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Mutational Analysis of Bovine Papillomavirus E6 Gene

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The bovine papillomavirus E6 gene can independently transform mouse C127 cells. To characterize E6 in greater detail, we created 16 site-directed mutations in E6, including substitution mutations in the cysteine codons of the four Cys-X-X-Cys motifs that are conserved in all papillomavirus E6 proteins. Proteins mutated in six of the seven cysteines tested, as well as those lacking the nonconserved C-terminus, were stable in transfected cells but were unable to induce morphological transformation, indicating that these amino acids play an important role in the function of E6.

Bovine papillomavirus type 1 (BPV-1), which can efficiently induce morphological transformation of certain established cells in culture, has been used extensively to study the molecular biology of papillomaviruses. By using BPV-1 DNA to transform mouse cells, two viral transforming genes, E5 and E6, have been identified. When expressed from a surrogate promoter, E5 can independently transform both NIH 3T3 and C127 cells (9, 14). In contrast, E6 has no effect on NIH 3T3 cells but is active on C127 cells (8). The BPV E6 gene product is a small, 16 kilodalton (kDa), positively charged protein that localizes to nuclei and an undefined membrane fraction (1). E6 is of particular interest because the homologous gene is consistently retained and expressed in human cervical tumors and tumor-derived cell lines that contain human papillomavirus (HPV) DNA (2, 10, 11). This has led to the hypothesis that E6 expression may be important for the development and maintenance of these human tumors. However, there is at present no in vitro quantitative bioassay for HPV E6. We have therefore chosen to analyze the function of BPV E6 in more detail. We have constructed several subtle BPV-1 E6 mutations and assayed their ability to encode a stable mutant protein product and to induce morphological transformation of C127 cells.

Figure 1 shows the linear amino acid sequence of the BPV-1 E6 protein, as well as the amino acids which are conserved among the sequenced papillomaviruses (PVs) (4). Of particular interest are the conserved Cys-X-X-Cys motifs, which occur four times in all PV E6s. It is possible that these motifs enable the E6 protein to form zinc-binding fingers, although the distance between the motifs is much greater than that found in the classic zinc-binding proteins that mediate DNA binding through these motifs (5).

Using oligonucleotide-directed mutagenesis (7), we introduced a number of amino acid substitutions or premature termination codons into BPV-1 E6 (Fig. 1). The mutations were synthesized in M13 and then subcloned immediately downstream from the Molony murine leukemia virus long terminal repeat in the construct pXH800, which has been described previously (8). Some of the less conservative substitutions were chosen because they led to the creation of novel restriction endonuclease sites. Mutations were confirmed by sequence or restriction enzyme analysis.

The mutations fell into three broad classes, which are indicated in Table 1. The first (class 1) are amino acid substitutions at one of the cysteines in the repeat motifs: mutants 139, 149, 238, 247, 359, 367, 368, and 460. Two different mutations of the second cysteine of the third repeat were constructed: mutants 367 and 368. In 367, a codon for histidine was substituted for the cysteine, since it has been suggested that both cysteine and histidine may be involved in the formation of metal-binding fingers (3). The 368 mutation is the most conservative substitution of serine for cysteine. The second class of mutations consists of substitutions in codons other than the cysteine repeats. Several were directed to noncysteine residues conserved among the PV E6 proteins (mutants 403, 438, and 457). The other mutations in the second class are those at amino acids which do not appear to be conserved (mutants 212, 228, and 473). The third class of mutations are the premature termination mutants 471 and 491. Mutation 471 is in the second cysteine of the fourth cysteine motif and deletes the last 11 amino acids. Mutation 491 is in an apparently nonconserved region and gives rise to an E6 protein truncated by only four amino acids.

Each mutant was cotransfected into C127 cells with the plasmid pSV2Neo, which expresses the gene for neomycin resistance (12). The two plasmids were transfected at an E6-to-*neo* gene ratio of 10:1 to ensure that the majority of geneticin-resistant colonies would also contain BPV-1 E6 sequences. The biological activity of each mutant was measured by two assays: focal transformation and anchorage-independent growth in soft agarose of cells selected with the transformation-independent marker. One day after transfection, the cells were reseeded. Some dishes received regular growth medium, were grown to confluence, and were scored for foci after 3 weeks (8). The cells in other dishes were selected in medium containing geneticin; the resistant colonies were pooled and then seeded into soft agar (13).

