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Genetic Analysis of the Activation Domain of Bovine Papillomavirus Protein E2: Its Role in Transcription and Replication

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The bovine papillomavirus protein E2 serves dual functions in viral transcription and in the initiation of viral replication. As a transcription factor, E2 can cooperatively interact with cellular proteins such as SP1 and stimulate transcription of distal promoters. In replication, E2 and the helicase E1 are the only viral proteins required for accurate replication of templates containing the viral origin. The amino terminus of E2 is a functionally separable domain critical for activation of both replication and transcription; its primary sequence is conserved between many strains of papillomavirus. We targeted conserved residues spanning the activation domain and constructed a series of 30 amino acid substitution mutants. These mutant E2 genes were analyzed for the ability to activate DNA replication and gene expression in cells. The majority of the substitutions affected the ability of E2 to support both viral replication and transcriptional activation, revealing substantial overlap of the functional determinants for these two processes. Replication and transcription activities are genetically separable, however, as mutations at amino acids 73 and 74 retained replication function but failed to activate transcription. Additionally, a mutation at position 39 substantially reduced replication activity but left transcriptional activation intact. Interestingly, over two-thirds of the mutations analyzed reduced function and protein accumulation, many in a temperature-dependent manner. The correspondence between the replication and transcription phenotypes of mutations spanning the activation domain may indicate that the entire region is folded into a single domain required for both functions.

Proteins that recognize specific DNA sequences generally determine when and to what extent activities such as DNA transcription, replication, recombination, or repair occur. For instance, many factors originally identified as transcriptional regulators have since been shown to play an important role in initiation of DNA replication (11, 19). The findings that a given protein may be a participant in multiple areas of DNA metabolism raise many interesting questions from both regulatory and evolutionary perspectives. "Evolution by descent" provides one possible explanation for this multiplicity of function: that active protein motifs could be reconfigured to function in different contexts. As replication and transcription are mechanistically related biochemical processes, it seems reasonable to expect that a domain functional in one system could find a novel use in the other pathway. For example, both processes require assembly of multicomponent DNA-protein complexes at an initiation site where certain domains may be used for protein-protein interactions with multiple partners.

Alternatively, the development of factors which regulate more than one process could be driven by functional necessity rather than evolutionary parsimony; DNA-binding proteins could thus act in multiple pathways to coordinate the regulation of different aspects of DNA metabolism. Such a function-driven model would allow for a wide variety of structural organizations, and one might find that the regulatory protein is a pastiche of different regions, each critical for a separate activity. In contrast, evolution by descent would predict that a protein will contain one or more discrete domains which serve similar purposes and have similar structures in their varied roles. Knowledge of the structural organization of such regulators may help distinguish between these theories.

A different possibility is that such a domain functions through identical contacts with another protein common to these multiple processes. It is clear, for instance, that alteration of chromatin structure to allow access to initiation sites may be important in the control of many aspects of DNA metabolism, and other regulatory systems or general factors are also shared by enzymatic processes. Such sharing of proteins between different branches of DNA metabolism could allow higher-level coordination. For example, the general transcription factor TFIIH, important for initiation at RNA polymerase II-dependent promoters, contains polypeptides whose activities are required for both transcription and DNA repair (15). To settle the issue of how and whether these common factors help to coordinate transcriptionally coupled DNA repair, one must understand how they function in both processes.

This question of how a region of a protein can regulate multiple functions is difficult to resolve without detailed knowledge of the structure and function of such a protein together with its interacting partners. For example, the cellular transcriptional transactivator NF-1/CTF plays a key role in adenovirus DNA replication and only its DNA-binding–dimerization domain is needed for DNA replication. This region of NF-1 is necessary and sufficient for interaction with the adenovirus DNA polymerase (4), and mutagenesis of this region has revealed amino acids capable of separating the DNA-binding, dimerization, and polymerase interaction properties of this domain, all of which are necessary for the replication function of NF-1 (1). A similarly detailed analysis of the transcriptional requirements is lacking, leaving open the possibility that trans-

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activation also requires more than DNA binding and dimerization from this domain.

