Mutational Analysis of Bovine Papillomavirus Type 1 E5 Peptide Domains Involved in Induction of Cellular DNA Synthesis

J. ALAN RAWLS, PAUL M. LOEWENSTEIN, AND MAURICE GREEN*

Institute for Molecular Virology, St. Louis University Medical Center, 3681 Park Avenue, St. Louis, Missouri 63110

Received 6 June 1989/Accepted 27 July 1989

Early gene E5 of bovine papillomavirus type 1 encodes a 44-amino-acid protein whose expression can transform immortalized mouse cell lines. We have previously reported that a chemically synthesized E5 peptide functions to induce cellular DNA synthesis upon microinjection into growth-arrested mouse cells. We further defined the two E5 domains essential for the full DNA synthesis induction activity by the analysis of E5 deletion and amino acid substitution mutant peptides. The first domain is the C-terminal 13-amino-acid core which is sufficient to activate DNA synthesis at high peptide concentration and contains two essential, highly conserved cysteine residues. The second domain is the 7-amino-acid hydrophobic sequence contiguous to the core domain which is sufficient to confer a 1,000-fold higher molar specific activity to the E5 peptide. A random hydrophobic sequence, but not charged amino acids, fulfills the function of the second domain.

Bovine papillomavirus type 1 (BPV1) is a fibropapilloma-virus that infects both epidermal keratinocytes and the underlying dermal fibroblasts, resulting in the production of benign tumors. E5 (44 amino acids; M_r , 5,978 [Fig. 1]) is the major oncogene of BPV1 (4, 11, 12-15). The N-terminal two-thirds of the protein (residues 1 through 30) are highly hydrophobic, while the C-terminal one-third is predominantly hydrophilic. Intracellular localization studies suggest that the E5 protein is associated with the Golgi apparatus as well as with the plasma and nuclear membranes (3, 14).

Clues to the functional role of the BPV1 E5 oncogene are provided by the demonstration that cellular DNA synthesis is induced by microinjection of chemically synthesized E5 peptides into mouse cell nuclei (6) and by transfection of mouse cells by the E5 gene promoted by the Rous sarcoma virus long terminal repeat (data not shown). Evidence was found for two functional regions: a 13-amino-acid C-terminal domain and a hydrophobic domain of unknown length. Here, we delineate the boundary and sequence requirements of the hydrophobic domain and examine the role of the conserved cysteine residues (Cys-37 and Cys-39) in inducing cellular DNA synthesis.

To determine the length of the hydrophobic region required to induce DNA synthesis at concentrations equimolar to that of the wild-type E5 peptide, we synthesized a library of E5 N-terminal deletion peptides with decreasing lengths of the hydrophobic domain. Each mutant peptide was microinjected into the nuclei of serum-starved quiescent C127 cells, incubated with [3 H]thymidine for 48 h, and autoradiographed (for details, see reference 6). The concentration needed to induce one-half maximal activity of the full-length E5 peptide (22% of cells activated) was determined and plotted against peptide length (Fig. 2). E5 mutant dm32-44 (the numbers designate the amino acid residues included in the peptide), which contains only the 13 hydrophilic C-terminal residues, induced half maximal activity at high peptide concentrations (3×10^{-4} M). The addition of one amino acid, tyrosine (dm31-44), did not appreciably increase the molar specific activity. However, a steep increase in specific activity was observed with the addition of hydrophobic residues 30 through 25. A virtual plateau occurs with

the addition of residues 24 to 1; the molar concentrations necessary for the activity of dm25-44 (5×10^{-7} M), dm16-44 (3×10^{-7} M), and E5 (2×10^{-7} M) are not significantly different within the limits of the microinjection assay. Photomicrographs illustrating the response of quiescent cells to microinjection with E5, dm25-44, p1-11 (simian virus 40 large T-antigen plasmid) (5), and the buffer control are shown in Fig. 3. These observations indicate that residues 25 to 44 encode information sufficient for efficient induction of cellular DNA synthesis.

The role of residues 25 to 30 appears to be dependent on the nonpolarity of this sequence. This hydrophobic stretch of amino acids appears to be sequence independent, as shown by the retention of activity when a random sequence of hydrophobic residues (E5 dm25-44R1) is substituted for residues 25 through 30 in the peptide (Table 1). Furthermore, substitution of charged residues (E5 dm25-44R2) reduced the DNA synthesis induction activity to that of background (Table 1). Of particular interest, our results on the sequence requirements for the inhibition of cellular DNA synthesis are consistent with data reporting the ability of E5 gene mutants to transform cells (9).

The nature of the enhancement function of the hydrophobic domain is not known. Several possibilities exist, including (i) targeting the peptide to a membrane structure at the site of activity, (ii) providing the optimal conformation for

FIG. 1. Amino acid sequence of BPV1 E5 protein. Superimposed on the sequence are the proposed hydrophobic augmentation (▨) and hydrophilic cellular DNA induction (■) regions suggested by our data.

* Corresponding author.

FIG. 2. Molar specific activity of BPV1 E5 deletion mutant peptides in the cellular DNA induction assay as a function of peptide length. The molar concentration of each peptide required to exhibit half maximal activity is plotted against the length of the peptide. DNA induction activity was determined by microinjection of the E5 peptides in quiescent C127 cells, exposure to [3 H]thymidine, and autoradiography. Peptide concentration was determined by absorbance at 280 nm.

peptide activity, and (iii) increasing the intracellular stability of the E5 protein. The role of the E5 N-terminal residues 1 to 24 is not known. Possibly, these hydrophobic amino acids further strengthen the association of E5 to a membrane target.

