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44-Amino-Acid E5 Transforming Protein of Bovine Papillomavirus Requires a Hydrophobic Core and Specific Carboxyl-Terminal Amino Acids

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The 44-amino-acid E5 protein of bovine papillomavirus type 1 is the shortest known protein with transforming activity. To identify the specific amino acids required for in vitro focus formation in mouse C127 cells, we used oligonucleotide-directed saturation mutagenesis to construct an extensive collection of mutants with missense mutations in the E5 gene. Characterization of mutants with amino acid substitutions in the hydrophobic middle third of the E5 protein indicated that efficient transformation requires a stretch of hydrophobic amino acids but not a specific amino acid sequence in this portion of the protein. Many amino acids in the carboxyl-terminal third of the protein can also undergo substitution without impairment of focus-forming activity, but the amino acids at seven positions, including two cysteine residues that mediate dimer formation, appear essential for efficient transforming activity. These essential amino acids are the most well conserved among related fibropapillomaviruses. The small size of the E5 protein, its lack of similarity to other transforming proteins, and its ability to tolerate many amino acid substitutions implies that it transforms cells via a novel mechanism.

The E5 gene of bovine papillomavirus type 1 (BPV1) encodes a short hydrophobic protein required for efficient in vitro transformation of established mouse cell lines. Frameshift mutations or the insertion of a translation termination codon in open reading frame (ORF) E5 markedly decreases the ability of the viral DNA to induce foci in mouse C127 cells (5, 9, 16, 20, 24). Detailed mutational and biochemical analysis of this gene indicates that the portion of ORF E5 downstream of the first methionine codon in the reading frame is translated to produce a 7-kilodalton polypeptide required for efficient focus formation (5, 9, 29). In addition, expression of this portion of the E5 gene from a strong heterologous promoter is sufficient to induce focus formation (27, 28, 32). The E5 protein is predicted to be only 44 amino acids long (Fig. 1). Its amino-terminal two-thirds consists almost exclusively of strongly hydrophobic amino acid residues, and the carboxyl-terminal third contains several charged and polar amino acid residues. There is no apparent similarity between the papillomavirus E5 proteins and other sequenced proteins including known viral or cellular transforming proteins.

An antiserum generated against the carboxyl-terminal third of the BPV1 E5 protein has been used to immunoprecipitate the 7-kilodalton E5 protein from BPV1-transformed mouse and hamster cells (5, 29, 33). In accordance with its predicted hydrophobic composition, the E5 protein is recovered predominantly in the membrane fraction of mechanically disrupted cells (29). Moreover, isolation of the E5 protein under nonreducing conditions results in the detection of E5 dimers and higher-order oligomers that can be converted to monomers by treatment with dithiothreitol (DTT) (5). We have proposed that the E5 protein oligomerizes via disulfide bonds at conserved cysteine residues near the carboxyl terminus of the protein (5).

The small size of the E5 transforming protein should permit the structural basis of its transforming activity to be understood at the amino acid level. Our previous studies have shown that insertion, deletion, and substitution mutations in the amino-terminal third of the protein are compatible with efficient focus formation and suggest that in this portion of the molecule there are few specific amino acids required for its focus-inducing activity (9). To systematically evaluate the carboxyl-terminal two-thirds of the E5 protein, we used saturation and site-directed mutagenesis procedures to generate missense mutations in this portion of the protein. Analysis of these mutants enabled us to discern the importance of individual amino acid residues for the biological and biochemical properties of the E5 protein.

MATERIALS AND METHODS

Mutagenesis. To generate mutations in the E5 gene, we used as a template the M13 clone MR7, which contains the *Eco*RI-to-*Bam*HI small fragment of BPV1 DNA (8). Single-stranded MR7 DNA substituted with uracil was prepared after infection of *Escherichia coli* RZ1032 (*dut ung*) as described by Kunkel (18). Mutagenic mixed (doped) oligonucleotides E5.1 and E5.2 were prepared essentially as described by Derbyshire et al. (7) to mutagenize the middle third and the COOH-terminal third of the protein, respectively. At certain positions, synthesis was done by using a mixture containing the wild-type nucleotide and a low level of the other three nucleotides. This generated a population of oligonucleotides of which each member had on average one change from the wild-type sequence. E5.1 is complementary to BPV1 nucleotides 3916 to 3951. At 28 positions, synthesis was done with mixtures containing the wild-type nucleotide (95.2%) and the other three nucleotides (1.6% each). Only the wild-type nucleotide was inserted at eight positions where nucleotide substitutions were predicted not to cause amino acid substitutions. E5.2 is complementary to BPV1 nucleotides 3963 to 4010, and all 48 positions were synthesized with mixtures containing the wild-type nucleotide

