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Transcriptional and Replicational Activation Functions in the Bovine Papillomavirus Type 1 E2 Protein Are Encoded by Different Structural Determinants

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A set of E2 proteins with mutations in the amino-terminal transactivation domain was made by a scheme called clustered charged-to-alanine scan. These mutant E2 proteins were tested for expression, stability, and compartmentalization in cells and for sequence-specific DNA binding, as well as in functional assays for transcriptional and replicational activation. We identified four groups of mutants. First, mutants K111A, K112A, and E176A were unable to activate replication and transcription because of oligomerization-induced retention of oligomers in the cytoplasm. Second, although fractions of the mutant proteins E74A and D143A/R172C existed in the oligomeric form, they were localized in the nucleus. Certain fractions of these proteins existed as a dimer able to form a specific complex and activate replication; however, these proteins were inactive in transcriptional activation. Third, mutants R37A and D122A were localized in the nucleus, existed in the dimeric form, supported replication efficiently, and were severely crippled in transcriptional activation. The fourth group of mutants did not differ considerably from the wild-type protein. The activation of transcription by the wild type as well as mutant E2 proteins was dependent on the concentration of input E2 expression vector DNA and had a bell-like shape. We suggest that the reduction of transcriptional activation at higher E2 concentrations, the self-squelching activity, is caused by oligomerization of the E2 transactivator and is one of the mechanisms for the regulation of E2 activity. Our results also show that transcriptional and replicational activation activities are encoded by different determinants in the E2 protein.

Transcriptional activators modulate the activity of the basal transcription machinery in eukaryotic cells, and they function at the assembly stage of the transcription initiation complex or stimulate the formation of the elongation complex (for reviews, see references 30 and 61). In addition, transcription factors are frequently involved in the modulation of DNA replication (for a review, see reference 12). Small DNA viruses, like papillomaviruses, provide a useful model system to study the functional and structural determinants of transcription factors involved in the regulation of transcription and replication. The 48-kDa full-length E2 protein of bovine papillomavirus type 1 (BPV1) was identified as a typical eukaryotic transcription activator, with all the properties of such proteins, including modular DNA binding and activation domains and the ability to activate heterologous promoters from DNA binding sites at a distance from the transcription start site. It has been demonstrated previously that E2 binding sites function synergistically in vivo and mediate E2-dependent induction of the expression of viral early genes of papillomaviruses (19, 21, 47, 51, 52, 60). That fact served initially as an explanation for the involvement of the E2 protein in the regulation of viral DNA replication as a transcriptional activator of the expression of viral early genes (13, 38). Later, however, it was shown that E2 protein directly participates in the replication process of viral DNA during each of the three stages of the viral life cycle. First, after the initial entry of the viral genome into the nucleus of the cell, papillomavirus DNA is amplified, i.e., papillomavirus DNA replicates faster than does cellular DNA (27). The E1 and E2 proteins are the only viral factors used for the initiation of DNA replication from the papillomavirus origin at this amplification stage of the viral life cycle (9, 40, 56, 57, 59). The minimal origin of replication has been identified as the essential *cis* element in this process and consists of three sufficient and necessary elements, an AT-rich region, E1 binding site, and E2 binding site (55). The second stage of the virus life cycle, the latent replication, is established when the optimal copy number of the viral genome is achieved. The establishment of stable extrachromosomal replication of the BPV1 genome requires at least six E2 binding sites in *cis* and E1 and E2 proteins in *trans*, in addition to the minimal origin (37). During the third stage, the vegetative stage of the papillomavirus life cycle, DNA amplification is initiated in terminally differentiated keratinocytes. It has been suggested that the E2 protein is directly involved in the regulation of this process (8). Therefore, the E2 protein is the master regulator of viral gene expression as well as all the stages of viral DNA replication.

