1 was replaced by the corresponding fragment of pE5XS-1 (data not shown).

DISCUSSION

These experiments examine the requirement of BPV ORF E5 for efficient transformation of mouse C127 cells. Mutations at two different positions located exclusively in this ORF characteristically cause a substantial drop in focus-forming activity in these cells, thus confirming the previously reported effect of mutations in ORF E5 (15–17). Further, the results strongly suggest that the transformation defect of the mutants is due to the inability of ORF E5 to be translated and not to an unanticipated effect of the mutation on some other ORF.

The genetic mapping experiment with the *Spe* I mutant indicates that the transformation defect is likely to be due to the constructed mutation in ORF E5. Although a formal genetic mapping experiment has not been performed with the defective mutants with sequence changes at the *Bst*XI site, several observations indicate that the transformation defect is actually due to the constructed mutations. Numerous different mutants with out-of-frame mutations at this site are defective; and in several cases, more than one independent isolate of the same defective mutant has been assayed. Moreover, in two cases, in-frame transformation-competent mutants (pE5DX-8, pE5DX-18) were derived from a defective out-of-frame one (pE5XL-2) by further sequence changes at the site of the original mutation.

The correlation between the ability of ORF E5 to be translated and of the viral mutants to transform C127 cells suggests that translation of this ORF is required for efficient focus formation by linear BPV DNA. The most likely basis for this requirement is that ORF E5 encodes a protein sequence essential for transformation. This interpretation is strengthened by the contrasting transforming activities of the two double mutants. Both mutants contain the same 4-bp insertion near the 5′ end of ORF E5, which causes a shift in reading frame. The mutants differ in the position of the downstream mutation and hence in the position at which the correct reading frame is restored. pE5XS-1, in which most of ORF E5 can be translated in the correct frame, transforms efficiently; pE5XA-1, in which most of the ORF is in the incorrect frame, is transformation-defective. Thus, translation of ORF E5 in the correct reading frame and not merely translation of this region of RNA seems to be required for transformation. However, this requirement is not absolute. In numerous transformation experiments with *Bam*HI-digested full-length viral DNA, the out-of-frame *Bst*XI mutants invariably displayed a marked quantitative defect in focus

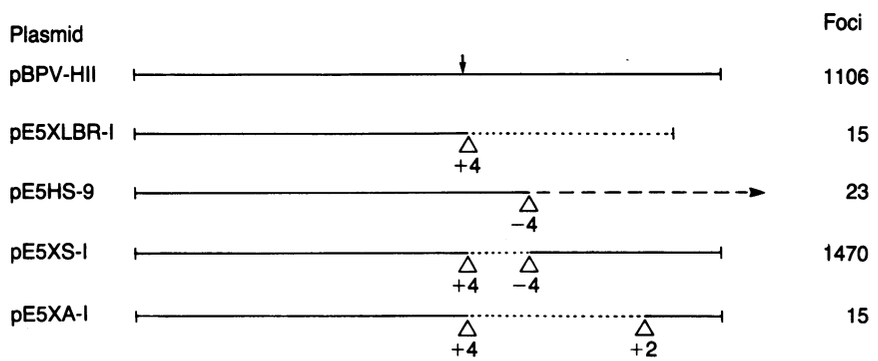

FIG. 4. Suppression of frame-shift mutation by a second, downstream mutation. The figure shows schematically the structure of ORF E5 in wild-type and mutant DNA. The downward pointing arrow on the top line indicates the position of the 5′-most ATG in this ORF (it is also present in all the mutants). The triangles represent the mutations at the *Bst*XI site (+4) and either the *Spe* I (−4) or the *Acc* I site (+2). The wild-type sequence at the *Acc* I site is ...GTATAC...; the mutant sequence is ...GTATATAC... . The dotted line represents the +1 reading phase of this DNA region relative to ORF E5; the dashed line represents the +2 reading phase (which continues 70 bases 3′ beyond the ORF E5 termination codon).

