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Genetic and biochemical definition of the bovine papillomavirus E5 transforming protein

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Mutations surrounding the first methionine codon of the E5 transforming gene of bovine papillomavirus (type 1) were analyzed for their effect on cellular transformation and on the synthesis of the 7-kd E5 polypeptide. Frameshift mutations upstream of this methionine codon (bp 3879) affect neither transforming activity nor the ability to synthesize full-size E5 protein. In contrast, frameshift mutations distal to this position result in the inhibition of cell transformation and prevent synthesis or accumulation of E5 protein in cells containing the mutant viral genomes. Several in-frame mutations distal to the first methionine codon have a minimal effect on transforming activity but alter the electrophoretic mobility of the E5 protein in a manner consistent with the generated genetic alteration (deletion, insertion or substitution). In all cases where the protein is detected, it fractionates with cellular membranes and forms dimers. These studies indicate that (i) the methionine codon at bp 3879 serves as the initiation codon for the mature E5 protein, (ii) changing the charge of the E5 amino-terminus (from neutral to positive) does not prevent the association of this hydrophobic polypeptide with cellular membranes, and (iii) E5 amino-terminal mutations do not interfere with the ability of this polypeptide to form homodimers. We conclude that the major focus-inducing activity of the intact BPV genome is due to the function of the small polypeptide encoded in the 3′ half of the E5 ORF.

Key words: papillomavirus/cell transformation/membrane protein/disulfide bond formation/frameshift mutations

Introduction

Bovine papillomavirus type 1 (BPV-1) provides a convenient and reproducible model system in which to study papillomavirus-mediated cell transformation. The development of an *in vitro* focus assay using immortalized mouse cells (Dvoretzky *et al.*, 1980) has permitted the detection and analysis of transforming functions in cloned BPV-1 DNA. Transfection studies using constructed viral mutants and subgenomic fragments of BPV-1 DNA have mapped two independent regions of BPV DNA that are directly involved in cell transformation: the E5 and E6 genes (DiMaio *et al.*, 1986; Groff and Lancaster, 1986; Sarver *et al.*, 1984; Schiller *et al.*, 1984, 1986; Yang *et al.*, 1985a,b). Focus formation is dramatically inhibited by insertion of a termination codon or frameshift mutations into open reading frame (ORF) E5 downstream of the first methionine codon (which is located at bp 3879 in the middle of the ORF). This suggests that the E5 ORF encodes a protein critical to the transformation process, a conclusion supported by the finding that many in-frame E5 mutants can transform cells efficiently (DiMaio *et al.*, 1986). An antiserum generated against a synthetic peptide corresponding to the carboxy-terminal amino acid sequence encoded by the E5 ORF recognizes a 7-kd membrane-associated polypeptide which is present only in BPV-transformed cells (Schlegel *et al.*, 1986). When the E5 ORF is placed downstream of a surrogate promoter in the absence of other BPV genes, it is capable of inducing foci in C127 and NIH-3T3 cells (although with markedly reduced efficiency relative to full-length viral DNA) and directing the synthesis of the 7-kd polypeptide in correspondingly lower amounts (Schlegel *et al.*, 1986; Schiller *et al.*, 1986). These results suggest that in these constructs the methionine codon at nucleotide 3879 can function as the initiation codon for the 7-kd protein. ORF E5 extends only 44 codons from this codon to the termination codon, and the predicted amino acid sequence of its protein product is extremely hydrophobic. These earlier studies did not determine whether ORF E5 is expressed in an analogous manner from the intact viral genome which transforms cells far more efficiently than the LTR/E5 constructs.

We initiated the genetic and biochemical experiments here with several objectives in mind: (i) to establish definitively that the 7-kd polypeptide is in fact the product of the 3′ half of the E5 ORF; (ii) to determine the effects of mutations in the protein coding domain on the two known biochemical properties of the 7-kd protein, membrane association and dimerization (Schlegel and Wade-Glass, 1987; Wade-Glass *et al.*, unpublished results); and (iii) to investigate whether or not the 7-kd protein expresses the efficient transforming activity genetically detected by mutational analysis of intact viral DNA. The results indicate that translation of ORF E5 initiates at the methionine codon in the middle of the ORF to produce a 7-kd protein which is required for efficient focus formation by the intact BPV genome.