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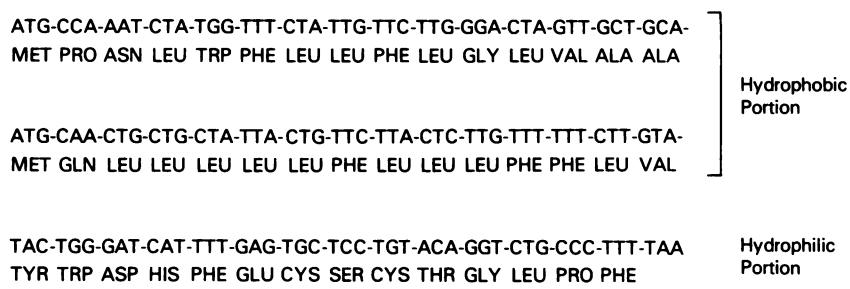

FIG. 1. Coding sequence of the E5 gene and predicted amino acid sequence of the E5 protein (6, 30).

(97.9%) and the three other nucleotides (0.7% each). In individual reactions, each primer was hybridized to MR7 single-stranded template and elongated in vitro with the large fragment of DNA polymerase. The elongation products were transfected into *E. coli* JM101, which selectively degrades the uracil-containing wild-type strand, thereby selecting for the strand synthesized in vitro.

From the reaction with primer E5.1, approximately 500 plaques were pooled, double-stranded DNA was prepared by primed synthesis with the M13 −20 universal primer (New England BioLabs, Inc., Beverly, Mass.), and the *Bst*XI-to-*Sal*I fragment containing the mutagenized segment was used to replace the analogous fragment in plasmid pBPV-142-6, which contains the full-length wild-type BPV1 genome cloned into the unique *Bam*HI site of pML2d (19, 26). Plasmid DNA from individual bacterial transformants was isolated and sequenced to identify mutations (25). From the reaction with primer E5.2, single-stranded bacteriophage DNA from individual plaques was sequenced, and the *Bst*XI-to-*Sal*I small fragment from mutants of interest was cloned individually into pBPV-142-6 as described above. Between 20 and 30% of the clones generated with either primer had mutations in the targeted regions. Substitution mutations were distributed throughout the length of the targeted regions except at the extreme 3′ end, which corresponds to the 5′ end of the oligonucleotides, and substitutions were not detected outside of the targeted regions. Of the mutations, 10 to 20% were small insertion and deletion mutations rather than substitutions and are not discussed further. Mutants 31F, 31S, 32S, 37S, 39S, and 37S39S were constructed with oligonucleotide primers with specific mismatches (34). Mutant pE5XL-2 has been described previously (9).

DNA transfer and cell culture. To assay the mutants for transforming activity, 100 to 200 ng of viral DNA was digested with *Bam*HI to separate the viral genome from the bacterial vector and transfected into C127 cells as previously described (10, 11, 13). Foci were counted 2 to 3 weeks after transfection. Morphologically transformed pooled cell lines were established from plates containing at least 50 foci induced by the transformation-competent mutants. Rare foci transformed by defective mutants were isolated in cloning cylinders and expanded into cell lines. To assay the long terminal repeat (LTR) constructs for transforming activity, we transfected 2 μg of undigested plasmid DNA as above. All cell lines were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and antibiotics.

Immunoprecipitation of E5 protein. Subconfluent cell cultures were metabolically labeled for 5 h with 0.5 mCi of [^{35}S]methionine and 0.5 mCi of [^{35}S]cysteine in 2 ml of serum-free Dulbecco modified Eagle medium deficient in methionine and cysteine. Total cell extracts or cell membranes were prepared as described previously and precipitated with rabbit anti-E5 peptide antiserum (29) by a modified double immunoprecipitation procedure (A. Burkhardt et al., submitted for publication). No proteins the size of the E5 protein were immunoprecipitated by preimmune serum (data not shown) (29). Immunoprecipitated proteins were suspended in sample buffer in the absence or presence of 100 mM DTT and were electrophoresed on sodium dodecyl sulfate–16% polyacrylamide gels. After electrophoresis, the gels were fixed, treated with Enlightening (Dupont, NEN Research Products, Boston, Mass.), dried, and exposed to film for 3 to 14 days at −70°C.

Genetic mapping experiments. For transformation-defective mutants 16R, 17G, 18R19K21F, 30L35S, 31S, 32S, 33V, 37R, 37S39S, 39R, and 39S, the fragment extending from the *Bst*XI site to the end of the transforming segment (containing ORF E5) was replaced with the corresponding fragment of wild-type DNA. This manipulation is predicted to restore the transforming activity if the mutation responsible for the defect is located in ORF E5 or in the downstream untranslated region. Nucleotide sequencing of the mutant fragments did not reveal any base changes outside of ORF E5. Focus-forming activities of wild-type viral DNA, the original E5 mutants, and the recombinant genomes were compared.

