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Amino Acids Critical for the Functions of the Bovine Papillomavirus Type 1 E2 Transactivator

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The N-terminal domain of the bovine papillomavirus type 1 E2 protein is important for viral DNA replication, for transcriptional transactivation, and for interaction with the E1 protein. To determine which residues of this 200-amino-acid domain are important for these activities, single conservative amino acid substitutions have been generated in 17 residues that are invariant among all papillomavirus E2 proteins. The resulting mutated E2 proteins were tested for the ability to support viral DNA replication, activate transcription, and cooperatively bind to the origin of replication with the E1 protein. We identified five mutated proteins that were completely defective for transcriptional activation and either were defective or could support viral DNA replication at only low levels. However, several of these proteins could still interact efficiently with the E1 protein. In addition, we identified several mutated proteins that were unable to efficiently cooperatively bind to the origin with the E1 protein. Although a number of the mutated proteins demonstrated wild-type activity in all of the functions tested, only 3 out of 17 mutated viral genomes were able to induce foci in a C127 focus formation assay when the mutations were generated in the background of the entire bovine papillomavirus type 1 genome. This finding suggests that the E2 protein may have additional activities that are important for the viral life cycle.

The papillomaviruses are small DNA viruses that infect basal epithelial cells and replicate in terminally differentiating keratinocytes. These viruses have been isolated from a wide range of vertebrates, and they exhibit both host species and tissue specificity. The viral E1 and E2 proteins are important for initiation of viral DNA replication and for regulation of viral transcription. The E1 protein is the viral replication initiator protein (25, 36, 40) and also appears to function as a transcriptional repressor (19, 27); the viral E2 proteins are transcriptional regulatory proteins that regulate the expression of the other viral gene products and, in addition, play an important role in DNA replication (reviewed in reference 13).

The E2 protein has been best studied for bovine papillomavirus type 1 (BPV-1), as this virus can infect and transform certain rodent cells within which the viral genome is replicated as an episome at a constant copy number (7, 18). This nonproductive virus-cell interaction is thought to be analogous to what has been found for the infected basal epithelial cells and dermal fibroblasts of a BPV-1-induced fibropapilloma and has permitted a detailed genetic analysis of the transcriptional regulatory and plasmid maintenance replication functions of the E2 protein(s).

The BPV-1 E2 open reading frame (ORF) encodes three polypeptides. The largest, E2-TA, is encoded by the entire ORF and functions as a transcriptional transactivator; two smaller polypeptides, E2-TR and E8/E2, are encoded by the 3′ half of the ORF and function as transcriptional repressors by antagonizing the function of E2-TA (2, 14, 16, 17, 32). The E2-TA protein activates transcription by binding to specific DNA binding sites that are located within enhancer elements in the viral genome (reviewed in reference 23). In addition to its transactivation function, full-length E2 is required for stable replication of viral DNA (36). The viral E1 protein can initiate DNA replication alone in vitro, but in vivo, the E2 protein is also required (41). E2 appears to have several functions in DNA replication: the viral replication origin contains a binding site for the viral E1 protein flanked on either side by E2 DNA binding sites (35, 37), and the E2-TA protein greatly increases the amount of E1 bound to the origin by cooperative binding (29, 31, 39, 40); the E2 protein also appears to augment replication by disrupting chromatin structure in the origin region (21) and by interacting with replication proteins such as replication protein A (20).

The E2 transactivator consists of two conserved domains linked by a flexible hinge region. The C-terminal domain is approximately 85 amino acids long and is required for DNA binding and dimerization (6, 22, 24). The N-terminal domain is approximately 200 amino acids long and is important for transcriptional activation (8, 11, 22). This domain is also critical for the replication function of the E2 protein and for cooperative binding to the replication origin with the E1 protein (38, 39). Unlike many transactivation domains, the E2 N-terminal domain seems to have a very constrained structure, as any deletion that has been made within this domain inactivates all E2-TA functions, presumably by disrupting protein conformation (22, 38, 39).

In this study, a number of amino acid substitutions have been generated in highly conserved residues of the BPV-1 E2 protein to determine what effect they might have on specific functions of the E2 protein. Very conservative amino acid substitutions were generated in an attempt to inactivate specific functions of the E2 protein without disrupting protein conformation. We have identified a number of amino acid residues in which mutations either inactivate the transactivation and DNA replication functions of the E2 protein or interfere with the ability of E2 to interact with the E1 protein. In the background of the viral genome, almost all of the E2 mutations gave rise to viruses defective in focus formation,

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indicating that the E2 protein may have additional, as yet unidentified functions in viral gene expression and/or replication.