The C-terminal one-third of E5, which possesses the core activity, includes a Cys-X-Cys motif conserved in the E5

TABLE 1. The effect of hydrophobic-hydrophilic substitutions in residues 25 through 30 on the cellular DNA induction activity of BPV E5 peptides

Material injected (residue sequence) ^a	Mean % labeled nuclei $\pm$ SD (no. of separate experiments)
E5 dm25-44 (25-L-L-F-F-L-V-30)	26 $\pm$ 5 (3)
E5 dm25-44 R1 (25-V-I-F-L-I-F-30)	24 $\pm$ 4 (3)
E5 dm25-44 R2 (25-Y-G-E-V-R-K-30)	9 $\pm$ 4 (3)
p1-11 ^b	40 $\pm$ 5 (3)
30 mM Tris, pH 8.5	4 $\pm$ 4 (3)

^a The DNA induction activity of mutants E5 dm25-44 R1 and E5 dm25-44 R2 was determined by microinjection into nuclei of quiescent mouse C127 cells, exposed to [3 H]thymidine, and autoradiographed. E5 dm25-44 R1 and E5 dm25-44 R2 have identical sequences to E5 dm25-44 except for the residues shown. All peptides were injected at a concentration of 10^{-6} M, and p1-11 was injected at 800 μ g/ml.

^b Simian virus 40 large T-antigen plasmid.

gene of papillomaviruses (1, 8, 10). To investigate the importance of these residues, Ala was substituted for Cys-37, Cys-39, or both. The mutations were built into the full-length E5 peptide backbone, as well as into the shorter active backbones, dm25-44 and dm27-44, which are more soluble than the full-length molecule. The peptides were microinjected into nuclei of quiescent C127 cells, and their ability to activate cellular DNA synthesis was determined. Substitution of both cysteine residues in all three peptide backbones resulted in the complete loss of activity (Table 2). When only a single cysteine residue was substituted, the loss was less dramatic (Table 2).

These data suggest that Cys-37 and Cys-39 play an important role in the DNA induction function. Analogous observations with E5 DNA mutants have been reported for the cell transformation function (9). The E5 protein has been reported to be present in the cell mainly as a dimer, presumably because of disulfide formation (2). Our data suggest that dimer formation may be essential for the function of E5 in DNA synthesis induction.

An important question is whether activation of cellular DNA synthesis is the sole basis of the ability of E5 to transform cells. Mutational analyses have defined structure-function requirements for cell transformation. Taken together, these results suggest a correlation between the requirements for transformation by E5 plasmids (2, 4, 7, 9) and those for induction of DNA synthesis by E5 peptides (6; data presented here). Recently Horwitz et al. (9) described several single and multiple amino acid substitution mutants upstream of E5 residue 25 which exhibit reduced foci for-

FIG. 3. [3 H]Thymidine incorporation after microinjection of synthetic BPV1 E5 peptides into mouse C127 cells. Cells were microinjected with the materials described below, labeled with [3 H]thymidine, and autoradiographed. Black nuclei indicate cells synthesizing DNA. The panels show an area of cells in which 100 have been injected with (left to right) E5-44, E5 dm25-44, p1-11 (simian virus 40), or 30 mM Tris, pH 8.5.

TABLE 2. The effect of cysteine substitutions on cellular DNA synthesis induction activity of BPV E5 peptides

Material injected ^a	Concn (M)	Mean % labeled nuclei $\pm$ SD (no. of separate experiments)
E5-44	1×10^{-6}	39 ± 11 (4)
E5-44 sm37,39	1×10^{-6}	6 ± 9 (4)
E5 dm25-44	6×10^{-6}	40 ± 11 (7)
E5 dm25-44 sm37,39	6×10^{-6}	9 ± 7 (3)
E5 dm27-44	5×10^{-5}	30 ± 13 (4)
E5 dm27-44	1×10^{-5}	27 ± 5 (4)
E5 dm27-44 sm37,39	1×10^{-5}	7 ± 4 (2)
E5 dm27-44 sm37	5×10^{-5}	21 ± 7 (5)
E5 dm27-44 sm37	1×10^{-5}	19 ± 5 (5)
E5 dm27-44 sm39	5×10^{-5}	18 ± 5 (5)
E5 dm27-44 sm39	1×10^{-5}	8 ± 2 (3)
p1-11 (simian virus 40 large T antigen)		38 ± 12 (7)
30 mM Tris, pH 8.5		5 ± 2 (7)

^a The DNA induction activity of the E5 peptides was determined by microinjection into quiescent mouse C127 cells. The double cysteine substitution mutants were injected at their respective maximum concentrations. p1-11 was injected at 800 μ g/ml.

mation. Our studies show that residues upstream of amino acid 25 are not required for activation of DNA synthesis in quiescent mouse cells. We can suggest several explanations for this difference. First, residues 16 to 24 may be essential for transport of the E5 protein to the nucleus, a function which is circumvented by microinjection directly into nuclei. Second, substitutions of these residues could alter the conformation of the active core, hydrophobic domain, or both. Third, it is possible that functions in addition to DNA synthesis induction are encoded in residues 16 to 24 and that these functions play a role in cell transformation. Further studies are needed to establish the relationship between E5 molecular functions required for cell transformation and those involved in the induction of cellular DNA synthesis.

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