Structural and mutational analyses of the E2 protein have revealed three distinct functional domains. The N-terminal part (residues 1 to 210) is an activation domain for transcription and replication. It is followed by the unstructured hinge region (residues 210 to 324) and the carboxy-terminal DNA binding-dimerization domain (residues 325 to 410) (15, 19, 31, 32). The structure of the carboxy-terminal DNA binding-dimerization domain has been solved by X-ray analysis and has revealed a novel dimeric DNA binding-dimerization motif (20). A similar organization of a DNA binding-dimerization domain has been determined for Epstein-Barr virus nuclear antigen 1 (5). However, very little, except for computer predictions, is known about the structural organizations and properties of the hinge region and the amino-terminal transactiva-

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tion domain of the E2 protein. The dimeric E2 protein binds an interrupted palindromic sequence, ACC(N_6)GGT; dimers are stable and highly resistant to urea denaturation; and a small 85-residue protease-resistant C-terminal core domain retains the ability to bind DNA (10, 14). E2 binding sites function synergistically in vivo because of cooperativity in the binding of E2 to clustered binding sites, which is mediated via the amino-terminal transactivation domain (35, 52). E2 is able to loop DNA when E2 binding sites are widely spaced on the template (22). The E2 protein binds multiple proteins, including cellular replication protein A, papillomavirus replication protein E1, cellular transcription activator Sp1, and basal transcription factors TATA-binding protein (TBP) and TFIIB (4, 25, 26, 28, 34, 39, 50). Sp1 and E1 are known to bind the N terminus of E2, while TBP and TFIIB interact with the C-terminal part of the E2 molecule. Very little, though, is known about the structural determinants of E2 involved in the direct interactions with these or other proteins. To identify structurally and functionally important determinants within the E2 protein, a number of deletion and in-frame insertion mutations have been made (13, 19, 58). The majority of these mutations abolished the replication and transcriptional activities of the E2 protein. These studies indicate that the E2 DNA binding-dimerization domain, in addition to the intact transactivation domain, is needed for this protein to function in replication and transcriptional activation. However, it was not determined which E2 mutations disrupt its activity directly and which inhibit E2 function indirectly by interfering with the DNA binding or stability of the E2 protein.

Here we characterize 15 different mutant E2 proteins with point mutations within the transactivation domain. These mutations were designed by a scheme called clustered charged-to-alanine scan (11). Analyses of these mutants showed that the structural determinants responsible for the transcriptional activation function in the E2 protein could genetically be separated from the structural determinants responsible for the activation of replication. In addition, our data indicate that oligomerization of the E2 protein mediated by the N-terminal transactivation domain could be one of the additional mechanisms regulating the activities of this protein in transcription and replication.

MATERIALS AND METHODS

Plasmids. E1 expression vector pCGEag, E2 expression vector pCGE2, and replication reporter pUCAlu have been described previously (56). Reporter plasmids pP2CAT and pSV3BS9CAT were kind gifts of Paul Szymanski. Plasmid pSV3BS9CAT contains three E2 high-affinity binding sites (binding site 9 [BS9]), three 21-bp GC-rich repeats, and an enhancerless simian virus 40 (SV40) early promoter (nucleotides 5172 to 103 from SV40) in front of the chloramphenicol acetyltransferase (CAT) gene. Plasmid pP2CAT contains BPV1 nucleotides 7476 to 94 (including the P2 promoter) in front of the CAT gene. Plasmid p53:E2 was generated by substituting the E2 transactivation domain for the p53 transactivation domain (amino acids 1 to 58) by using *Xba*I and *Sca*I restriction sites in pCGE2epi (55) (see Fig. 1A). Plasmid VP16:E2 has been described previously (25) and contains 80 C-terminal amino acids from VP16 fused to the C terminus of E2 (starting from amino acid 250) in the context of pCG.

To generate mutant E2 proteins, the *Xba*I-*Kpn*I fragment from pCGE2 was cloned into pUC19. E2 protein mutants were created by the PCR-based method of Mikaelian and Sergeant and then inserted back into pCGE2 at the *Xba*I-*Kpn*I sites (33). All mutants were verified by sequencing. The introduced mutations at the nucleic acid and protein levels are presented in Table 1.