The bovine papillomavirus (BPV) transactivator E2 provides a model for studying the means by which a protein can regulate multiple processes and the relationship between the functions of such a factor and its structure. E2 acts as an essential transcription and replication factor in the viral life cycle. E2 plays a direct role in the initiation of replication, interacting with the viral helicase E1 to cooperatively assist binding at the origin of replication (26, 33, 34, 36, 43, 46, 47). As E1 has been shown to interact with the large subunit of cellular DNA polymerase α (28), it is possible that E2 affects replication largely by recruiting E1 to the origin, where E1 interacts with components of the cell's replication machinery. This cooperative binding is dependent upon the amino-terminal region of E2, and without these residues the shortened forms act as repressors of both replication and transcription (2, 5, 7, 20, 24, 26). E2's role as an auxiliary protein in the initiation of replication may be multifaceted as E2 can counteract the repressive effects of chromatin on BPV replication (22), and it has been shown to interact directly with cellular replication protein A, a single-stranded DNA-binding protein (21). E2's ability to bind to proteins is also important for its role as a transcriptional activator. For example, a cooperative interaction between E2 and the cellular transcription factor SP1 is necessary for efficient transactivation of both viral and synthetic promoters (23) and E2 can also cooperate with a variety of different transcription factors, including the TATA-binding protein (14, 30, 42).

The E2 protein has a modular structure characteristic of many site-specific transcriptional activators, consisting of an amino-terminal activation region separated from a carboxyl-terminal DNA-binding–dimerization domain by a proline rich, protease-sensitive "hinge" region (12, 16, 17, 25). Deletion analysis of E2 has determined that the activation region is necessary for both replication and transcription in transient transfection assays, while deletions in the hinge affect replication and not transcription of complex promoters (45). Interestingly, the hinge has been shown to be sufficient for interaction with the general transcription factor TATA-binding protein in an interaction which is necessary for activation of a subset of simple promoters that are sensitive to TATA-binding protein levels (37). These previous studies established that the transactivation region is necessary for both replication and transcription by E2; however, the question of whether distinct functional or structural domains within this region are separable remained. Our study was undertaken to address this question, and since completion of our work, other findings on the effects of conservative amino acid substitutions of absolutely conserved residues in the BPV type 1 E2 amino terminus have been reported (3). That study found many residues to be equally important for the transcriptional activation and replication functions of E2 and a single change that allowed separation of these activities.

We examined 30 single amino acid substitutions made throughout this region of E2, by targeting both residues that are absolutely conserved and many that are highly homologous between the different papillomaviruses. The results of our analysis changing most residues to alanine also demonstrate that the functional determinants of replication and transcription overlap significantly. Individual residues are capable of separating the replication and transcriptional functions of E2, although we did not find discrete regions of the activation domain that separate function. In addition to two changes at residue 73 that retain wild-type replication activity but are completely defective in transcriptional activation assays, we found a change at position 39 that exhibits the opposite separation of function. In contrast to many other transcriptional activation domains, this region is surprisingly susceptible to single amino acid mutations. Almost two-thirds of the mutations significantly affect E2's function, many in a temperature-sensitive manner.

MATERIALS AND METHODS

Plasmids. The BPV E1 and E2 proteins were expressed from the cytomegalovirus promoter in the plasmid backbone pCG(ATG$^-$) (39). E1 expression vector pCGEag has been described previously (44). The BPV sequences from nucleotides 2608 to 4451 containing the E2 open reading frame were cloned between the polylinker *Bam*HI site and the *Bst*XI site in the rabbit β-globin polyadenylation sequence of pCG(ATG$^-$). For ease of manipulation, the E2 open reading frame contained two silent restriction enzyme sites engineered by oligonucleotide-directed mutagenesis: a *Hin*dIII site at 2838 and an *Xho*I site at 3210; a *Bam*HI site was also added immediately upstream of the initial methionine. Oligonucleotide-directed mutagenesis was used in this background to create single amino acid substitutions as indicated in the figure legends. A single mutation, DG24, was generated by random chemical mutagenesis and screened in *Saccharomyces cerevisiae* for a transactivation phenotype as described by Myers et al. (27). pKSO(46) and P2CAT(38) have been previously described.