The *Eco*RI-to-*Xho*I fragment of the Moloney murine sarcoma virus LTR was ligated to the ORF E5-containing *Xho*I-to-*Eco*RI fragment of plasmid RX18, which has an *Xho*I site at BPV1 nucleotide 3874 (this fragment also contains the bacterial plasmid vector) (5). In the resulting plasmid, LTR-RE5, the E5 gene is the only BPV1 gene and it is in the correct orientation to be transcribed from the LTR. The 518-base-pair *Sma*I-to-*Xho*I fragment from the LTR was removed from LTR-RE5 to generate LTR-ΔRE5. A frameshift mutation at the *Bst*XI site of LTR-ΔRE5 was generated by sequential digestion with *Bst*XI and mung bean nuclease prior to religation, producing LTR-ΔRE5-fs1. DNA sequence analysis revealed that this mutant contained a 4-base-pair deletion. To replace the wild-type E5 gene in LTR-ΔRE5 with the mutant genes, we replaced the *Bst*XI-to-*Sal*I small fragment from LTR-ΔRE5 with the corresponding fragment prepared from mutants 30L35S, 31S, 32S, 33V, and 37S39S.

RESULTS

Oligonucleotide-directed saturation mutagenesis. To generate a large number of missense mutations in the carboxyl-terminal two-thirds of the E5 protein, we used a modification of standard M13-based oligonucleotide-directed mutagenesis procedures that employs mixed populations of mutagenic oligonucleotide primers and biological selection against the

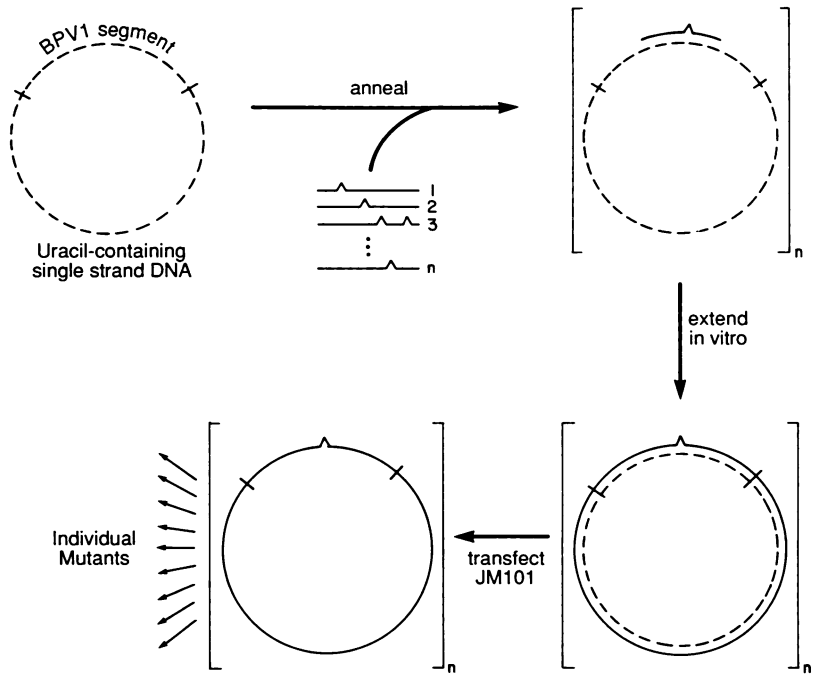

FIG. 2. Mutagenesis procedure. Uracil-containing single-stranded M13-MR7 bacteriophage DNA was prepared in the appropriate *E. coli* host (18). A mixture of doped oligonucleotide primers prepared as described in Materials and methods was hybridized to the phage DNA and extended in vitro with the Klenow fragment of DNA polymerase and the four deoxynucleoside triphosphates. Extension products were transfected into *E. coli* JM101, which selectively degrades the uracil-containing wild-type strand, and single phage plaques were isolated and analyzed by DNA sequencing to identify mutations. Dashed lines indicate uracil-containing DNA. Solid lines indicate DNA not substituted with uracil. Indentations indicate a base not complementary to the wild-type base present in the template DNA. Populations of intermediates that differ only as a consequence of the different mutagenic primers are surrounded by brackets.

wild-type strand (Fig. 2) (see Materials and Methods). The majority of mutants obtained contained single nucleotide substitutions, and a smaller proportion contained multiple mutations (data not shown). Almost all possible nucleotide substitutions were generated, and missense mutations were produced at 22 of the 27 amino acid positions targeted. In addition, several mutations were generated at specific positions by standard oligonucleotide-directed mutagenesis. Mutants predicted to encode structurally altered E5 proteins were assayed for focus formation in C127 cells.