Cells and transfections. Transient-replication assays were carried out as described by Ustav and Stenlund (56). CHO cells were electroporated with 100 ng of pUCAlu DNA, 500 ng of pCGEag, and 250 ng of wild-type or mutant pCGE2. Replication assays were quantitated with a PhosphorImager SI (Molecular Dynamics). For transcription assays, CHO cells were electroporated with 250 ng of the respective reporter and various amounts of pCGE2 or derivatives. Forty hours later, cells were harvested and lysed by freezing-thawing, and CAT assays were performed by the thin-layer chromatography method in the linear range (43). The acetylated form of chloramphenicol was quantitated with a liquid scintillation counter. Activities were normalized to the total amount of protein in the lysate, as determined by the Bradford assay (6). The values shown are the averages of at least three independent transfection experiments.

TABLE 1. Generated point mutations in the E2 coding sequence

Starting position	Codon		Change at the amino acid level	Name of mutant
	Wild type	Mutated[a]		
2641	CAA	**GC**A	Gln-12→Ala	Q12A
2644	GAA	G**C**A	Glu-13→Ala	E13A
2665	GAG	G**C**G	Glu-20→Ala	E20A
2716	AGA	**GC**A	Arg-37→Ala	R37A
2722	GAG	G**C**G	Glu-39→Ala	E39A
2746	AGG	**GC**G	Arg-47→Ala	R47A
2809	AGA	**GC**A	Arg-68→Ala	R68A
2827	GAA	G**C**A	Glu-74→Ala	E74A
2875	GAA	G**C**A	Glu-90→Ala	E90A
2938	AAG	**GC**G	Lys-111→Ala	K111A
2941	AAA	**GC**A	Lys-112→Ala	K112A
2971	GAT	G**CT**	Asp-122→Ala	D122A
3034	GAC	G**CC**	Asp-143→Ala	D143A
3133	GAG	G**C**G	Glu-176→Ala	E176A
3121	CGC	**T**GC	Arg-172→Cys	R172C

[a] Mutated nucleotides are in bold letters.

DNA binding assay. For the preparation of COS-7 extracts, cells transfected by electroporation with expression plasmids were removed from the semiconfluent 100-mm-diameter plates with a rubber policeman, washed, and lysed in 100 μl of lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.1 mM EDTA, 0.35% Nonidet P-40, 10 mM dithiothreitol, and protease inhibitors) on ice for 30 min. The cell debris was removed by centrifugation, glycerol was added to a final concentration of 20%, and the extracts were aliquoted and stored at −70°C. The amounts of E2 protein in cell extracts were determined by enzyme-linked immunosorbent assay (ELISA) using bacterially expressed E2 protein as a standard. Equal amounts of lysates containing E2 mutant proteins were used in DNA binding assays. For gel shifts, respective amounts of cell extracts were incubated in 10 μl of binding buffer (10 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 15% glycerol, 5 mg of bovine serum albumin [BSA] per ml, 1 μg of leupeptin per ml, 1 μg of aprotinin per ml) at room temperature for 15 min in the presence of 1 μg of sonicated salmon sperm DNA and 0.2 ng of end-labeled probe. The double-stranded high-affinity BS9 of BPV1 (5′-ACAAAGTACCGTTGCCGGTCGAA-3′) was used as a probe. Protein-DNA complexes were separated from unbound DNA on 6% PAAG (80:1) in 0.25× Tris-borate-EDTA. Gels were dried and exposed to X-ray film. Quantitative analysis was done with a PhosphorImager SI (Molecular Dynamics). For pronase digestion experiments, increasing amounts of pronase were added; the reaction mixtures were incubated for an additional 10 min at room temperature before or after DNA binding, loaded onto a polyacrylamide gel, and processed as described above.

Immunoblotting of E2. Comparisons of the expression levels and estimations of the intactness of E2 mutant proteins in COS-7 and CHO cells were done by Western blot (immunoblot) analysis using rabbit anti-E2 polyclonal antibody. Cells from a semiconfluent 60-mm-diameter dish were lysed 36 h after electroporation in 200 μl of Laemmli sample buffer and boiled for 10 min, and Western blotting was done as previously described (18). In addition, COS-7 cell extracts prepared for the DNA binding assay were also tested for the concentration of E2 protein by immunoblotting.