Transfections. Cell culture and transfections of simian kidney epithelial cell line CV-1 were performed essentially as described previously (31, 44) but with the following alterations. Equal amounts of the different E2 expression plasmids were linearized with *Asp*700, and the E1 construct was hydrolyzed with *Xho*I. After extraction and precipitation, plasmids were resuspended in 0.1× TE, pH 8, and quantitated by UV spectroscopy to normalize concentrations. For transient replication assays, samples were introduced into the cells by electroporation with different amounts of the E2 expression construct, as indicated in the figure legends; 2.5 μg of the E1-expressing vector; 1 μg of supercoiled pKSO; and 20 μg of carrier DNA. Transient transfections for chloramphenicol acetyltransferase reporter assays were performed with various amounts of E2, 2.5 μg of P2CAT, and 20 μg of carrier DNA. After electroporation, the cells were plated to four 35-mm-diameter dishes and assays for activity and protein accumulation were performed in parallel at 48 h posttransfection.

Transcription and replication activities. Replication samples were harvested by the Hirt lysis method with modifications as previously described (40). Samples were then digested with *Xba*I or *Bam*HI to linearize the DNA and with *Dpn*I to cut unreplicated DNA into small fragments. One-third to one-half of each sample was analyzed by electrophoresis, transferred to Nytran membranes (Schleicher & Schuell), and hybridized to random-primer-labeled pKSO by the method of Church and Gilbert (8). The blots were analyzed by autoradiography and quantitated with a PhosphorImager (Molecular Dynamics). To test the relative transcriptional activity of the E2 mutants, samples were harvested in 1 ml of 25 mM Bicine (pH 7.8)–0.5% Tween 20–20 mM EDTA, subjected to one freeze-thaw step to ensure complete lysis, and centrifuged at 14,000 × *g* for 5 min. The supernatant fractions were normalized to total protein levels by the bicinchoninic acid assay (Pierce) and assayed for chloramphenicol acetyltransferase activity as described previously (13). For detection of E2 protein, cells from one-fourth of a transfection experiment were lysed with 2× Laemmli buffer. Samples were boiled, fractionated by electrophoresis, and detected after transfer to Immobilon (Millipore) by using anti-E2 monoclonal antibody B202(26) and protein A-conjugated horseradish peroxidase. Bands were visualized by electrochemiluminescence.

RESULTS

To identify essential regions of the amino terminus of E2, we compared the amino acid sequences of E2 proteins from 10 strains of papillomavirus. We designed a set of 30 single amino acid substitutions that span this region (Fig. 1), changing residues conserved for charge or shape, as well as amino acids that are absolutely conserved, to distribute mutations across the activation region. The first 200 amino acids of E2 showed approximately 30% homology in this comparison. Many of the functions of this domain are likely conserved, as E2 proteins from the papillomaviruses of different species can complement each other in critical functions, such as replication (6, 9, 28, 47) and transcription (42). The degree of sequence and functional similarity between the papillomaviruses indicates that a mutational analysis of the conserved amino acids would likely target residues important for function.

Titration of E2 expression vector into transient replication and transcription assays. As it was not initially clear whether an equivalent amount of E2 protein is needed for function in

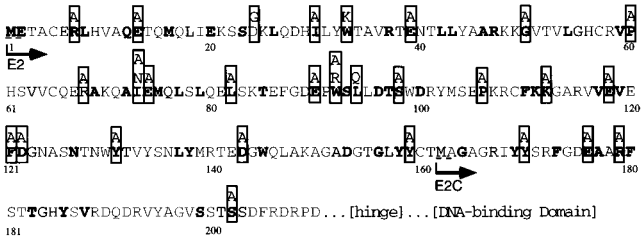

FIG. 1. Schematic diagram of the activation domain of BPV E2 showing the mutations analyzed in this study. The amino acids in boldface are identical in 9 or 10 papillomaviruses included in a comparison published previously (23) or are absolutely conserved for charge or shape (12). Residues in black are less highly conserved: at least 70% identical or 80% conserved. Amino acids targeted for mutation are boxed, and the changes made are indicated above the sequence within the box. Residues in gray are not highly conserved.

replication or transcription assays, we performed E2 titrations across a wide range of input expression vector levels in both assays. If less E2 is required to maximally stimulate replication than transcription, or vice versa, a mutation that simply reduces protein accumulation could appear to separate the two functions of E2. Our analyses indicate that both transcription and replication assays saturate at equivalent levels of the input wild-type expression vector. A representative titration is shown in Fig. 2A. The results of these titrations with parallel Western blots (immunoblots) (Fig. 2B) show that the functional assays were already saturated with E2 at levels at which we were able to detect the E2 protein in transfected cells. While the activities of E2 reached a plateau with very small amounts of the expression vector transfected into the cells, E2 protein accumulation continued to increase. This occurred even past 10 μg of input DNA, at which level activation of both assays by E2 was substantially reduced, most likely by squelching (data not shown). Importantly, wild-type E2 expression and mutant E2 expression were similar under replication and transcription assay conditions and thus E1 does not affect levels of E2 accumulation (Fig. 2B). Because the replication and transcription assays were saturated by amounts of E2 protein too low to be detected by our monoclonal antibody, our attempts to directly and quantitatively correlate protein accumulation with activity in the linear range of the titration curve were unsuccessful. For this reason, assays comparing the mutants and the wild type were performed at an input E2 vector DNA level that was past saturation but not sufficient to squelch the functional assays.