MATERIALS AND METHODS

Plasmids and mutagenesis. Single point mutations were introduced into the E2 nucleotide sequence by one of three techniques: oligonucleotide reconstruction, PCR-generated mutagenesis, or Transformer site-directed mutagenesis. Mutations were generated in the background of the pTZE2sh shuttle expression vector described previously (38). For those mutations generated by oligonucleotide reconstruction (Q-12 to N, E-20 to D, W-33 to F, R-37 to K, and E-39 to D; the corresponding mutant proteins are designated N12, D20, F33, K37, and D39) the multiple cloning site within the pTZE2sh vector was first modified to remove the *Sph*I site. Double-stranded oligonucleotides were used to reconstruct the E2 sequence from the *Sph*I site at nucleotide 2621 to the *Avr*II site at nucleotide 2766 with individual point mutations. As a control, wild-type E2 was reconstructed in the same fashion. Six E2 mutations (V-59 to L, P-60 to G, P-106 to G, K-111 to R, K-112 to R, and D-122 to E; the corresponding mutant proteins are designated L59, G60, G106, R111, R112, and E122) were generated by PCR techniques. The first five mutations lie within the E2 nucleotide sequence between the *Avr*II site at nucleotide 2766 and the *Kas*I site at nucleotide 2944. The last mutation (D-122 to E) lies between the *Kas*I site and the *Dra*II site at nucleotide 3259. PCR primer pairs were designed with terminal recognition sequences for *Avr*II and *Kas*I or for *Kas*I and *Dra*II, and each pair contained one primer with a point mutation and another that corresponded to the wild-type sequence. E2 DNA fragments were amplified by PCR, cleaved with the appropriate restriction enzymes, and cloned into the corresponding sites in pTZE2sh. Six E2 mutations (I-73 to L, W-92 to F, G-156 to A, Y-159 to F, F-173 to Y, and V-188 to L; the corresponding mutant proteins are designated L73, F92, A156, F159, Y173, and L188) were generated using the Transformer (Clontech) mutagenesis procedure, which is based on the technique first described by Deng and Nickoloff (3). Briefly, primers containing point mutations in the E2 sequence were annealed in combination with a selection primer to denatured template pTZE2sh plasmid DNA. The selection primer also contained a nucleotide substitution that converted the unique *Sal*I site in the vector sequences to *Xho*I. A mutant DNA strand was synthesized from the primers with T4 DNA polymerase and T4 DNA ligase, and the populations of mutation-bearing and wild-type plasmids generated were used to transform the *mutS Escherichia coli* strain BMH 71-8 (Clontech), which is deficient for mediating mismatch repair. Mutant plasmids were then selected from the amplified plasmid pool on the basis of resistance to digestion by *Sal*I and susceptibility to digestion by *Xho*I. Introduction of the correct mutation was confirmed for all plasmids by DNA sequence analysis.

Each of the E2 N-terminal mutations was cloned into the pC59 E2 expression vector (42) and the entire BPV genome, p142-6 (28), by exchanging the *Bst*EII (nucleotide 2405)-to-*Bst*XI (nucleotide 3889) fragment containing wild-type E2 sequences with the corresponding fragment from each of the mutated pTZE2sh plasmids.

Protein extracts. ^{35}S-labeled proteins were expressed from the pTZE2sh plasmids described above, using the TNT coupled reticulocyte lysate system (Promega). An aliquot of lysate was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and the radiolabeled E2 proteins were quantitated with a PhosphorImager (Molecular Dynamics).

Transient expression assay. Transfections were carried out by using the calcium phosphate technique as previously described (32). Briefly, 0.3 μg of mutated or wild-type C59-E2 DNA and 2 μg of an E2-responsive chloramphenicol acetyltransferase (CAT) reporter plasmid, pTKM-11 (34), were cotransfected into subconfluent 60-mm^2 dishes of monkey CV-1 cells. Following a 4- to 6-h incubation, cells were subjected to glycerol shock and then grown in Dulbecco modified Eagle medium (Gibco/BRL)–10% fetal bovine serum with 5 mM sodium butyrate. Cell lysates were prepared 24 to 48 h later. CAT assays were carried out with equivalent quantities of protein from all dishes. Each plasmid was tested in several independent studies.

Transient replication assay. Transient replication assays were carried out as described by Ustav and Stenlund (36). In brief, 5 μg of either mutated or wild-type C59-E2 plasmid DNA, 5 μg of E1 expression plasmid (pCGEag-1235 from A. Stenlund), and 2 μg of replicon DNA were introduced into mouse C127 cells by electroporation. A 3.2-kb fragment of the BPV-1 genome containing positions 4786 to 7946 and 1 to 83 was used as a replicon. This fragment was cleaved from the prokaryotic vector sequences and recircularized before electroporation. At 3 and 5 days posttransfection, cells were lysed and low-molecular-weight DNA was isolated. DNA samples were digested with *Dpn*I and *Hin*dIII or with *Mbo*I and *Hin*dIII, and the products were separated on a 0.8% agarose gel in Tris-borate-EDTA buffer. The DNA products were transferred to nytran membranes (MSI; Magna Graph) and probed with a fragment from the long control region (nucleotides 6958 to 6936) that was generated by PCR and ^{32}P labeled by the random priming technique.