Glycerol density-gradient centrifugation. Extracts (100 μl) of COS-7 cells transfected with E2 or mutants were layered on the top of 5-ml linear glycerol gradients (10 to 30%; 10 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 100 mM NaCl, 1 mM dithiothreitol, and protease inhibitors). The samples were centrifuged in a Beckman SW55 rotor at 45,000 rpm for 15 h at 4°C. Marker proteins, alcohol dehydrogenase (150 kDa), and BSA (66 kDa) were run in parallel gradients. The gradient was dripped into 14 fractions, and the bottom of the tube was washed with 250 μl of buffer containing 10% glycerol. Collected fractions were subjected to quantitative ELISA analysis and sequence-specific gel shift assay. The final wash was done with 50 μl of Laemmli buffer in order to solubilize the precipitated protein, which was then subjected to sodium dodecyl sulfate (SDS)-PAAG and Western blot analysis with monoclonal antibody 1E2 (epitope, amino acids 180 to 190).

Immunofluorescence. For immunofluorescence analysis, COS-7 cells were transfected by electroporation with 150 ng of E2 expression vector pCGE2. After that, cells were allowed to adhere to coverslips, grown for 48 h, and fixed in cold (−20°C) acetone-methanol (1:1) for 10 min. Coverslips were washed three times with phosphate-buffered saline (PBS), and primary antibody was added at a concentration of 10 ng/μl (diluted in PBS containing 1 μg of BSA per μl). Coverslips were incubated for 1 h at room temperature and washed three times with PBS, and fluorescein isothiocyanate-conjugated goat anti-mouse secondary

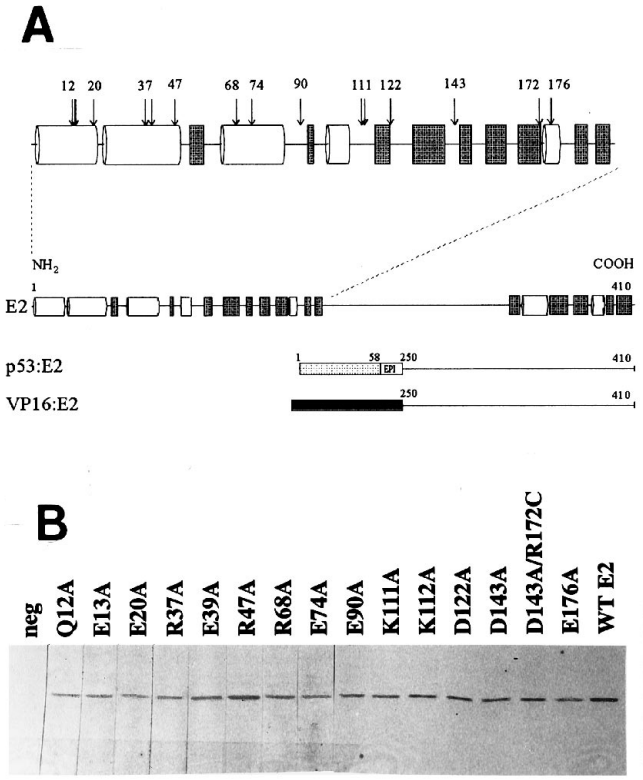

FIG. 1. (A) Schematic representations of designed E2 point mutations and chimeric E2 proteins. The prediction of the secondary structure (cylinder, alpha-helix; shaded box, β-sheet) was made by the method of Rost and Sander for the N-terminal part of the E2 molecule (41). The structure of the C-terminal part of the E2 protein is presented according to the X-ray structure (20). The locations of mutations are indicated by arrows. (B) Immunoblot analysis of the expression of E2 proteins in CHO cells. Cell lysates were prepared from cells transfected with a wild-type (WT) or mutant E2 expression plasmid. Thirty microliters of cell lysate was loaded in each lane. The negative (neg) control lysate was prepared from cells electroporated with carrier DNA only. The mutant used is indicated above each lane.

antibody (Sigma) was added at the concentration recommended by the manufacturer. After 1 h, coverslips were washed three times with PBS and mounted on glass slides in 50% glycerol. Cells were examined on a Olympus Vanox-S AH2 microscope. Monoclonal antibody 3F12 (epitope, amino acids 199 to 207) was used as the primary antibody.