As protein can be detected in our system only when E2 is in vast excess, we chose to compare mutant phenotypes by using a consistent amount of the input E2 expression vector. Although this amount of DNA results in different levels of accumulation of each mutant protein, this should not prevent direct comparison of the activities of these mutants. As is clear from the titration in Fig. 2, our detection limit for E2 is at input vector levels that are higher than necessary to maximally stimulate the functional assays.

Analysis of E2 mutants for replication function. When recombinant expression vectors for E1 and E2 are transfected into cultured cells, these two proteins are sufficient to direct replication of BPV origin DNA-containing plasmids (44). Transient transfections of cultured mammalian cells were used to compare the reporter replication levels achieved by wild-type E2 and the 30 mutant proteins when each was coexpressed with E1. A representative replication assay is shown in Fig. 3A. Under our assay conditions, an origin containing the palindromic E1-binding motifs flanked by two E2-binding sites was replicated efficiently in an E2-dependent manner (data not shown). The majority of the substitutions in the activation domain reduced E2 function. Data from this experiment and others are quantitatively presented in Fig. 3B. The range of data for each mutant is indicated by vertical bars above and below columns which show the average of three to six independent electroporation experiments.

Mutations at amino acids 30, 39, 60, 68, 74, 90, 94, 122, and 179 reduced the replication signal substantially. The range of data for these changes never exceeded 50% of wild-type levels, and the average reduction varied from approximately 2- to 10-fold. Additionally, changes at 33, 82, 92, 106, 112, 121, 131,