Biological activity of ORF E5 mutants. To generate mutations in the middle third of the E5 gene, we used a mixture of mutagenic oligonucleotides that spanned codons 15 to 25. The wild-type amino acids in this portion of the protein and the missense mutations generated with this primer are shown in Fig. 3. The right-hand column of the figure shows the averaged results of several C127 cell focus formation assays in which the transformation efficiencies of the mutant DNAs are compared with that of wild-type viral DNA. All mutants that solely had hydrophobic amino acids substituted with different hydrophobic amino acids transformed efficiently, as did mutant 15T17H, which had alanine 15 and glutamine 17 replaced with threonine and histidine, respectively. Membrane fractions of pooled cell lines transformed by selected transformation-competent mutants were assayed for the presence of E5 protein by immunoprecipitation with an anti-E5 polyclonal antibody. All cell lines examined contained E5 protein which associated with cellular membranes and displayed a mobility similar to that of wild-type E5 protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, except for the protein produced by mutant 15V21F22V, which exhibited a slightly slower mobility (Fig. 4A). These mutant proteins also formed dimers (data not shown).

In contrast to these results with hydrophobic substitution mutants, all mutants containing lysine or arginine in this region were defective for focus formation. The low level of transforming activity seen with these mutants was comparable to that seen with E5 frameshift mutants or mutant 17TC, which has a translation termination signal at codon 17, and was evidently due to the presence of the BPV1 E6 transforming gene. Cell lines expanded from rare foci induced by the mutants containing an arginine or a lysine contained membrane-associated E5 protein which migrated with increased mobility compared with the wild-type protein on sodium dodecyl sulfate-polyacrylamide gels (Fig. 4B). This increased mobility was presumably due to the altered structure or charge of these small proteins. Mutant 17G, which has glutamine 17 replaced with glycine, displayed a less severe transformation defect than did mutations which placed strongly basic residues in this region. Morphologically transformed cell lines established from rare foci induced by this mutant contained substantial amounts of membrane-associated E5 protein (Fig. 4C), although there was considerable variability in the amounts of protein in independent cell lines.

To generate mutations in the hydrophilic carboxyl-terminal third of the E5 protein, we used a mixture of mutagenic oligonucleotides spanning codons 29 to 44. Figure 5 shows the missense mutations generated in this segment of the E5 protein and their effects on C127 cell focus formation. Transformation was not significantly inhibited by any of the mutations we isolated at leucine 29, histidine 34, glutamic acid 36, serine 38, threonine 40, and glycine 41. At several of

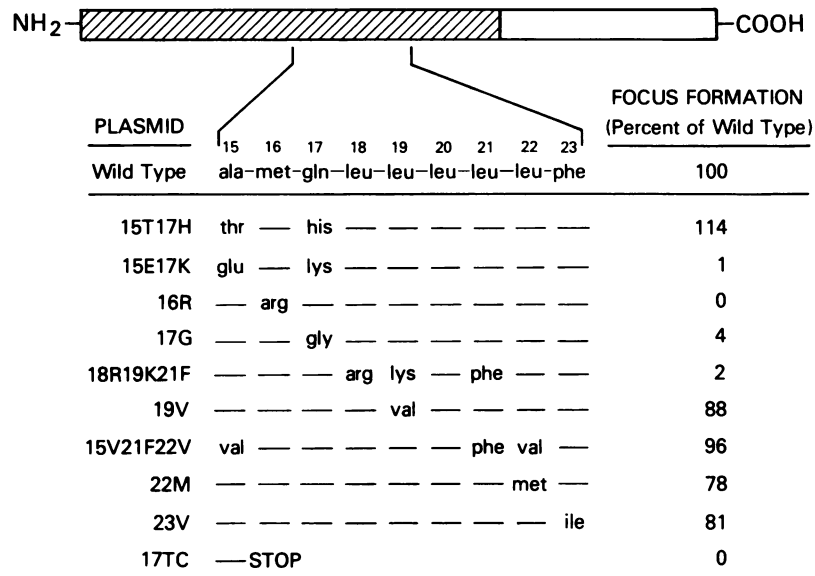

FIG. 3. Transformation efficiency of mutants with substitutions in the middle portion of the E5 protein. Top bar represents the E5 protein. The open area is the carboxyl-terminal hydrophilic portion of the protein, and the shaded area is the amino-terminal hydrophobic portion. Expanded sequence shows the amino acids targeted for mutagenesis in the hydrophobic region. The leftmost column indicates the names of mutant plasmids. The central section shows the predicted amino acid substitution(s) encoded by the corresponding mutants. STOP indicates a translation termination codon. The right-hand column indicates the percentage of wild-type focus formation induced by the mutant plasmids (average of at least two independent experiments). Wild-type viral DNA induced about 3,000 foci per μg.