E1-E2 DNA binding assay. E1-E2 cooperative DNA binding was measured in a DNA-protein coimmunoprecipitation assay. The DNA probe was generated from plasmid KS+/origin, which contains BPV-1 nucleotides 7781 to 7946 and 1 to 83 cloned in the *Bam*HI-to-*Hin*dIII sites of Bluescript KS+ (31). Plasmid KS+/origin was cleaved with *Bam*HI, *Hin*dIII, and *Afl*III to release a 250-bp fragment containing the origin and a 400-bp fragment from the vector that was used as a control for nonspecific binding. These fragments were end labeled with [^{32}P]dCTP and the Klenow fragment of DNA polymerase.

In the binding reaction, 15 μl of reticulocyte lysate containing the E1 protein was added to a buffer containing 10 μg of sonicated salmon sperm DNA and final concentrations of 28 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.4), 70 mM magnesium acetate, and 1.4 mM dithiothreitol in a total volume of 200 μl. A final NaCl concentration of 170 mM was obtained by the addition of buffer C-300 (20mM HEPES [pH 7.9], 25% [vol/vol] glycerol, 0.3 M NaCl, 1.5 mM $MgCl_2$). Where indicated, 50 μl of reticulocyte lysate containing in vitro-translated E2 protein was also added. Insoluble protein was removed from the reaction mixture by centrifugation before the addition of 200 ng of DNA probe. After 60 min of incubation at room temperature, protein A-Sepharose beads containing prebound affinity-purified antiserum SSQN (31) were added to the binding reaction mixture, which was then and incubated at 4°C for 1 h. Immune complexes were washed three times in NT-200 (50 mM Tris [pH 7.4], 200 mM NaCl, 0.1% Triton X-100). The beads were suspended in 250 μl of NT-200, a 25-μl aliquot was removed, and proteins analyzed by SDS-polyacrylamide gel electrophoresis. The DNA-protein-antibody complexes on the remaining beads were dissociated with 1% SDS. Ten micrograms of carrier tRNA was added, and DNA was isolated by phenol extraction and ethanol precipitation. DNA fragments were analyzed after separation on polyacrylamide gels and were quantitated with a PhosphorImager (Molecular Dynamics).

Immunoprecipitation and immunoblot analysis. Thirty-six micrograms of pC59E2mut or pC59E2wt DNA was transfected into 150-mm-diameter plates of subconfluent COS-7 cells, using Lipofectamine (Gibco). At 40 to 48 h posttransfection, protein was extracted in radioimmunoprecipitation assay buffer (150 mM NaCl, 20 mM morpholine propanesulfonic acid [MOPS; pH 7], 1 mM EDTA, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS). Cell lysates were precleared with Pansorbin (fixed *Staphylococcus aureus*; CalBiochem), and 1 mg of protein was incubated with monoclonal antibody B202 (E. Androphy). Immune complexes were collected with protein A-Sepharose and washed with radioimmunoprecipitation assay buffer. Released proteins were resolved on an SDS–8% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore Immobilon-P). The membrane was blocked in 5% Carnation dried milk, 1× TNET buffer (10 mM Tris-HCl [pH 7.5], 2.5 mM EDTA, 50 mM NaCl, 0.1% Tween) and incubated with antibody B202. Following washes in TNET, the membrane was incubated with a horseradish peroxidase-conjugated antiserum against mouse κ chain (Caltag). The lot of antiserum used for Fig. 2 also showed cross-reactivity with the immunoglobulin heavy chain. Proteins were detected by using an enhanced chemiluminescence system (Amersham).

Focus formation assay. C127 cells were transfected by the calcium phosphate technique. Briefly, 100-mm-diameter dishes of cells were transfected with 1 μg of 142-6 DNAs (E2 mutations in the background of the cloned BPV-1 genome) that had been cleaved at the *Bam*HI site to separate BPV-1 and pML2d sequences and religated at a concentration of 5 μg/ml to favor intramolecular recircularization. Cells were stained with methylene blue 2 to 3 weeks posttransfection.