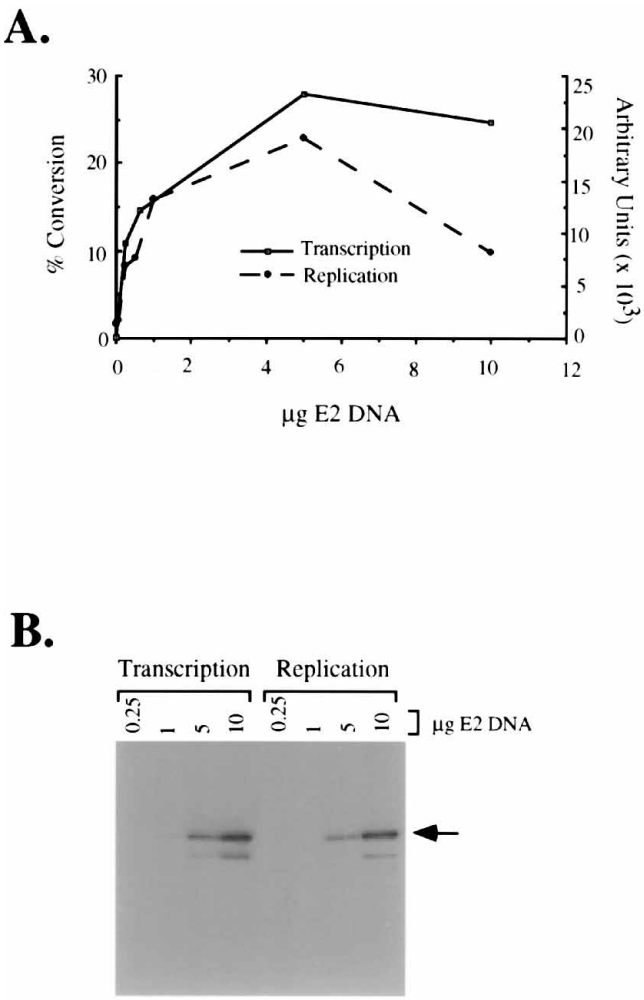

FIG. 2. Effect of input E2 expression vector levels on transcription and replication assays. (A) Replication and transcription saturate at similar E2 levels. Titration of the wild-type E2 expression vector under transient replication conditions is indicated by the dashed line, and transient transcriptional activation titration is indicated by the solid line. The amount of the E2 expression vector in each sample is indicated below the *x* axis. Transcriptional activity is expressed as a percentage of the total amount of chloramphenicol acetylated by transfection extracts in a standard chloramphenicol acetyltransferase assay; units are on the left vertical axis. Replication is expressed in arbitrary units on the right axis. (B) Western analysis of E2 protein with increasing amounts of input expression vector. In parallel with replication and transcription assays, the amount of E2 accumulating in the cells was analyzed at the input E2 expression vector level indicated above each lane. Accumulation under transcription conditions is on the left, and replication conditions (in the presence of E1) is on the right. Full-length E2 is indicated by the arrow. Below full-length E2 on this blot is a faster-migrating species that is a major proteolysis product of E2 that sometimes appears in these cell extracts.

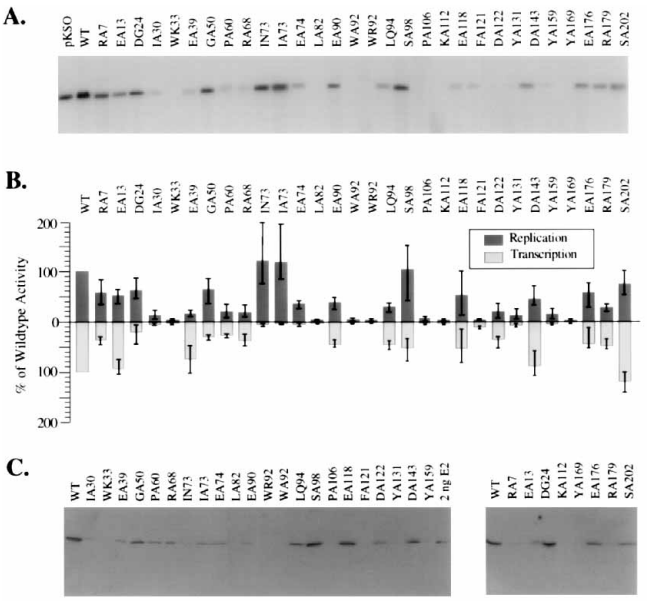

FIG. 3. Functional analysis of the mutant E2 proteins in transient transfections. (A) Representative transient replication assay of BPV origin-containing plasmid pKSO. As a marker, 50 pg of linearized pKSO was run in the far left lane. The mutation analyzed is indicated above each lane and abbreviated as follows: RA7 represents an arginine-to-alanine change at position 7. The amount of replication of pKSO supported by wild-type E2 and wild-type E1 is indicated by the designation WT above the second lane from the left. Each sample was electroporated with 5 μg of the indicated E2 expression plasmid and the E1 vector as described in Materials and Methods. (B) Summary of the quantitation of replication and transcriptional data. Above the axis, three to five independent replication assays are averaged and expressed as a percentage of wild-type (WT) activity. Below the axis, transcriptional activation of the reporter P2CAT by the mutants is compared with the wild-type protein's activity. Three to six independent transcriptional activation assays are averaged. For both assays, the range of data is indicated by vertical bars. (C) Immunodetection of E2 proteins. In parallel to the above assays, cells were also analyzed for accumulation of the mutant E2 proteins. On the left is accumulation of mutant E2 proteins done in parallel with a representative transcription assay. On the right are eight mutants analyzed for accumulation in parallel with a representative replication assay. All assays were performed with 5 μg of the E2 expression vector.

159, or 169 consistently reduced replication activity 5- to 50-fold; however, these mutant proteins are difficult to detect in parallel Western analyses (Fig. 3C), and it is therefore problematic to interpret their functional defects. The high percentage of the single amino acid substitutions resulting in a significant effect on the replication activity of E2 validates the assumption that conserved residues are critical for function in these transient assays.