RESULTS

Expression of mutant proteins. To identify the structural and functional determinants of the E2 protein involved in the activation of transcription and/or replication functions, a set of mutant E2 proteins was constructed. Single conserved charged residues in the amino-terminal transactivation domain were replaced with alanine. The introduced point mutations are described in Fig. 1A on the putative secondary structure of the E2 protein and summarized in Table 1 (41). We used two different cell lines (CHO and COS-7) to test the stabilities and expression levels of mutant proteins in vivo. The expression vector was delivered into cells by a standard electroporation procedure (56), cells were lysed, and the expression level and stability of each mutant protein were tested by ELISA and Western blot analysis. All mutant E2 proteins were expressed at similar levels compared with the wild-type E2 protein in CHO (Fig. 1B) and COS-7 (data not shown) cells. No degradation of mutant proteins in the cell lysate was observed. These data indicated that the constructed mutant proteins were stable and expressed at approximately the same level.

The activities of mutants in transient-replication assays. We used two different origin-containing plasmids (pUCAlu and pP2CAT) in transient-replication assays with CHO cells to study the abilities of E2 mutants to activate replication. The expression plasmid for mutant E2 proteins was cotransfected with the origin plasmid and E1 expression vector pCGEag into CHO cells by electroporation, and episomal DNA was extracted by alkaline lysis, purified, digested with *Dpn*I and linearizing enzyme, and analyzed by Southern blotting as described earlier (56) (Fig. 2). All mutant E2 proteins, except for K111A, K112A, and E176A (Fig. 2, lanes 21 to 24, 31, and 32), supported the replication of origin-containing plasmid pUCAlu in CHO cells in the transient-replication assay. Similar results were obtained with origin-containing plasmid pP2CAT (data not shown). Eight independent replication assays were quantitated and normalized to wild-type E2 protein activities (see Fig. 4B). Two mutants, R37A and E90A, seem to be up-mutations and activate the replication of the BPV1 origin 1.6 and 2.2 times better, respectively, than does the wild-type protein.

The activities of E2 mutants in transcriptional activation of promoters. It has been shown that different promoters can be activated to different extents by the BPV1 E2 protein, depending on the promoter content (17, 54). The activities of mutants were tested in CAT assays using two different E2-dependent reporter plasmids. Plasmid pP2CAT contains the BPV1 URR and the native P2 promoter, including the transcription initiation site (nucleotide 89). Reporter plasmid pSV3BS9CAT contains an SV40 early promoter in which the 72-bp enhancer is replaced by three E2 binding sites (BS9) in front of the basal promoter driving the CAT coding sequence (Fig. 3A). The reporter plasmid either alone or with increasing amounts of the E2 expression vector was transfected by electroporation into CHO cells. Forty hours later, the extracts were prepared for CAT assays (43). The CAT activities in the extracts of cells transfected with mutant E2 proteins were normalized to the basal activity without added E2 expression plasmid and to the total amount of protein. We tested the transactivation function of all mutants at various concentrations of transfected expression vectors (0.0004 to 3 μg of DNA per 7×10^6 cells). We observed that the level of activation of promoters by wild-type E2 and E2 mutants was dependent on the amount of the input DNA of the E2 expression vector (Fig. 3B to E). The extracts of cells transfected with expression constructs for E2 mutants E74A, K111A, K112A, E176A, and D143A/R172C showed