these positions, multiple mutants with different substitutions were examined, including some in which the structure of the substituted amino acid markedly differed from wild type. On the other hand, mutations that did interfere with transformation were isolated at seven different positions (marked with the asterisks in Fig. 6). The nonconservative substitutions tyrosine 31 to serine, tryptophan 32 to serine, and aspartate 33 to valine all resulted in marked transformation defects,

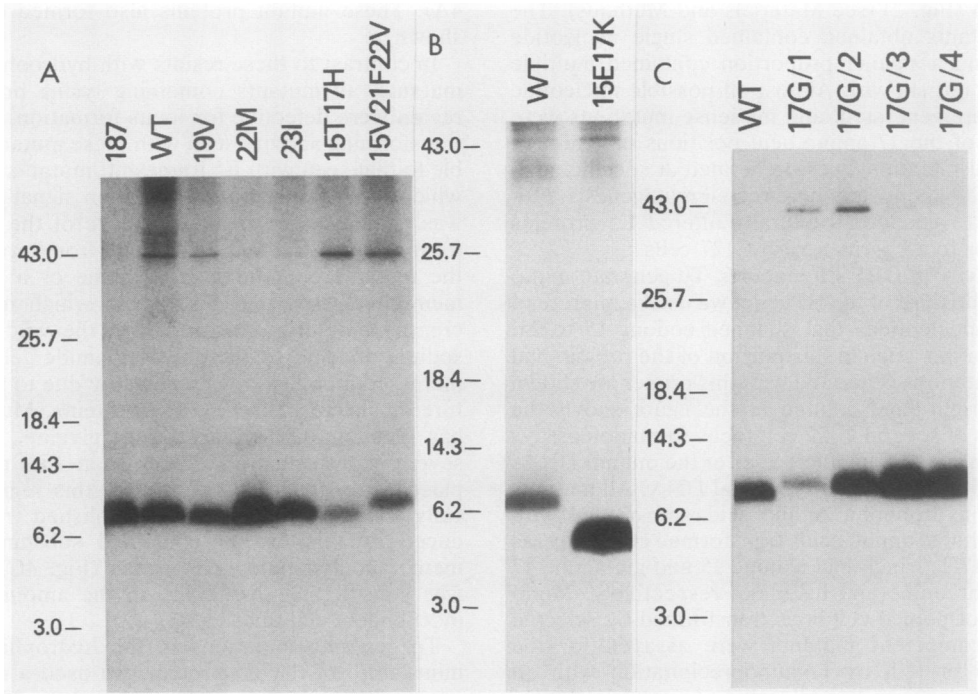

FIG. 4. Production of E5 protein in cell lines transformed by mutants with substitutions in the middle third of the protein. Cell lines were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine, and E5 protein was immunoprecipitated from membrane fractions and eluted in the presence of 100 mM DTT. (A) Pooled cell lines transformed by indicated transformation-competent mutants. 187, cDNA clone that expresses wild-type E5 protein. WT, Wild-type viral DNA. (B and C) Cell lines expanded from individual rare foci induced by the indicated transformation-defective mutants. 17G/1 to 4 are four different cell lines induced by mutant 17G. The molecular weights ($\times 10^3$) of protein markers are indicated on the left of each panel.