Transcriptional phenotypes of activation domain mutants. The activity of the 30 E2 mutants was tested in transient transcription assays as well. In this assay, wild-type E2 protein reproducibly stimulated transcription of a reporter containing the entire BPV upstream regulatory region, P2CAT, by approximately 50-fold. A summary of the numerous transfection experiments is represented below the horizontal axis in Fig. 3B. The results of these transcription assays mirror the replication data in many ways. The majority of single amino acid substitutions affect E2's function, and changes across this region severely reduced transactivation in these transient experiments. As a brief overview, IA30, IA73, IN73, and EA74 dropped transactivation approximately 10- to 50-fold. The nine mutant proteins listed above that have substantial replication defects but are not easily visible in Fig. 3C also reduced transactivation by E2 more than 10-fold. This set of mutants substantially overlaps those with severe replication defects. Additionally, changes at amino acids 7, 24, 50, 60, 68, 90, 94, 122, 176, and 179 consistently reduced activity two- to fivefold. A panel of mutants were also compared by using synthetic E2-responsive promoter pSP1E2, containing only E2- and SP1-binding sites and the TFIID-binding site of the herpesvirus thymidine kinase promoter (23). The results were qualitatively the same (data not shown).

Accumulation effects of single amino acid substitutions. Nearly all of the 30 mutants tested did not accumulate to wild-type levels (Fig. 3C). Our high E2 detection threshold (Fig. 2B) implies that in all cases in which the E2 protein was detected, sufficient E2 was present for maximal activity, if the protein was not otherwise affected by the mutation. Indicative of this point, while both IA73 and IN73 accumulated to significantly lower protein levels than wild-type E2 in these assays, each was as active as wild-type E2 in transient replication assays. We also titrated the input expression vector levels for approximately half of the mutants over a wide concentration range (e.g., well beyond the E2 vector levels that would saturate the wild type) and performed transcription and accumulation assays in parallel to demonstrate that the functional defects were not simply due to reduced amounts of E2 (data not shown). As mentioned above, while mutations at nine residues which have functional defects were not detected in the Western analysis shown in Fig. 3C, there were nine additional mutants with reduced replication activity and 14 proteins defective as transcriptional activators whose accumulation was readily observed. We do not understand why many of the mutants affect E2 accumulation. E2 is capable of stimulating transcription from the cytomegalovirus promoter used to direct expression of E2, despite the absence of known E2-binding sites in this promoter (29). The ability of E2 to activate promoters lacking E2-binding sites at high vector levels has been previously observed (16, 18, 23), although the magnitude of this effect in published reports varies greatly. While E2's ability to stimulate its own expression in these transient assays may be partly responsible for the changes in protein accumulation, it is likely that additional effects on protein synthesis or stability are involved. This latter point is indicated by the fact that many proteins which lack transcriptional activity accumulated to a variety of levels. For instance, IA73 protein levels were higher than WK33 levels, although both failed to activate transcription.

Separation of transcription and replication functions. Mutations at BPV type 1 E2 residues 39, 73, and 74 differentially affect transcription and replication and demonstrated that these two activities of the protein are genetically unlinked (Fig. 4). Over a range of E2 vector levels, the EA39 mutant was equivalent to the wild type in transcriptional activation levels, yet in side-by-side assays at these levels it was defective for replication. Reciprocally, mutation IA73 had very little transcriptional activity yet retained wild-type replication activity in transient assays at all E2 vector input levels (Fig. 4). While the data in Fig. 2B show that levels of wild-type E2 were not affected by E1, we were concerned that mutant E2 protein levels may so be affected, leading to an apparent separation of function that was due simply to differential E2 protein stability under replication conditions. Western analysis done in parallel with these titrations demonstrated that the level of expression of each mutant was consistent in replication and transcription assays, indicating that neither protein is simply differentially stable in the presence or absence of E1 or some other factor inherent to the assays (data not shown). IN73 showed the same phenotype as IA73, and EA74 showed a similar, although less dramatic, separation of function (Fig. 3B). Because these pro-

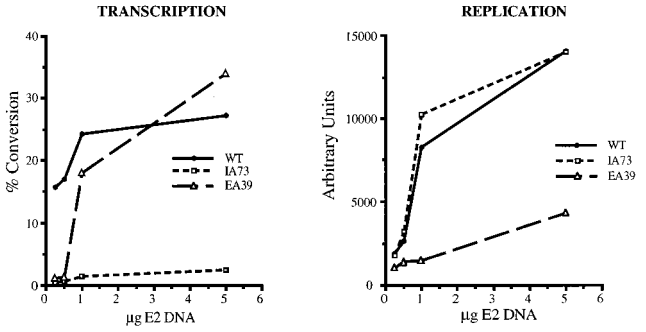

FIG. 4. Mutant phenotypes for E2 alleles which are replication positive and transcription negative or replication negative and transcription positive. Transcriptional activity and replication activity for EA39 and IA73 were compared with those of the wild-type (WT) E2 protein while E2 DNA levels were varied from 0.25 to 5 μg.

teins can function to activate BPV replication, it seems likely that this change affects a region or residue critical only for transcriptional activation.