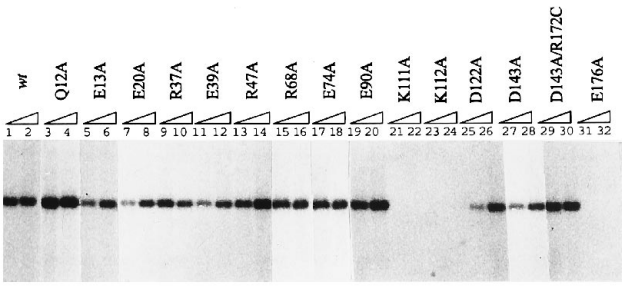

FIG. 2. The abilities of mutant E2 proteins to activate the replication of the BPV1 origin in CHO cells. Cells were electroporated with 100 ng of reporter plasmid pUCAlu, 500 ng of pCGEag, and 250 ng of pCGE2, which expresses wild-type (*wt*) E2 or derivatives. Cells were harvested either 36 or 48 h (◿) after electroporation. Episomal DNA was digested with *Dpn*I and linearizing enzyme *Hin*dIII and analyzed by Southern blotting.

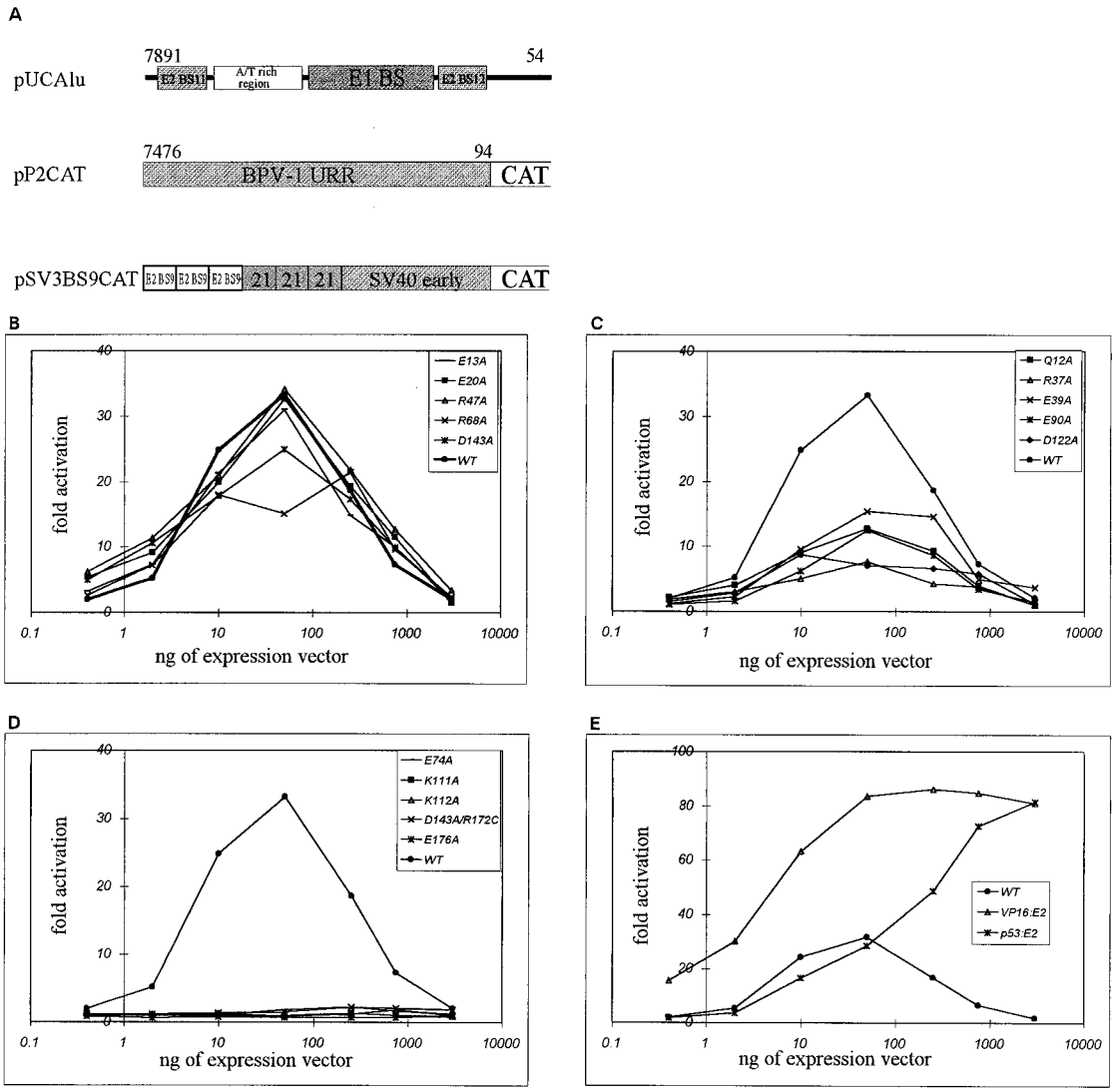

FIG. 3. (A) Structures of the reporter plasmids used in transcription and replication activation assays. The numbers indicate positions in the BPV1 URR sequences. (B to E) Transcription activation assays with E2 protein mutants. CHO cells were transfected with increasing amounts of the expression vector for E2 mutants or chimeric E2 proteins, as indicated, and with 250 ng of reporter plasmid pSV3BS9CAT. Normalized CAT activities were determined 40 h after transfection. The symbol for each mutant proteins is indicated in the corresponding panel. (B) Mutants with nearly wild-type protein properties in transcription activation assays. (C) Mutant proteins whose transcriptional activities have decreased to approximately 50% of that of the wild-type protein. (D) Inactive mutant proteins. (E) Transcriptional activation properties of chimeric proteins p53:E2 and VP16:E2.