Results

The studies summarized in the introduction strongly suggest that the initiation codon for the E5 protein is the first ATG at bp 3879. To test this hypothesis, several frameshift mutations were constructed that bracketed this ATG. These mutants were evaluated for their transforming activity as well as their ability to synthesize a full-size E5 polypeptide. The mutants RV9 (bp 3838) and RX18 (bp 3875) with frameshift mutations between the end of ORF E2 and the first E5 methionine codon (bp 3879) transform efficiently (Figure 1). Moreover insertion of an additional 8 bp into RV9 to generate a third frameshift mutant did not impair focus-forming efficiency (data not shown). In contrast, individual frameshift mutations at three different positions downstream of the first methionine codon cause substantial transformation defects. Stable pooled cell lines were established with wild-type (wt) viral DNA and with each of the mutants shown in Figure 1. Metabolically labelled extracts from these lines were immunoprecipitated with anti-E5 protein antiserum to examine the correlation between E5 protein expression and cell transformation

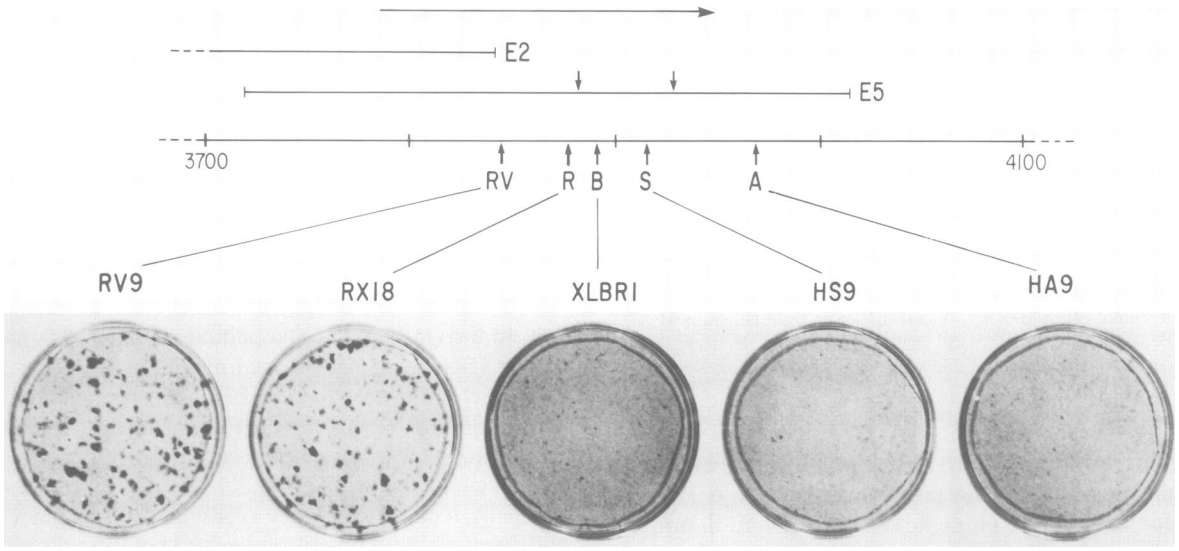

Fig. 1. Location and biological activity of the ORF E5 frameshift mutations. The distal portion of the BPV-1 'early' region (bp 3700–4100) encodes the 3′ end of the E2 ORF and the entire E5 ORF. The arrows indicated within the E5 ORF mark the position of the two methionine codons. Frameshift mutations were constructed (see Materials and methods) at five restriction endonuclease sites within the E5 ORF: RV = *BclI*; R = *RsaI*; *B* = *Bst*XI; S = *Spe*I; A = *Acc*I. Frameshift mutations 5′ to the first methionine codon had little effect on focus-forming activity (compared with wild type, not shown) whereas those positioned distal to this methonine codon resulted in dramatically reduced activity.