Temperature sensitivity of the E2 mutants. Amino acid substitution mutants can be temperature sensitive for activity, particularly if the mutant residues are critical for stabilizing local secondary structural motifs or are present at surfaces involved in protein-protein interactions. The previous report of a temperature-sensitive mutant that contained an insertion in this region of E2 (10) and the accumulation defects discussed above led us to investigate whether some of the mutant phenotypes in this study could be temperature dependent. Many of the mutants which showed low accumulation did prove to be temperature sensitive for both transcription and replication activities, and defects previously detected at 37°C were rescued at 33.5°C. IA30, YA131, and YA159 showed a drop in both replication and transcriptional activities between 10- and 30-fold when assayed at 39.5°C compared with activities measured at 33.5°C (Fig. 5A). Additionally, while IN73 failed to activate transcription at either the permissive or the restrictive temperature, IA73 regained transcriptional activity at 33.5°C (Fig. 5A). Mutant IA73, then, separates functions at the nonpermissive temperature but functions in both assays under permissive conditions.

Temperature-sensitive mutants that affect protein folding can have a shortened half-life at restrictive temperatures, as the mutation destabilizes a region of the protein and it becomes accessible to proteolysis. We thus investigated whether two mutants, one representing a strong temperature-sensitive phenotype and another with weak temperature sensitivity, display differential accumulation at different assay temperatures. Figure 5B shows that, indeed, for mutants IA30 and RA68, protein accumulation levels were affected by cell growth temperatures. We suspect that for these mutants, protein stability or possibly protein folding pathways are affected.

DISCUSSION

Mutagenesis of single amino acids throughout the activation domain of BPV E2 revealed a congruence of determinants necessary for replication and transcriptional activation by E2. We altered one residue roughly every 5 to 10 amino acids throughout the amino terminus of E2, and most of the changes significantly affected replication and transcription functions. We believe that this result is most simply interpreted to mean that the activation domain of E2 forms a single structure wherein many residues are important for both functions. Although many of the mutant proteins accumulated to reduced levels, all functional assays were done at protein levels that would saturate activity if the proteins were not functionally impaired. This conclusion is valid for 21 of the mutants whose accumulation was detectable in this study. We have shown that many changes that affect protein levels are also temperature sensitive, consistent with the model in which the residues that we mutated are important for the stability or synthesis of a single structure. This substantiates the designation of domain to the amino-terminal part of the protein.

It is important to test the subcellular localization of these point mutants, as three of the amino acid substitutions analyzed here, PA106, KA112, and EA118, fall near or within a recently characterized nuclear localization signal in E2's activation domain (35). It is possible that residues outside of this region are needed for nuclear transport, as the basic region alone is not sufficient to direct transport of a heterologous protein. Further study is necessary to determine whether incorrect localization is responsible for the failure of some of the point mutants to function. However, unless residues throughout the activation domain are shown to be needed for localization, this knowledge would be unlikely to affect our conclusions.

While most of the mutations that reduced one function affected the other equally, we isolated substitutions at three residues that differentially affected replication and transcription. Mutations at E2 positions 73 and 74 failed to activate transcription but were still capable of transactivating DNA replication. Additionally, we found that residue 39 is critical for replication but not for transcriptional activation by E2. As each of these residues is bordered by a substitution within 11 amino acids affecting both functions equally, this genetic analysis did not distinguish discrete functional subdomains as identified by primary sequence blocks important for one function and not the other. It seems possible that surfaces within the tertiary structure which are formed by long-range interactions may yet define replication- or transcription-specific structures. However, our genetic data are equally consistent with the notion that the E2 structural motifs utilized in replication may also be used in the protein's transcriptional mode. Specific amino acids within such a common surface might differ in importance for the interactions between E2 and its distinct partners in replication and transcription.