RESULTS

Sequence conservation among the papillomavirus E2 proteins. Papillomaviruses have been identified in birds and mammals, and more than 70 different human types have been identified. A comparison of the predicted amino acid sequences of the papillomavirus E2 proteins show that there are two conserved domains: a C-terminal domain of about 85 amino acids and an N-terminal domain of about 200 amino acids. In all cases that have been examined, the N-terminal domain has been shown to be important for transcriptional regulation and viral DNA replication. To identify amino acid residues in this domain that are critical for E2 function, the predicted amino acid sequences of 22 different papillomavirus E2 proteins were compared (Fig. 1). The viruses belong to each of the five recently defined evolutionary supergroups (1). Excluding the initiation methionine, 17 amino acid residues were found to be identical among the papillomavirus E2 proteins, indicating that they may be crucial for protein structure and one or more viral functions. (Many more residues were highly, but not absolutely, conserved.) Mutations were generated in these residues in an attempt to interfere with specific protein functions. Where possible, very subtle changes were made in these amino acids by replacing them with chemically similar residues in order to minimize possible alterations in global protein conformation, as the structure of the E2 transactivation domain

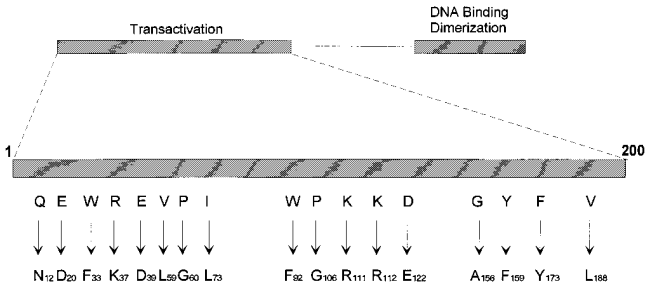

FIG. 1. Positions of the amino acid substitutions in the N-terminal domain of the E2 protein. The predicted amino acid sequences of 22 different papillomavirus E2 proteins (BPV-1, -2, and -4, deer, European elk, Cottontail rabbit, and rhesus monkey papillomaviruses, and human papillomavirus types 1a, 5, 5b, 6b, 8, 11, 16, 18, 31, 33, 35, 39, 42, 47, 51, and 58) were compared, and mutations were generated in those residues that were absolutely conserved.

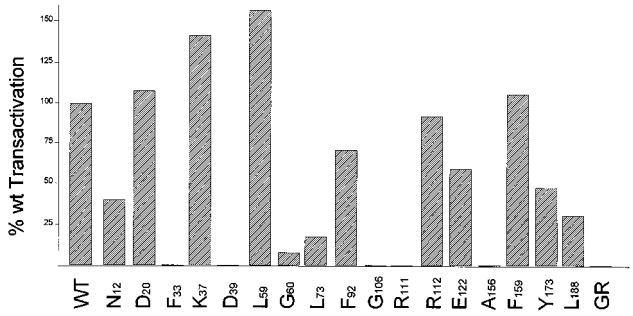

FIG. 3. Transcriptional activation by E2 proteins containing N-terminal mutations. CV-1 cells were cotransfected with pTKM-11, an E2-responsive CAT reporter plasmid, and the series of C59 E2 expression plasmids. CAT activity was determined in each case and is expressed relative to wild-type (WT) C59 activity, which was given a value of 100%. Each plasmid was tested in seven independent experiments. Wild-type E2 activity ranged from 13 to 54% acetylated chloramphenicol. GR, the G105R111 protein.

appears to be quite constrained (Fig. 1). An additional E2 protein (G105R111) containing two mutations that occurred erroneously during cloning, an E-to-G substitution at position 105 and a K-to-R substitution at position 111, was also analyzed in this series of experiments. For these studies, proteins were expressed either in vitro by coupled in vitro transcription and translation or in vivo from a vector that expresses the E2 gene products from a simian virus 40 promoter (42). To ensure that the proteins expressed in vivo were stably expressed, the C59 series of plasmids were transfected into COS-7 cells, and the levels of the mutated E2 proteins were determined by a combined immunoprecipitation-Western blot (immunoblot) analysis. This analysis demonstrated that all of the mutated proteins were expressed at levels comparable to that of the wild-type E2 protein (data not shown). A representative blot showing two of the mutants is presented in Fig. 2.

Transcriptional activation function of the E2 proteins. The BPV-1 E2 protein activates transcription by binding to specific E2 binding sites located within enhancer elements of a promoter and increases the rate of transcriptional initiation, presumably by interacting with one or more components of the basal transcriptional machinery or other proximal promoter factors (10, 23, 32). To determine whether the conservative amino acid substitutions that were generated in the E2 protein had an effect on transcriptional activation, CV-1 cells were cotransfected with the C59 vectors expressing the series of mutated E2 proteins and a E2-responsive CAT plasmid, pTK-11. This highly responsive plasmid expresses the CAT gene from a herpes simplex virus thymidine kinase promoter with six tandem E2 binding sites located upstream (34). As can be seen in Fig. 3, six of the mutated E2 proteins (F33, D39, G106, R111, A156, and G105R111) were severely impaired in the ability to activate transcription from pTK-11. All other mutated proteins exhibited transactivation levels that were between 10% of wild type and levels that were comparable to or greater than that of wild-type E2. Thus, amino acid substitutions such as lysine to arginine, glutamic acid to aspartic acid, and tryptophan to phenylalanine are sufficient to inactivate the transactivation function of the E2 protein.