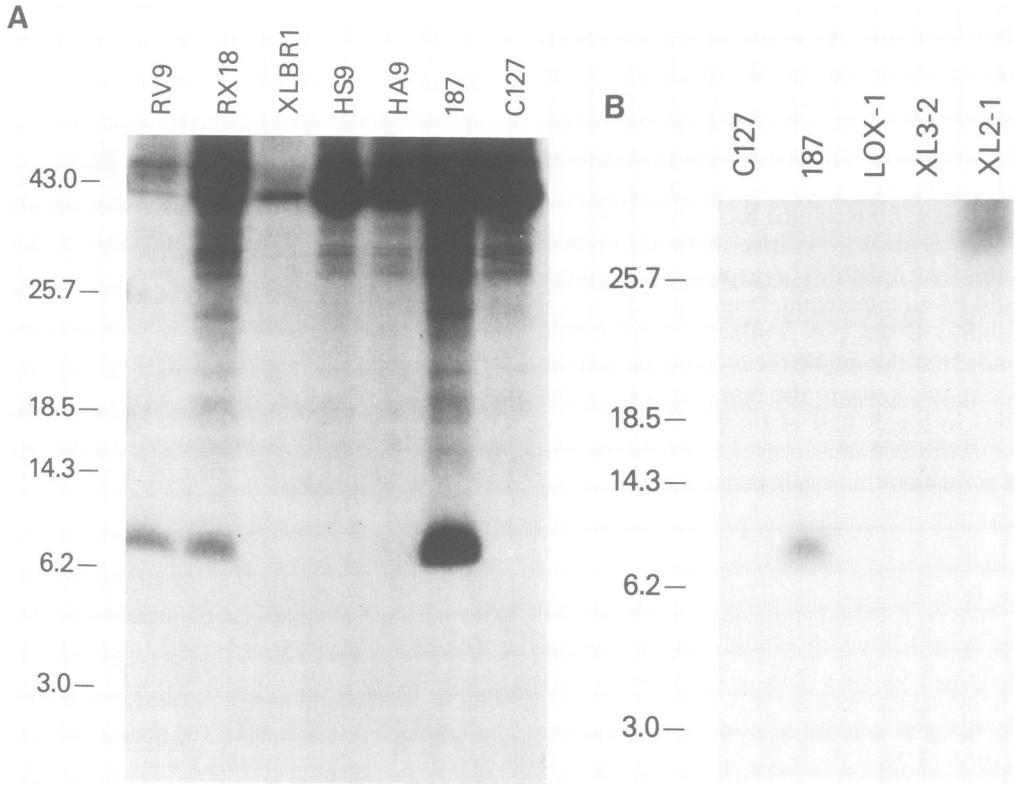

Fig. 2. Relationship between frameship muations in the E5 ORF and the detectable expression of the 7-kd E5 protein. **(A)** Pooled C127 cell lines transfected with the mutant DNAs described in Figure 1 were generated and analyzed by immunoprecipitation for E5 protein as described in Materials and methods. Cell lines transfected with DNA containing frameshift mutations prior to the first methionine codon (i.e. RV9 and RX18) synthesized E5 protein which exhibited the same electrophoretic mobility as wt E5 protein (from 187 cells). E5 protein was not detectable in cells transfected with DNA containing frameshift mutations after this methionine (XLBR1, HS9, HA9). Non-transfected C127 cells are included as a negative control. **(B)** Three independent, morphologically transformed cell lines (LOX-1, XL3-2 and XL2-1) were established from rare foci induced by the defective ORF E5 frameshift mutants (pE5DX-16 or pE5XL-2, see Materials and methods). In these cell lines, transformation is presumably mediated by the E6 ORF. Each cell line expresses BPV-1 RNA homologous to the ORF E5 which is quantitatively and qualitatively similar to that seen in cells transformed by wt BPV-1 DNA (data not shown). However, as shown here, immunoprecipitation studies with anti-E5 antibodies fail to detect E5 protein in these cell lines. Comparisons with cell lines which are positive (187) and negative (C127) for E5 protein are included.