The results of these experiments are shown in Table 1. The wild-type E6 efficiently transformed C127 cells in both assays. However, most class 1 mutations, which affect the conserved cysteines in each of the four motifs, completely abolished the transforming activity, including the three conservative cysteine-to-serine changes and mutation 367, which results in a cysteine-to-histidine substitution. In one case (mutant 238), a cysteine-to-glycine substitution in the second cysteine motif resulted in detectable but significantly reduced transforming activity. The second cysteine in this motif is followed by a cysteine four amino acids downstream

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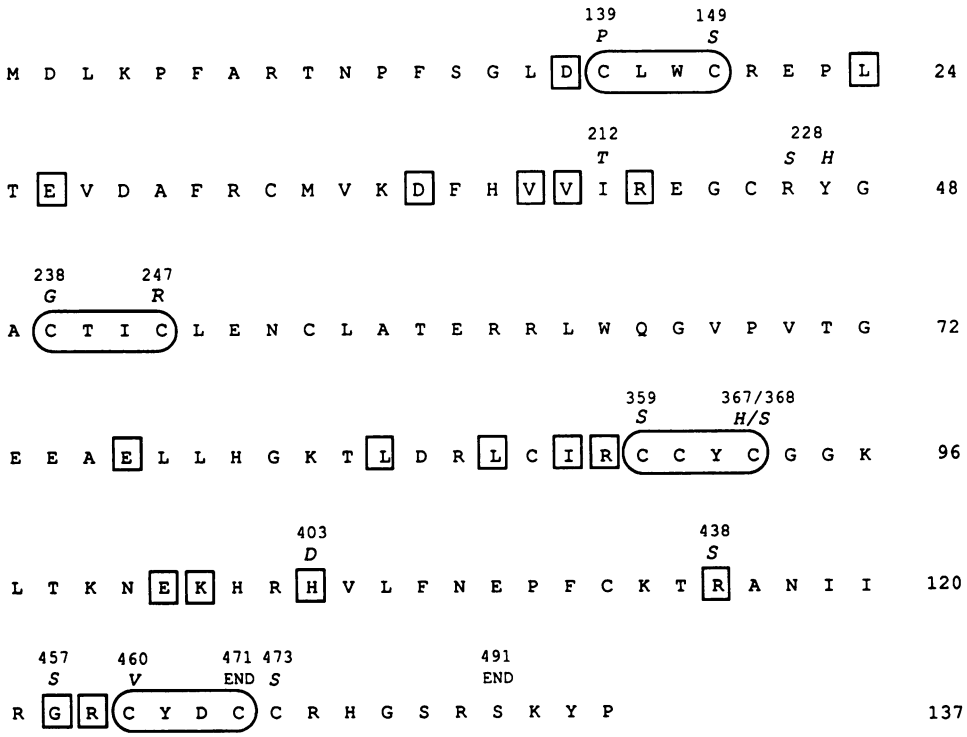

FIG. 1. Linear sequence of the 137 amino acids of BPV-1 E6 protein. The conserved Cys-X-X-Cys motifs are shown enclosed in elliptical boxes. Other amino acids which are conserved between BPV-1 and the E6 of sequenced HPVs are shown enclosed in rectangular boxes. The numbers on the right refer to the number of the right-hand amino acid residue on that line. The mutants generated in this study are named according to the first nucleotide altered, numbering from the *Hpa*I site. The mutant amino acid is indicated above the position at which the substitution was made.

(Cys-X-X-X-Cys), which perhaps can substitute inefficiently for the mutated motif.

Results obtained with the second class of mutants indicate that other conserved amino acids are also essential for the transforming activity of E6 but that some nonconserved amino acids may be mutated without any detectable loss of biological activity. Mutations 212 and 473, which both affect nonconserved amino acids, had almost wild-type transforming activities. Mutant 228, which has a substitution of two apparently nonconserved amino acids, displayed reduced activity. Interestingly, the histidine substitution created a His-X-X-Cys motif that could potentially form an alternative metal-binding site. The three mutants which carried substitutions in conserved amino acids (403, 438, and 457) were transformation defective.

Both premature-termination mutations also destroyed the biological activity of E6. This might be expected of mutant 471, which has a deletion of one of the conserved cysteines, but is perhaps surprising for mutant 491, which has a deletion of only the carboxy-terminal four amino acids, none of which appear to be conserved. DNA sequence analysis confirmed that no other mutations were present in the E6 sequences in this construct.