As anticipated by current models which posit that E2 enhances DNA replication, at least in part, by specific interactions with the viral E1 protein, the replication and transcrip-

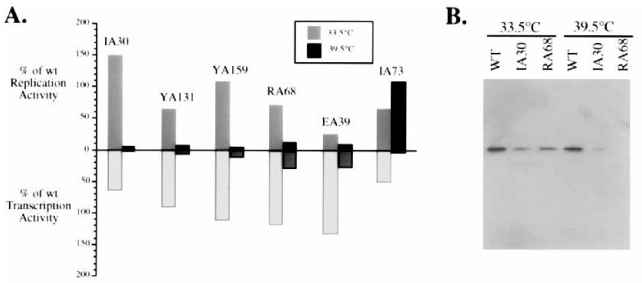

FIG. 5. Temperature-sensitive phenotypes of activation domain mutants. (A) Replication, above the axis, and transcription, below the axis, are expressed as a percentage of wild-type (wt) E2 activity. Gray bars represent the activity of E2 in cells grown at 33.5°C, and black bars represent the activity of cells grown at 39.5°C, in either case, after transfection with 5 μg of the indicated E2 mutant. (B) Western analysis of accumulation of mutants IA30 and RA68 under permissive and restrictive conditions. In parallel with the functional assays summarized in panel A, protein accumulation was assayed for two representative E2 mutants.

tion functions could be genetically separated. Since E1 is not required for E2's transcriptional effects, particular residues important for its interaction with E1 should be uncovered by such an analysis. For example, the glutamic acid residue at position 39 may play a critical role in stabilizing a surface important for interaction with E1, as suggested by recent studies with the human papillomavirus type 16 E1 and E2 proteins (32). Our data also demonstrate that the activation domain needs distinct determinants for transcription. Together, these mutations substantiate the model in which the activity of E2 as a replication protein is independent of its activity as a transcription factor. The congruence between the results of our study and those of Sakai et al. (32) is striking. In that work, a mutational analysis of the human papillomavirus type 16 E2 gene also revealed that the same two residues, E39 and I73, when mutated, isolated the replication and transcription functions of the protein. A greater understanding of the extent and significance of this separation of function within E2's activation domain awaits experiments that address the biological significance of a single protein providing two regulatory activities. Moreover, detailed information about the three-dimensional structure of this region of the protein will allow investigation of how many of the conserved and critical residues analyzed actually are exposed on the surface of the protein and which form the internal framework of the domain unlikely to participate in protein-protein interactions.

Our study confirms and extends the results of a recent analysis of conservative amino acid substitutions of 17 residues within the amino terminus of BPV type 1 E2 that are invariant among papillomavirus strains. Brokaw et al. (3) found a number of residues to be important for activation of transcription and replication, interaction with E1, and focus formation by the viral genome. They made five single amino acid substitutions that substantially reduced activation of both transcription and replication by E2, showing that this part of the protein is easily perturbed by small changes. Brokaw et al. showed that approximately 40% of their conservative changes reduced activation of transcription and replication, whereas in our study approximately 80% of the substitutions affected function. In contrast to the mutations analyzed in this report, Brokaw et al. did not find clear genetic separation of transcription and replication functions by mutations at residues 73 and 39. As their substitutions were conservative, it is important to compare the mutants from both studies side by side. The major apparent conflict between our reports concerns the extent to which E2 accumulation is affected by mutation. Here again the specific mutations may be relevant, but we suspect that procedural differences may be more important. While our work looked at accumulation in cells analyzed in parallel for protein function, Brokaw et al. used COS-7 cells for their accumulation data, a cell line different from the lines used for their functional comparisons. Additionally, they used significantly more of the expression plasmid to examine protein accumulation than they used for functional assays. The use of COS-7 cells allowed simian virus 40 amplification of their expression construct, further increasing their expected E2 accumulation. It is therefore possible that in their studies the cells were saturated for protein expression and accumulation, thus minimizing differences in accumulation that might be apparent at the levels of E2 used in functional assays performed with another cell line.

The work presented here extends the observation that E2 is highly perturbable by single amino acid changes, a result that contrasts with those of many previous genetic studies of transcriptional activation domains. Many transcriptional activators have multiple regions that can function independently as activation domains, and consequently single residue substitutions have either little or no effect upon the protein's function (41). Additionally, the activation domains of a number of transcription factors, including GAL4, GCN4, VP16, and p53, seem to consist of multiple, repeating subdomains which have additive effects on transcription. In many of these proteins, repeats of as few as eight amino acids function as well as the entire activation domain when linked to a DNA-binding–dimerization domain (41). Our mutagenesis indicates that E2 probably does not fit this paradigm of a transactivator.

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