Table I. NH_2-terminal E5 mutants

BPV-1	M	P	N	L	W	F	L	L	F	L	G	L	V	A	A
E5XS-1	M	P	[T	S	R	M	V	S	I	V	L	G	I]	A	A
E5DX-8	M	P	[T	G]	W	F	L	L	F	L	G	L	V	A	A
E5DX-18	M	P	TSRG ▽		W	F	L	L	F	L	G	L	V	A	A
E5XL-1	M	P	TSRPRG ▽		W	F	L	L	F	L	G	L	V	A	A
142-BTS-4	M	P	▽		W	F	L	L	F	L	G	L	V	A	A

(Figure 2A). The E5 protein detected in cell lines transformed by RV-9 and RX-18 (transformation-competent mutants) co-migrates with the E5 protein produced in cells transfected with a cDNA expressing wild-type ORF E5 (cell line 187). Cell lines containing transformation-defective mutants were obtained by co-transformation with the Tn*5* neomycin-resistance gene and selection in G418. Using the anti-E5 immunoprecipitation assay described, the E5 protein is not detectable in these cell lines (Figure 2A). We also established morphologically transformed cell lines from rare individual transformed foci induced by two different ORF E5 mutants with frameshift mutations at the *Bst*XI site. Again, immunoprecipitation experiments failed to reveal the presence of an ORF E5 protein in these cells (Figure 2B). Northern blot experiments documented that the amount of RNA homologous to ORF E5 in these transformed cell lines is comparable to that in wild-type transformed cells which express readily detectable ORF E5 protein (data not shown). These data and previously published results (DiMaio *et al.*, 1986; Groff and Lancaster, 1986; Schiller *et al.*, 1986; Yang *et al.*, 1985b) indicate that the 5′ half of this reading frame is not required for cellular transformation, that the 3′ half of ORF E5 has to be translated in-frame for efficient transformation, and that the ATG at bp 3879 probably represents the initiation codon for the 7-kd protein and for the genetically defined transforming activity (see Discussion).

To demonstrate that the amino acid residues encoded by the 3′ end of ORF E5 were indeed part of the mature E5 polypeptide, we determined the apparent size of the E5 protein synthesized by several transformation-competent in-frame mutants with sequence changes immediately distal to the first methionine codon. The wild-type E5 protein exhibits an estimated mass of 7 kd when electrophoresed on a 16% polyacrylamide−SDS gel (Schlegel *et al.*, 1986). Table I lists the mutants and the predicted alterations in the amino acid sequence of the protein. The E5 protein was immunoprecipitated from cells transformed by these mutants and analyzed by SDS−PAGE (Figure 3). One mutant (142-BTS) contains a 6-bp deletion in the E5 ORF and produces an E5 polypeptide which migrates demonstrably faster than the wt E5 protein. Mutants XS-1 and DX-8 (which contain 33- and 6-bp substitutions, respectively) produce an E5 protein which migrates considerably faster than wild-type E5 protein. The basis for this observed alteration is not known but could result from proteolytic cleavage of the mutant E5 protein at a newly generated arginine. Mutants DX-18 and XL-1, which contain 6- and 12-bp insertions, respectively, do not produce E5 proteins with appreciable alterations in electrophoretic mobility as assayed under these conditions. However, clear increases in apparent size can be demonstrated using different electrophoretic conditions (−DTT, Figure 3).