It is possible that the lack of transforming activity observed in some of these mutants might be due simply to the instability of the mutant protein. To evaluate this possibility, C127 cells transfected with the mutants were analyzed for the presence of E6 protein. Pooled cultures of geneticin-resistant cells were metabolically labeled with [^{35}S]cysteine, and cell extracts were immunoprecipitated with anti-E6 antibodies as previously described (1). An E6 protein of the

TABLE 1. Mutant classes

Class and mutant	Mutation	Mutation in conserved region	Transformation	
			No. of foci/μg	Growth in agar[a]
pXH800 (wild type)			68	+++
Mutations in Cys-X-X-Cys motifs				
139	Cys-17 to Pro	Yes	0	−
149	Cys-20 to Ser	Yes	0	−
238	Cys-50 to Gly	Yes	14	+
247	Cys-53 to Arg	Yes	0	−
359	Cys-90 to Ser	Yes	0	−
367	Cys-93 to His	Yes	0	−
368	Cys-93 to Ser	Yes	0	−
460	Cys-124 to Val	Yes	0	−
Other substitutions				
212	Ile-41 to Thr	No	64	+++
228	Arg-46 to Ser, Tyr-47 to His	No	20	+
403	His-105 to Asp	Yes	0	−
438	Arg-116 to Ser	Yes	0	−
457	Arg-123 to Ser	Yes	9	−
473	Cys-128 to Ser	No	26	+++
Premature-termination mutations				
471	11aa[b] truncation		0	−
491	4aa truncation		0	−

[a] Size of colonies rated from + (small) to +++ (large); −, no significant growth.

[b] aa, Amino acid.

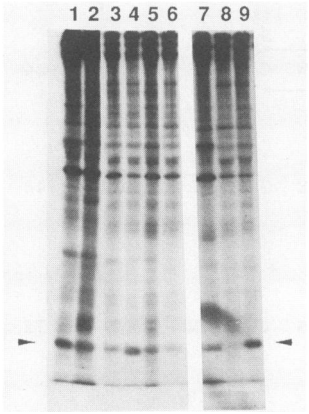

FIG. 2. Immunoprecipitation of representative E6 mutant proteins. Lanes 1 to 7 are immunoprecipitates from mutants 149, 212, 247, 491 (premature termination), 359, 367, and 471 (premature termination), respectively. The pSV2neo negative control and pXH800 positive control are shown in lanes 8 and 9, respectively. The arrows indicate the location of the E6 protein. Lanes 7 to 9 were run on a separate gel.

expected size was precipitated from cells transfected with all the mutants. Figure 2, which displays representative results, shows an E6-specific 16-kDa protein in cells transfected with all the mutants except 471 and 491 (the two premature-termination mutants), which, as expected, encoded mutant E6 proteins that migrated slightly faster (Fig. 2).

We have created a series of BPV-1 E6 proteins carrying subtle alterations in conserved and nonconserved amino acids. All of the mutant proteins are readily detectable in transfected C127 cells, so it is unlikely that the negative biological results were obtained because of protein instability. Two functionally important features of BPV-1 E6 have been revealed by the transformation studies described here. First, the conserved cysteines in the Cys-X-X-Cys repeats are essential for the transforming function of E6. For six of the seven cysteines in these repeats that were tested, mutation of only one cysteine completely abolished the transforming activity of E6, while mutation of the seventh cysteine (mutant 238) resulted in reduced activity. These results imply that this repeat structure is of particular importance to the function of BPV-1 E6 and, presumably, the E6 proteins of other PVs. It should also be noted that some conserved amino acids in addition to the cysteine repeats were found to be essential for activity. Second, at least some of the four C-terminal amino acids are required for biological activity, although this region of E6 is apparently not conserved. We are now in a position to correlate the transforming activity of the mutants with other known properties of the E6 protein, such as subcellular localization (1) and control of viral genome copy number (3). We have recently determined that a bacterially synthesized E6 protein binds zinc in vitro (2a). In preliminary experiments, all of the mutant E6 proteins described herein bound the metal. This would be the expected result if either one or a pair of Cys motifs could complex a molecule of zinc (6). Construction of multiply mutated E6 genes will be required to conclusively determine the role of the Cys repeats in zinc binding.

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