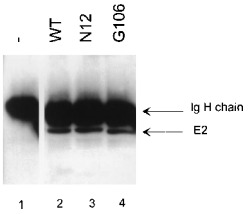

FIG. 2. Expression of mutated E2 proteins. Shown is a representative Western blot of protein extracts of COS-7 cells transfected with vector alone (lane 1), C59-E2 (wild type [WT]; lane 2), C59-E2N12 (lane 3), and C59-E2G106 (lane 4). E2 proteins were detected as described in Materials and Methods. Ig, immunoglobulin.

Replication properties of the mutated E2 proteins. To determine the effect of the E2 N-terminal amino acid substitutions on viral DNA replication and to discern whether the transactivation and replication properties of the E2 protein are separable, the ability of each of the mutated E2 proteins to support DNA replication in a transient DNA replication assay was analyzed. A 3.2-kb fragment of the BPV-1 genome encompassing the long control region and sequences encoding the L1 and L2 late proteins (nucleotides 4786 to 7946 and 1 to 83) was cleaved from prokaryotic vector sequences and recircularized to serve as the replicon. Wild-type or mutated C59E2 DNA was cotransfected into mouse C127 cells by electroporation with an E1 expression plasmid (pCGEag1235) (36) and the replicon DNA. At 3 and 5 days posttransfection, low-molecular-weight DNA was isolated and digested with *Hin*dIII and *Dpn*I. The resulting DNA fragments were then analyzed by Southern blot hybridization with a BPV-1 DNA probe (p142-6). *Dpn*I cleaves DNA that has been methylated as a result of growth in *E. coli*; however, DNA that has undergone replication in eukaryotic cells is no longer methylated and is resistant to cleavage by *Dpn*I. Therefore, after a single cleavage by *Hin*dIII, replicated BPV-1 DNA migrates as a linear 3.2-kb fragment.

As shown in Fig. 4, six E2 proteins (F33, D39, G106, R111, A156, and G105R111) consistently exhibited a dramatic reduction in replication activity compared with the wild-type E2 protein, each displaying between 0 and 10% of the activity of wild-type E2. The remaining proteins exhibited different degrees of impairment in replication function, ranging from 20 to 100% of the activity of the wild-type E2 protein. The six proteins that were most defective in replication (F33, D39, G106, R111, A156, and G105R111) were all inactive in transcriptional transactivation. Therefore, none of the mutations were able to absolutely separate the replication and transactivation properties of the E2 protein. The E2 L73 protein demonstrated wild-type activity in replication assays but exhibited only about 20 to 30% activity in transcriptional activation. In addition, while the mutated proteins F33, R111, and G105R111 were completely defective for transcriptional activation, they did support a low level of DNA replication. This