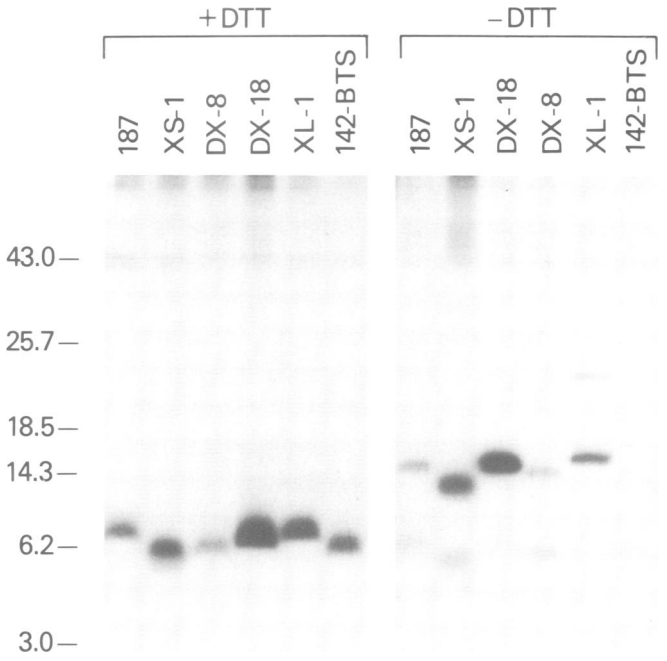

Fig. 3. SDS gel electrophoresis of wt and in-frame mutant E5 proteins in the presence and absence of dithiothreitol. Cells lines transfected with each of the mutuant DNAs shown in Table I were surveyed for the presence of E5 protein by immunoprecipitation using anti-E5 protein antibodies. Immunoprecipitated E5 protein was then electrophoresed on SDS−polyacrylamide gels in the presence (+DTT) or absence (−DTT) of 100 mM dithiothreitol. In the presence of DTT, wt E5 protein migrates as a monomer of ~7 kd; the mutant proteins exhibit altered electrophoretic mobilities which are discussed in the text. In the absence of DTT, both wt and mutant E5 proteins are present as dimers. One cell line, XL-1, also shows a detectable level of E5 trimer formation.

It has recently been shown that a proportion of the E5 polypeptide can exist in dimer form, apparently as a consequence of disulphide bond formation between cysteine residues located at its carboxy-terminus (Schlegel and Wade-Glass, 1987; Wade-Glass *et al.*, unpublished results). To determine if the amino-terminal mutations might indirectly affect dimer formation, membrane-derived E5 protein was electrophoretically separated under non-reducing conditions (−DTT, Figure 3). In all cell lines containing detectable E5 protein, a substantial fraction of the E5 protein was present as apparent dimers. It should be noted that small amounts of monomer E5 are also present under non-reducing conditions. The dimer forms (~15 kd) reflect the summed alterations in predicted size for the monomer E5 such that the mutant dimers of E5 show more obvious changes in electrophoretic mobility than do the monomers. Specifically, the DX-18 and XL-1 mutant dimers now show demonstrable retardation in mobility which was not obvious with the monomeric forms. The XS-1 substitution mutant still exhibits an increased mobility. Also of interest is the observation that some E5 molecules can apparently form multimers larger than dimers. The XL-1 E5 protein, for example, can apparently form trimers (at the 22-kd position). Multimers larger than trimers have also been observed with wild-type E5 protein (Wade-Glass *et al.*, unpublished results).

Although all of these in-frame mutants retain efficient transforming activity, they should produce E5 proteins with quite different amino-terminal charges. For example, XL-1 generates two

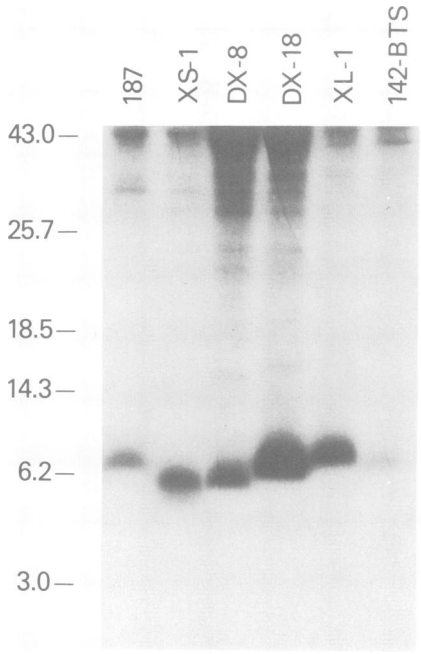

Fig. 4. Membrane association of wt and in-frame mutant E5 proteins. Membrane fractions were prepared from each of the designated cell lines (see Materials and methods) and equivalent amounts of protein were immunoprecipitated with anti-E5 protein antiserum. All mutant E5 proteins were membrane-associated and displayed the same electrophoretic mobility as seen with E5 derived from total cell extracts.

new arginine residues in a region which was previously neutral. Since the presence of a charged amino-terminus might seriously interfere with the ability of the E5 molecule to interact with the hydrophobic cellular membranes, we examined membrane fractions of cells transformed by each of the above in-frame mutants (Figure 4). E5 protein was detected in the membrane fractions from all of the cell lines, demonstrating that the new positive charges did not prevent membrane association. Again, the protein expressed by the deletion mutant 142-BTS, showed the anticipated increase in electrophoretic mobility. The XL-1 mutant (which inserts four amino acids) displays a detectably slower mobility. The 11-amino acid substitution of XS-1 results in the smaller E5 protein described in Figure 3.