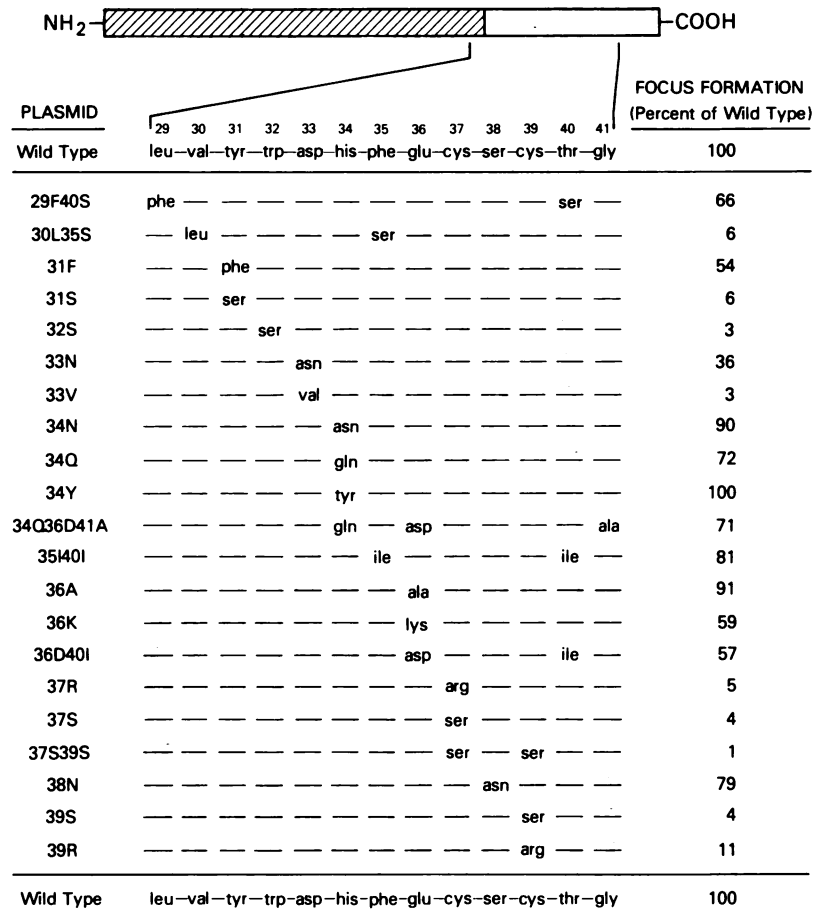

FIG. 5. Efficiency of transformation by mutants with substitutions in the carboxyl-terminal third of the E5 protein. See the legend to Fig. 3. The expanded sequence shows the amino acids targeted in the carboxyl-terminal third of the E5 protein.

although at two of these positions relatively conservative changes (tyrosine to phenylalanine or aspartic acid to asparagine) were phenotypically silent. The transformation-defective mutant 30L35S contained two substitutions, valine 30 to leucine and phenylalanine 35 to serine, whereas in a different mutant (35I) the change of phenylalanine 35 to isoleucine was silent. Mutations that changed either of the cysteines (positions 37 and 39) to serine or arginine resulted in transformation defects, as did a double mutation in which both cysteines were replaced by serine.

The E5 protein was examined in cell lines established with each of the defective carboxyl-terminal mutants and with several of the transformation-competent ones to ensure that the defects were not due to lack of expression or dimerization or to grossly abnormal localization of the protein. In all cases, a membrane-associated E5 protein was detected that comigrated with the wild-type species on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 7). Dimer formation was similar to that of the wild type except for those mutants with mutations at the cysteines (Fig. 7). Dimer formation by the E5 proteins that contained mutations at the cysteine residues was examined in the membrane fraction because in this fraction essentially all the wild-type protein was present as dimers (Fig. 7C). Approximately 50% of the E5 protein was in the monomeric form in cells transformed by mutants with a single cysteine residue, and no dimeric E5 protein was detected in cells transformed by mutant 37S39S, which had both cysteines changed to serines (Fig. 7C).

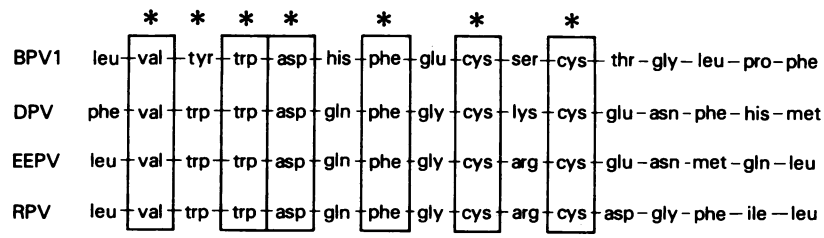

FIG. 6. Comparison of the carboxyl-terminal amino acid sequences of BPV1, deer papilloma virus (DPV), European elk papilloma virus (EEPV), and reindeer papilloma virus (RPV). Amino acids that are identical in all four sequences are boxed. Amino acids in BPV1 that were changed in defective mutants are marked with asterisks.