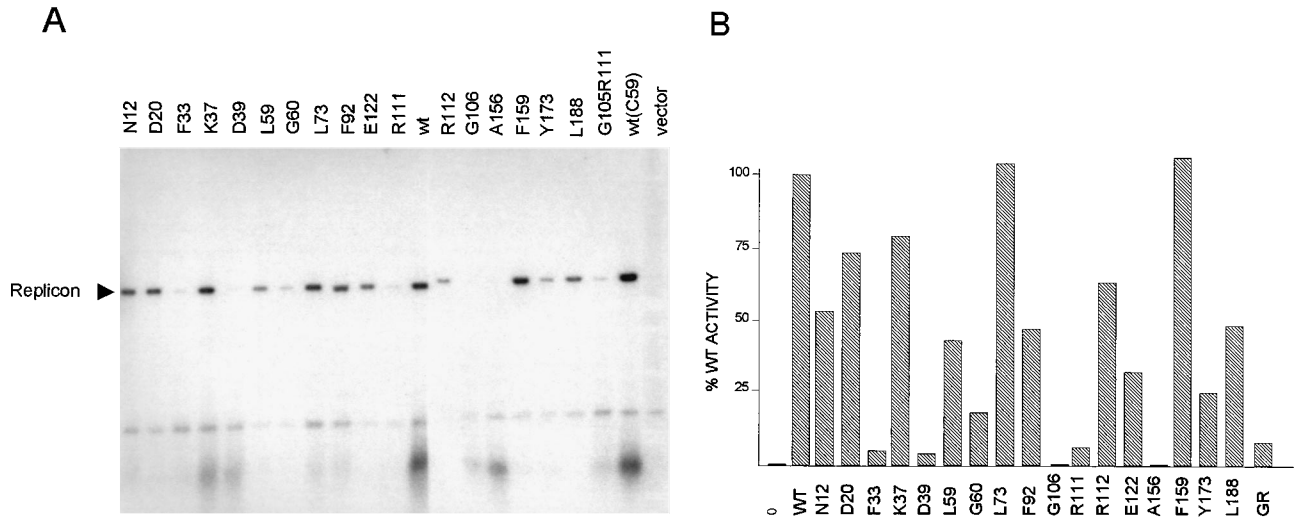

FIG. 4. Transient replication properties of the mutated E2 proteins in C127 cells. (A) Representative transient replication assay. In each lane, cells were electroporated with p716 replicon DNA, pCGEag-1235 E1 expression vector, and either C59 vector without the E2 insert (vector), wild-type (wt) pC59, or mutated pC59 constructs, as indicated. (B) Average amounts of replication obtained in four assays (two time points in two independent experiments). Replication activity was quantitated with a PhosphorImager and is expressed relative to wild-type C59 (WT) activity, which was given a value of 100%. GR, the G105R111 protein.

low level was confirmed by digestion with *Mbo*I (data not shown). This finding suggests that the activities of the E2 protein required for transactivation and replication are only partially overlapping.

Interaction of the E1 and E2 proteins. The BPV-1 replication origin consists of an E1 binding site flanked on either side by E2 binding sites. The E1 and E2 proteins bind with relatively low affinities to their respective binding sites, but an E1-E2 complex binds the origin region with high affinity. To establish whether the mutated E2 proteins could bind cooperatively to the BPV-1 replication origin with the E1 protein, a DNA-protein coimmunoprecipitation assay was carried out (Fig. 5). Both E1 and E2 proteins were generated by in vitro transcription and translation, and the amounts of the mutated E2 proteins were quantitated and adjusted to equal concentrations. The E1 protein was added to a mixture of three DNA fragments, one of which contained the origin sequences (nucleotides 7781 to 7946 and 1 to 83), and DNA-protein complexes

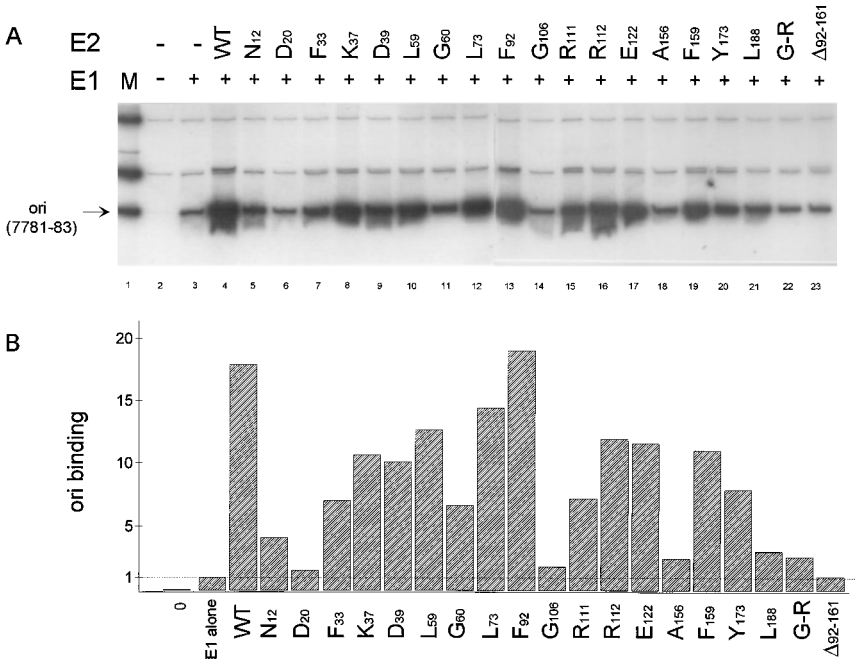

FIG. 5. Cooperative binding of E1 and E2 to the replication origin. (A) Representative E1-E2 coimmunoprecipitation experiment. Lane 1 represents the input DNA, with the origin (ori)-specific fragment indicated by an arrow (positions are indicated in nucleotides). In lane 2, neither E1 nor E2 protein was added to the assay. In lane 3, only E1 protein was added. In the remaining lanes, either wild-type (WT) E2 protein (lane 4) or mutated E2 proteins (lanes 5 to 23) were added. G-R, the G105R111 protein. (B) Average amounts of E1 origin binding obtained in two different experiments. Origin binding was quantitated with a PhosphorImager and was calculated as the fold increase above the origin binding of E1 protein alone.