Discussion

This genetic and biochemical analysis demonstrates that the 3′ but not the 5′ half of ORF E5 is essential for efficient cellular transformation and specifies the primary structure of the protein immunoprecipitated by the anti-E5 antiserum. The endpoint of the mutation in the transformation-competent mutant RX-18 is 3 bp upstream of the first methionine codon, whereas the endpoint of the mutation in the defective mutant XLBR-1 is 4 bp downstream of this codon. Thus, the boundary between the nonessential and the essential protein-coding portions of ORF E5 coincides with the position of the first methionine codon, suggesting that this codon serves as the initiation codon for the E5 ORF transforming activity and for the 7-kd protein. These results also exclude other potential mechanisms for expression of the ORF E5. For example, because RV-9 and RX-18 contain frameshift mutations downstream of the end of ORF E2, their ability to efficiently transform cells indicates that ORF E5 is not expressed via simple ribosomal frameshifting at or near the E2 stop codon. Moreover, because there are no potential splice acceptor sites between the RX18 mutation and the essential 3′ portion of the reading frame, the transformation competence of this mutant implies that splicing of the 3′ end of ORF E5 to an upstream exon is not essential for efficient transformation. Based on these results and on the studies summarized in the Introduction, we conclude that the primary transforming activity of intact BPV DNA is expressed by the ORF E5 7-kd protein that initiates at the methionine codon in the middle of the reading frame (bp 3879). The proposed ORF E5 initiator codon and adjacent nucleotides conform well to the consensus sequence described by Kozak (1984). The presence of several hundred bases 5′ to this methionine codon in all characterized BPV RNAs in transformed cells raises the possibility that expression of ORF E5 may be under translational control.

The DNA sequence of ORF E5 predicts that the E5 transforming protein is only 44 amino acids long and extremely hydrophobic. The protein's electrophoretic mobility in SDS–polyacrylamide gels and its membrane association are consistent with these predictions. Although post-translational modifications have not been entirely ruled out, the E5 protein is not labelled metabolically with P-32 orthophosphate or fatty acids (palmitic or myristic acid) and does not associate with or exhibit kinase activity (R.Schlegel, unpublished results). The biochemical activities of the E5 protein that are essential for its transforming activity have not been established, but membrane association and dimerization are retained by all the transformation-competent mutants. Sequence analysis of other papillomaviruses also suggests the importance of these properties. The genome of related fibropapillomaviruses, including the deer papillomavirus and BPV type 2, also contain ORFs downstream of ORF E2 with the potential to encode homologous, short, hydrophobic proteins, as do the genomes of some human papillomaviruses such as HPV-6, HPV-11 and HPV-31. The conservation of the carboxy-terminal Cys-X-Cys sequence in the E5 proteins of the bovine, deer, and human viruses suggests that oligomerization via disulfide bond formation is an important property of the E5 protein. Specific mutagenesis of these cysteine residues and the hydrophobic portion should indicate the importance of these conserved sequence elements for oligomerization, membrane association, and the efficient transformation of mammalian cells.

The studies reported here help define the BPV-1 E5 protein as the shortest transforming protein yet characterized. Its size and the absence of homology between the papillomavirus E5 ORFs and other characterized oncogenes suggest that the E5 polypeptide may mediate cell transformation via a novel mechanism. Although other viral or cellular gene products may well contribute to the transformed phenotype in this system, the small size of the E5 polypeptide makes it an ideal target for mutational and biochemical analysis.

Materials and methods

Mutant construction

The frameshift mutations were constructed in plasmid pBPV-H11 which consists of the 5437-bp *Hin*dIII to *Bam*HI subgenomic transforming fragment of BPV-1 cloned in the bacterial vector pBRd (DiMaio *et al.*, 1982). To construct RV9, pBPV-H11 DNA was partially digested with *Bcl*I. The ends of the molecules were then treated with T4 DNA polymerase in the presence of dGTP, dATP and dTTP followed by treatment with nuclease S1 to generate blunt ends. After ligation, an isolate containing a new *Eco*RV site at the end of ORF E2 was identified by restriction analysis and designated RV-9. To construct RX18, pBPV-H11 was linearized by partial *Rsa*I digestion in the presence of ethidium bromide, and 8-bp *Xho*I linkers were inserted into the molecules. An isolate with a unique

*Xho*I site located at the *Rsa*I site in ORF E5 was identified by restriction analysis and designated RX18. The DNA sequences of both the RV9 and RX18 mutations were determined by standard techniques. The mutations in XLBR1 and HS9 have been described previously (DiMaio *et al.*, 1986). The mutation in HA9 is the *Acc*I mutation originally constructed in the double mutant XA-1 (DiMaio *et al.*, 1986). It was separated from the upstream mutation by subcloning the mutant *Spe*I to *Sal*I small fragment into pBPV-H11.