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BPV   ...ATG-CCA-AAT-CTA-TGG-TTT-CTA-TTG-TTC-TTG-GGA-CTA-GTT-GCT-GCA
      ...met pro asn leu trp phe leu leu phe leu gly leu val ala ala

XS-1  ...ATG-CCA-ACC-TCG-AGG-ATG-GTT-TCT-ATT-GTT-CTT-GGG-ATT-GCT-GCA
      ...met pro thr ser arg met val ser ile val leu gly ile ala ala

BPV   ATG-CAA-CTG-CTG-CTA-TTA-CTG-TTC-TTA-CTC-TTG-TTT-TTT-CTT-GTA
      met gln leu leu leu leu leu phe leu leu leu phe phe leu val

BPV   TAC-TGG-GAT-CAT-TTT-GAG-TGC-TCC-TGT-ACA-GGT-CTG-CCC-TTT-TAA
      tyr trp asp his phe glu cys ser cys thr gly leu pro phe
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FIG. 5. Predicted amino acid sequence of putative protein product of ORF E5. The first, third, and fourth pairs of lines show the nucleotide sequence of the 3′ half of ORF E5 and the predicted amino acid sequence of its putative protein product, beginning with the first methionine encoded by the reading frame. The second pair of lines shows the sequences of the corresponding region of the double mutant pE5XS-1, with the out-of-frame segment underlined.

formation. When the DNA of mutant pE5XL-2 is circulated *in vitro* prior to transfection, it usually displays a similar transformation defect. In some experiments, however, the circularized DNA induces the appearance of a small number of minute, slowly enlarging foci (D.D., unpublished data). The basis for this variability is unclear, but perhaps under some assay conditions other viral transforming genes (for example ORF E6) can partially compensate for a defective ORF E5.

Some of the in-frame mutations are predicted to cause significant changes in the amino acid sequence of the putative ORF E5 protein. For example, mutant pE5XL-1 has the potential to encode a protein with the insertion of two arginines and a proline, and there is an 11 amino acid substitution between the proteins encoded by mutant pE5XS-1 and wild type (Fig. 5). Although some of the in-frame mutations may cause defects in other viral activities, the focus-forming activity of ORF E5 in C127 cells is not impaired by mutations that would result in amino acid substitutions near the first methionine. The portion of ORF E5 downstream of the *Spe* I site, on the other hand, seems essential for efficient focus formation. The predicted amino acid sequence for this portion of the ORF contains an uninterrupted stretch of hydrophobic residues (Fig. 5), a sequence that suggests membrane association. The corresponding region of the two other sequenced genomes of fibropapillomaviruses, those of bovine papillomavirus type 2 and the deer fibromavirus, have the potential to encode a short polypeptide of similar composition (ref. 16 and W. Lancaster, personal communication).

Translation of ORF E5 from the AUG just 5′ of the *Bst*XI site would result in the production of a 44 amino acid hydrophobic polypeptide. This AUG is not the most 5′ one in any characterized BPV message (25, 26), and it is not known whether translation of ORF E5 initiates at this codon or whether the ORF E5 polypeptide is a domain of a larger protein initiated upstream. If the latter is the case, ORF E5 may encode a C-terminal hydrophobic domain that causes the larger protein to be membrane-associated. Such a protein could be translated from an as-yet-undetected spliced mRNA or it could be synthesized via frameshift suppression of the termination codon at the 3′ end of ORF E2. However, it does not appear that upstream sequences are absolutely required for the transforming activity of ORF E5 in some plasmid constructions (15). Biochemical identification and characterization of the ORF E5 gene product are obviously required to determine its structure and localization. The availability of the mutations that cause predictable alterations in the amino acid sequence of the ORF E5 protein should facilitate this analysis. Moreover, the mutants described here should be a useful reference set for determining the requirement for BPV ORF E5 in other biological activities of the viral DNA.

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