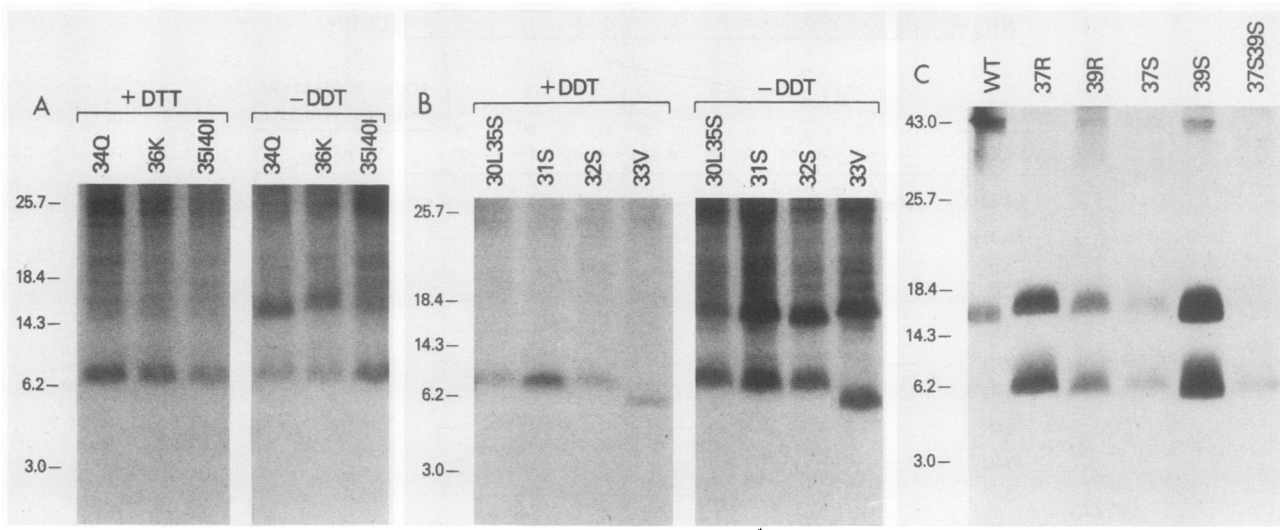

FIG. 7. Production of E5 protein in cell lines transformed by mutants with substitutions in the carboxyl-terminal third of the protein. Lanes +DTT are immunoprecipitations from membrane fractions eluted in the presence of DTT. Lanes −DTT are immunoprecipitations from total cell extracts eluted in the absence of DTT. (A) Pooled cell lines transformed by indicated transformation-competent mutants. (B) Cell lines expanded from individual rare foci induced by indicated transformation-defective mutants. (C) Cell lines expanded from rare foci induced by indicated defective mutants with substitutions at the cysteine residues. Protein was extracted from membrane fractions and electrophoresed without DTT treatment. The molecular weights ($\times 10^3$) of protein standards are shown on the left of each panel.

Genetic mapping experiments. For most of the defective mutants, a small region of DNA containing the E5 gene was replaced with the corresponding region from wild-type viral DNA. Each of these recombinants transformed cells at essentially wild-type levels (data not shown), demonstrating that the mutations in ORF E5 were responsible for the defects in focus formation. We also examined the transformation efficiency of wild-type and mutant E5 genes expressed from a retroviral LTR in the absence of all other BPV1 genes. Wild-type E5 expressed from the LTR induced C127 cell foci, an activity abolished by a frameshift mutation just downstream of the ORF E5 initiation codon (Table 1). Several missense mutations that caused defects in the context of the full-length viral genome were subcloned into the LTR vector, and these constructs were tested for transforming activity. In each case, the E5 missense mutation severely inhibited focus formation (Table 1). These results strongly suggest that these mutations in the E5 gene cause transformation defects by directly affecting the transforming ability of the E5 protein. The fact that most of these LTR constructs are not totally defective for focus formation implies that these missense mutations do not completely inactivate the E5 protein and suggests that dimer formation is not absolutely required for focus formation.

TABLE 1. Focus formation by the LTR-E5 clones

Mutant	Mutation	Foci (% of wild type)[a]
LTR-ΔRE5	Wild type	100
LTR-ΔRE5-fs1	Frameshift	<1
LTR-ΔRE5-30L35S	Val-30 → Leu Phe-35 → Ser	<1
LTR-ΔRE5-31S	Tyr-31 → Ser	1
LTR-ΔRE5-32S	Trp-32 → Ser	1
LTR-ΔRE5-33V	Asp-33 → Val	2
LTR-ΔRE5-37S39S	Cys-37 → Ser Cys-39 → Ser	3

[a] LTR-ΔRE5 induced approximately 40 foci per μg.

DISCUSSION

Saturation mutagenesis is a powerful tool with which to study the structural basis of biological phenomena, but some previously described methods require the use of specialized cloning vectors, physical enrichment of mutant products, or a target region flanked by unique cleavage sites for restriction endonucleases (7, 17, 22, 23). The method of saturation mutagenesis we describe here does not have these limitations and thus appears to be generally applicable. A similar approach has been developed independently (1, 23a).

Our earlier studies indicated that few specific amino acids in the N-terminal third of the E5 protein are required for efficient focus formation or membrane association (5, 9). This is illustrated most clearly by the transformation-competent mutant E5XS-1 in which amino acids 3 to 13 are substituted. The analysis of the mutants reported here demonstrated that amino acid substitutions at many positions in the carboxyl-terminal two-thirds of the molecule do not interfere with efficient focus formation and membrane association. Overall, some amino acid substitutions are tolerated at 26 of the 32 positions where mutations have been isolated.