The in-frame mutations were all constructed in the full-length BPV genome and have been described previously (DiMaio *et al.*, 1986), except for 142BTS-4. To construct this mutant, pBPV-142-6 was linearized with *Bst*XI, and nucleotides were removed from the ends of the molecule by sequential digestion with T4 DNA polymerase and nuclease S1. Following ligation and bacterial transformation, 142BTS-4 was isolated and the sequence of the mutation was determined. This mutant induces foci approximately one-third as efficiently as the wild-type (data not shown). The mutation in pE5XL2 is in the full length viral genome but is otherwise identical to pE5XLBR1; the pE5DX-16 mutation is a different frameshift mutation at the *Bst*XI site but is also in the full length viral genome (DiMaio *et al.*, 1986).

DNA transfer

Viral DNA (50–200 ng) was separated from the bacterial vector and transferred into C127 cells as described by DiMaio *et al.* (1985). Morphologically transformed, pooled cell lines were established by pooling foci from plates containing at least 50 foci. To introduce the transformation-defective frameshift mutants into cells, 200 ng of viral DNA and 56 ng of pKOneo were transferred into C127 cells. After selection with G418 (Colbere-Garapin *et al.*, 1981; Southern and Berg, 1982) for 3 weeks, cell lines were established by pooling G418-resistant colonies from plates containing at least 50 colonies 3 weeks following biochemical selection. In addition, transformed cell lines were established from individual foci induced by pE5XL2 (cell lines XL2-1 and XL3-2) or pE5DX-16 (cell line LOX-1).

Cell culture

The cell line used as a source of wild-type E5 protein was YC-C88-A (now designated 187) which contained a cDNA plasmid with ORFs E2, E3, E4 and E5 of BPV-1 expressed by the SV40 early promoter (Schlegel *et al.*, 1986). This cell line and the lines containing the mutant BPV-1 DNAs were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum.

Immunoprecipitation of E5

Subconfluent cell cultures were metabolically labelled with 0.5 mCi of [^{35}S]-methionine and 0.5 mCi of [^{35}S]cysteine in 2 ml of serum-free DMEM (deficient in methionine and cysteine) for 5 h at 37°C. Cells were washed with cold phosphate-buffered saline, harvested by scraping, and solubilized in a modified RIPA buffer (20 mM MOPS, 150 mM NaCl, 1 mM EDTA, 1.0% NP-40, 1% deoxycholate and 0.1% SDS, pH 7.0) containing phenylmethylsulfonylfluoride and 1% aprotinin. Nuclei were removed by centrifugation in an Eppendorf microfuge for 30 s. A modified double immunoprecipitation procedure was devised that markedly reduces the precipitation of non-specific protein (Glass, submitted). In brief, two rounds of immunoprecipitation using 20 μl anti-E5 antiserum and 100 μl Protein A Sepharose (Pharmacia) were performed. Following multiple washes with ice-cold RIPA buffer, the Protein A Sepharose beads were resuspended in sample buffer with or without 100 mM dithiothreitol, heated at 100°C for 5 min, and electrophoresed on a 16% polyacrylamide–SDS gel. Gels were then fixed with glacial acetic acid/methanol, treated wtih Enlightning (New England Nuclear), dried, and exposed to Kodak XOmat AR film for 3–5 days at −70°C.

Preparation of cell membranes

Radiolabelled cells were washed and swollen in a hypotonic citrate buffer (Schlegel and Benjamin, 1978), and then disrupted in a Dounce homogenizer with a type B pestle. Nuclei were pelleted by centrifugation at 3000 r.p.m. for 15 min in a Sorvall RT6000B centrifuge. The supernatants were then centrifuged at 100 000 *g* in a Beckman Type 50 Ti rotor for 45 min at 4°C. The membrane-containing pellets were resuspended in RIPA buffer and analyzed by immunoprecipitation and electrophoresis as described above.

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