essentially basal levels of CAT activity, independent of the amount of input E2 expression vector, with pSV3BS9CAT (Fig. 3D) or pP2CAT reporters (data not shown). In the case of active mutants, the optimal concentration of E2 expression vector was within 80 to 100 ng of pCGE2 DNA per transfection and resulted in activation of more than 30 times the basal level (Fig. 3B to E). In all cases, higher-than-optimal E2 concentrations resulted in a reduction in transactivation (Fig. 3B and C), most likely caused by the self-squelching activity of E2. This activity for the E2 protein has been described previously, and the mechanism for this is unknown (39). The suppression of activation at higher E2 concentrations is specific for the E2 transactivation domain because it is not observed for p53:E2 and VP16:E2 chimerical proteins, in which the E2 transactivation domain is replaced by the activation domains of p53 and VP16, respectively (Fig. 3E). The expression levels of wild-type and hybrid p53:E2 and VP16:E2 proteins in CHO cells were comparable (data not shown).

The activities of R37A, D122A, and D143A were dependent on the promoter context of the reporter plasmid used. Point mutations R37A and D122A (Fig. 3C and 4B) resulted in a sharp decrease in transactivation to 20% of that of the wild-type protein from reporter pSV3BS9CAT; however, with pP2CAT as the reporter, these mutants were moderately active (Fig. 4B). In the case of D143A, the same tendency was observed; however, the corresponding relative activities were higher. The third group of mutants (Q12A, R68A, and E90A) were 50% active with both reporters compared with wild-type E2.

The results described above show that point mutations K111A, K112A, and E176A create proteins inactive for both functions, transcriptional and replicational activation. E2 pro-