TABLE 1. Focus formation of C127 cells by BPV-1 containing mutations in the N terminus of the E2 protein

E2 protein	No. of foci/μg of DNA[a]
No DNA	0, 0
Wild type	115, 189
N12	0, 0
D20	120, 70
F33	0, 0
K37	0, 0
D39	0, 0
L59	12, 3
G60	0, 0
L73	0, 0
F92	0, 0
G106	0, 0
R111	0, 0
R112	0, 0
E122	0, 0
A156	0, 0
F159	97, 116
Y173	0, 0
L188	0, 0
G105R111	0, 0

[a] Results from two experiments.

were immunoprecipitated with an E1-specific antiserum, SSQN. The coprecipitated DNA was extracted and analyzed by polyacrylamide gel electrophoresis (Fig. 5A, lane 3). As can be seen in lane 4, addition of the E2 protein to this assay increased the amount of E1 origin-specific binding by 17.5-fold. In Fig. 5A, lanes 5 to 23, the assay was carried out with the series of mutated E2 proteins, and the results from two different experiments are represented in Fig. 5B. An E2 protein with a large deletion in the transactivation domain (E2Δ92–161) was used as a negative control, as it has been shown to be unable to interact with the E1 protein (39). This analysis showed that two proteins (G106 and A156) that were completely defective in both transcriptional activation and DNA replication were also defective in cooperative origin binding, enhancing E1 binding by approximately twofold. The double mutant G105R111 was also relatively defective, but the remaining three proteins that were defective in the in vivo assays (F33, D39, and R111) could function in the origin binding assay, enhancing E1 binding about 7- to 10-fold. However, somewhat surprisingly, two proteins (D20 and L188) that could function in the replication and transactivation assays were unable to efficiently enhance binding of the E1 protein to the replication origin. The remaining proteins could enhance E1 origin binding between 4- and 19-fold. Therefore, residues important for interaction with the E1 protein are different, in some cases, from those required for transactivation and replication.

BPV-1 focus formation assay. Although the 17 residues that were mutated in the transactivation domain are identical among the papillomavirus E2 proteins, a number of the mutated E2 proteins (N12, K37, L59, L73, F92, R112, E122, F159, and Y173) can still function in transient transactivation, replication, and cooperative origin binding assays. Previous studies have shown that mutations in the E2 ORF are pleiotropic and can affect viral transcription, replication, and cellular transformation properties of the viral genome (4, 5, 12, 26). To determine whether the mutated E2 proteins have additional defects, the mutated E2 ORFs were cloned into the background of the entire viral genome and assayed for the ability to induce focus formation in C127 cells. As shown in Table 1, all of the mutated proteins except D20, F159, and L59 were unable to induce focus formation in the background of the viral genome. D20 and F159 gave rise to approximately the same number of foci as the wild-type genome, whereas L59 resulted in greatly reduced numbers. Cells transformed by D20, L59, and F159 contained episomal viral genomes at copy numbers of 17, 49, and 35%, respectively, of wild-type copy number (data not shown). Therefore, all of the mutated E2 proteins except F159 are defective in some aspect of E2 function. The activities of the mutated E2 proteins are summarized in Table 2.

DISCUSSION

The full-length BPV-1 E2 protein is a multifunctional protein that plays a central role in regulation of viral transcription and DNA replication. In this study, we identified a number of amino acid residues that are critical for one or more functions of the E2 transactivator. Three mutated E2 proteins, one of which contained a double mutation that occurred erroneously during cloning procedures, were either completely inactive or severely impaired in all of the E2 activities that were analyzed. The doubly mutated protein, G105R111, may have a disrupted conformation, as the amino acid change at position 105 is not conservative. The two singly mutated E2 proteins contain either a proline-to-glycine substitution at amino acid 106 or a glycine-to-alanine substitution at residue 156. Because proline and glycine residues often play significant roles in protein sec-

TABLE 2. Summary of activities of mutated E2 proteins

E2 protein	Activity[a]			
	Trans-activation	Repli-cation	Cooperative origin binding with E1	Trans-formation
Active in all assays				
Wild type	+	+	+	+
L59	+	+	+	+/−
F159	+	+	+	+
Defective in:				
All assays				
G106	−	−	−	−
A156	−	−	−	−
G105R111	−	−	−	−
Transformation				
N12	+	+	+	−
K37	+	+	+	−
G60	+	+	+	−
L73	+	+	+	−
F92	+	+	+	−
R111	+	+	+	−
E122	+	+	+	−
Y173	+	+	+	−
Transactivation, replication, and transformation				
F33	−	−	+	−
D39	−	−	+	−
R111	−	−	+	−
Efficient cooperative origin binding				
D20	+	+	−	+
Efficient cooperative origin binding and transformation				
L188	+	+	+/−	−

[a] +, efficient activity; −, low or no activity; +/−, intermediate activity.

ondary structure, it seems plausible that the structure of E2 proteins containing either G-106 or A-156 may have been globally disrupted. Moreover, proline residue 106 lies within the nuclear localization signal of the E2 transactivator, and a recent study has shown that the G106 E2 protein is cytoplasmic (30). This would explain why the G106 protein was unable to function in the in vivo assays but not why it was unable to cooperatively bind the replication origin with the E1 protein in vitro.