The hydrophobic nature of the first two-thirds of the E5 protein and not its precise amino acid sequence appears essential for efficient focus formation. In the hydrophobic middle third of the protein, the sequence requirements for efficient transformation parallel those predicted for proper membrane insertion of a transmembrane protein. Like many transmembrane proteins, the wild-type E5 protein and all the transformation-competent mutants contain a stretch of 20 or more amino acids that is strongly hydrophobic (2). Although many variant hydrophobic sequences are compatible with membrane association and focus formation, mutations that introduce a strongly basic amino acid into this region of the protein result in defective transformation. Theoretical considerations indicate that insertion of an arginine or a lysine into a lipid bilayer is energetically unfavorable (12), and these amino acids are rarely found in the transmembrane domain of a protein that spans the membrane once (31).

These results suggest that the very hydrophobic middle third of the E5 protein is involved in membrane association and that this interaction is essential for transformation. Although by crude cell fractionation techniques the mutant E5 proteins with new basic residues are membrane associated, the precise localization of the protein may be abnormal, as for a mutant vesicular stomatitis virus G protein containing an inserted arginine (3). On the other hand, the structural alterations caused by insertion of an arginine or a lysine may interfere directly with the function of the E5 protein. A transformation defect is also caused by the substitution of glycine for glutamine 17. It is possible that there is a requirement for a hydrophilic amino acid at this position, since it is occupied by glutamine in the wild type and by histidine in the competent mutant 15T17H.

The occurrence of numerous hydrophilic and charged amino acids in the carboxyl-terminal third of the BPV1 E5 protein suggests that it plays a different role in the function of the protein than does the hydrophobic portion. In fact, at several positions, including histidine 34, glutamate 36, serine 38, and threonine 40, the introduction of a strongly basic or a nonconservative amino acid is tolerated. These data suggest that the structure of the E5 protein required for it to exert its transforming function is not dependent on the amino acids at these positions. Although we isolated phenotypically silent mutations at leucine 29 and glycine 41, the substituted amino acids, phenylalanine and alanine, respectively, are of similar size and hydrophobicity to the wild-type amino acids. Although it can be inferred that the wild-type amino acids are not absolutely required at these positions, we did not determine whether amino acids differing in size, charge, or hydrophobicity would be tolerated.

There is considerable similarity between the predicted E5 amino acid sequences of BPV1 and the other sequenced fibropapillomaviruses, deer papillomavirus, elk papillomavirus, and reindeer papillomavirus, all of which have biological properties similar to those of BPV1 (Fig. 6) (4, 15, 21). However, at the six positions where mutations did not cause defects, none is occupied by the same amino acid in all four viruses. This is consistent with the conclusion that these residues are not essential for the transforming activity of the E5 protein. At the three carboxyl-terminal amino acids of the protein, there is little similarity among the four sequenced papillomaviruses, suggesting that these amino acids are also of minor importance.

The amino acids at the positions where mutations result in defects are highly conserved among the sequenced fibropapillomaviruses (Fig. 6). Positions 32 and 33 are identical for the four papillomaviruses, and position 31 is occupied by tryptophan in all of them except BPV1, where it is a tyrosine, which is similar to tryptophan in terms of size, hydrophobicity, and aromaticity. Although conservative substitutions are tolerated at two of these three positions, a substitution at any of these three amino acids can result in transformation defects. Positions 30 and 35 are also invariant among the sequenced fibropapillomaviruses and are both changed in the defective mutant 30L35S. These results indicate that the small region from amino acids 30 to 35 probably plays an important role in the function of the E5 protein. The two cysteine residues are also invariant, and substitution of either of them to serine or arginine resulted in a severe transformation defect. The apparent lack of dimer formation by a mutant devoid of cysteines provides further evidence that these residues form interchain disulfide bonds and mediate E5 oligomer formation. However, the ability of this mutant to transform at a low level when it was expressed from the LTR suggests that dimer formation is not absolutely required. The inability of E5 proteins containing a single cysteine to transform cells efficiently, even though significant dimer formation occurs, suggests that the cysteines play a role in transformation in addition to simply mediating dimer formation. Although we did not directly assess the stability of the mutant E5 proteins, none of the mutations appears to cause gross destabilization.

The inhibition of transformation by diverse mutations in the carboxyl-terminus of the protein indicates that this segment is essential for transforming activity. The importance of the carboxyl-terminal segment was also suggested by the results of recent microinjection experiments demonstrating mitogenic activity of carboxyl-terminal E5 peptides (14). The essential amino acids in the carboxyl terminus may contact another protein, maintain an active configuration of the E5 protein, or contribute directly to an enzymatic or some other function. The small size of the E5 protein and the large proportion of positions that can accommodate amino acid changes argue against the existence of a catalytic site composed exclusively of E5 sequences. However, this small peptide may modulate the function of a larger protein. Potential targets for productive interaction with the E5 protein are proteins regulating cell growth and differentiation such as proteins involved in membrane signal transduction, generation of cytoplasmic second messengers, and regulation of gene expression.

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