Three mutated E2 proteins were found to be completely defective for transcriptional activation, and their replication functions were severely impaired. These proteins contain either a tryptophan-to-phenylalanine change at residue 33, a glutamic-to-aspartic acid change at position 39, or substitution of arginine for lysine at position 111. Such changes are probably less likely to disrupt conformation of the E2 protein, and it is possible that these residues are crucial for interaction with cellular transcription and replication factors. All three proteins are able to efficiently interact and cooperatively bind with the E1 protein to the replication origin, which also suggests that structural integrity has not been impaired. Previous reports have suggested that two regions at the amino-terminal end of the transactivation domain that are predicted by computer analysis to form acidic amphipathic helices may be important for transcriptional activation (8, 11, 15, 22). Early studies on acidic transcriptional activators suggested that an amphipathic helix with negative charges on one face may be important for activation function (33). However, more recent studies indicate that activation domains may be relatively unstructured and that aromatic and hydrophobic residues may be critical for the functions of these regions (reviewed in reference 9). The E2 N-terminal domain differs from most transactivation regions in that the structural integrity of this domain appears to be tightly constrained; any deletion, and as described above even some point mutations, inactivate all known functions of the 200-amino-acid domain. This structural constraint may be due to the multifunctional nature of the E2 protein. While the sequence similarity of the first 90 amino acids of the N-terminal domain is only about 25 to 60% conserved among various papillomavirus E2 proteins, all of the proteins examined are predicted to form two alpha helices in the first 90 amino acids. Two of the point mutations that inactivate the E2 transactivation function (F-33 and D-39) are located in the first of these sequences and, if this region did in fact form an alpha helix, would be predicted to lie in close proximity on the same face of the helix. The third substitution to inactivate the transactivation function of E2 replaces a lysine with an arginine residue at position 111. It is not possible to predict whether this would be in a similar region to that of W-33 and E-39 in the tertiary protein structure; however, this residue is located in a region of the E2 transactivator that has recently been identified as a nuclear localization signal. Residues K-111 and K-112 lie within this signal, but mutation to arginine does not disrupt nuclear transport (reference 30 and unpublished data). The mutated E2 proteins that are defective for transactivation and replication functions should prove invaluable for analyzing the functional significance of protein-protein interactions.

It was not possible to completely separate the transcriptional activation and replication properties of the N-terminal domain with the mutants described in this study. However, E2 proteins F33 and R111 were completely defective for transcription but could support DNA replication at very low levels. This may be because the E2 protein seems to have several auxiliary roles in replication (cooperative binding with the E1 protein, disruption of chromatin, and interaction with replication factors), and the point mutations may have inactivated only one or two of these functions. For example, all three mutated proteins are still able to bind cooperatively to the origin with the E1 protein. It is also possible that interaction with the E1 protein at the replication origin can, at least partially, restore some E2 activity. Conversely, the E2 L73 protein supports replication at wild-type levels but is quite reduced in transcriptional activation. These findings suggest that the transactivation and replication properties of the E2 proteins probably result from, at least partially, overlapping functions.

With the exception of the three proteins for which the protein conformation may have been disrupted, only two mutated E2 proteins (D20 and L188) were unable to efficiently enhance binding of E1 to the replication origin. However, both proteins were able to support viral DNA replication in vivo. This finding implies that E1-E2 cooperative binding is not crucial for DNA replication, at least in a transient assay. As described above, this could be because the E2 protein seems to have several auxiliary roles in replication (cooperative binding with the E1 protein, disruption of chromatin structure, and interaction with replication factors). E2 proteins defective in only one function may still be able to support replication to some degree. Overexpression of the E1 and E2 proteins may also compensate for the reduction in E1 and E2 cooperative binding. The replication properties of viral genomes containing these E2 point mutations are currently being determined. Because the E1 protein is able to repress E2-mediated transactivation from the viral P_{89} promoter (27), presumably by forming an E1-E2 complex at the replication origin, studies are in progress to determine whether the transactivation properties of the E2 D20 protein can be repressed by the E1 protein.

A number of the mutated E2 proteins had no dramatic phenotype in the replication, transactivation, or E1 interaction assays, yet the mutated residues are invariant among papillomavirus E2 proteins. In addition, almost all of the viral genomes containing the E2 mutations were defective in the ability to induce foci in C127 cells, indicating that they were defective in some aspect of E2 function. The E2 proteins in these viruses may be defective in regulation of specific viral promoters, which could result in inefficient expression of other viral gene products, including the E2 proteins. Further analyses of these proteins may discover more subtle or specific defects in regulation of viral transcription and replication and may reveal additional functions of the E2 protein in the viral life cycle.

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