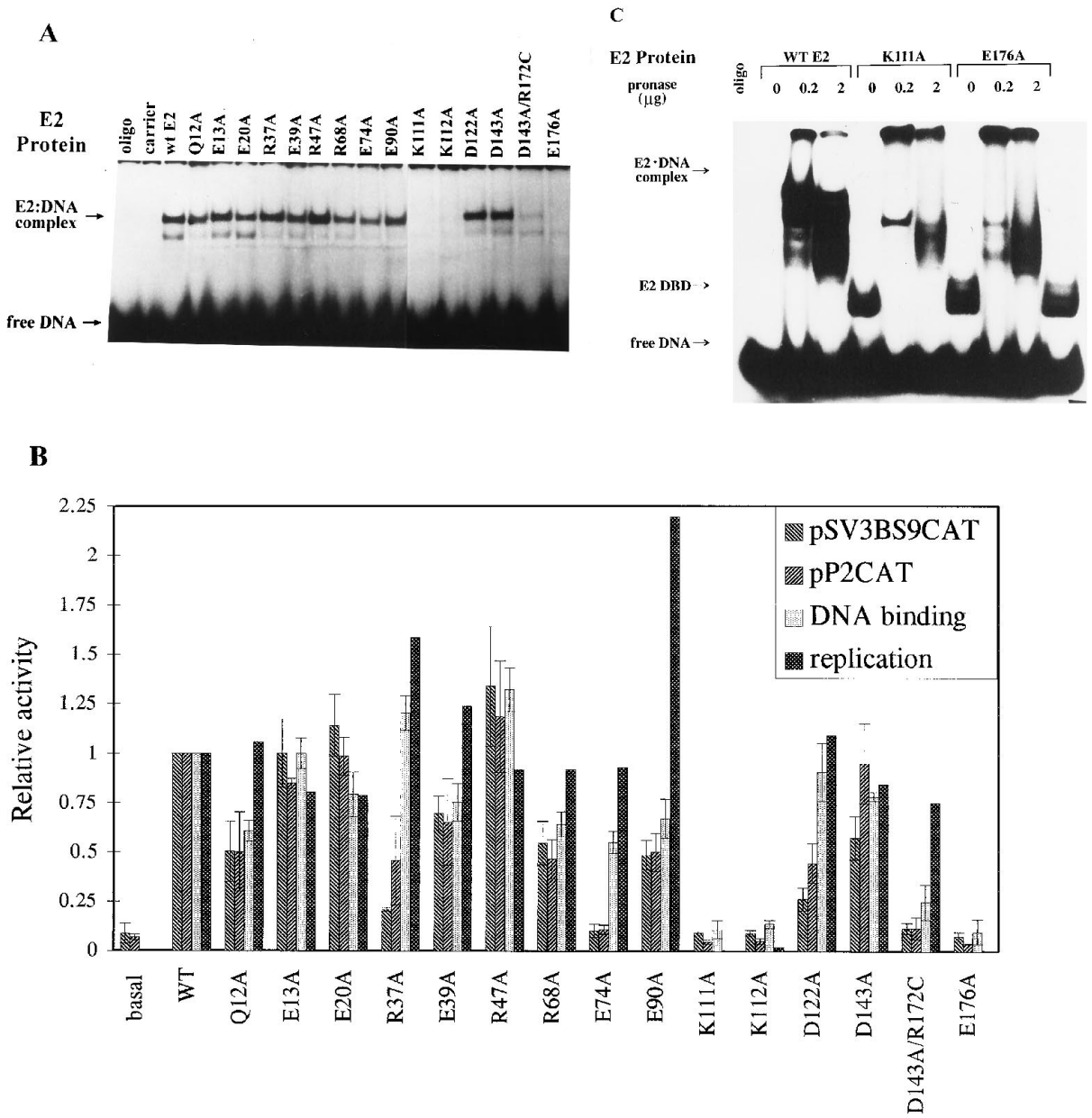

FIG. 4. (A) DNA binding assay with mutated E2 proteins. The sequence-specific binding of E2 to one DNA palindromic target was determined by gel retardation assays. The E2 proteins were expressed in COS-7 cells. Band shift assays were performed with 2 μl of cell extract and 0.5 ng of radiolabeled BS9 for 15 min at room temperature. Protein-DNA complexes were resolved on 6% PAAG in 0.25× Tris-borate-EDTA. oligo, oligonucleotide; wt, wild type. (B) Comparison of the activation of transcription from two reporter plasmids, replication, and DNA binding abilities of E2 protein mutants. The radioactive signals of gel shift and replication assays were quantitated with a PhosphorImager. The wild-type (WT) E2-specific signal in all assays was set at 1.0. For transcription activation assays, CHO cells were electroporated with 250 ng of the indicated reporter and 250 ng of pCGE2 or a derivative. Normalized CAT activities were determined 40 h after transfection. In all cases, the values shown are the results (± standard errors) of at least three independent transfection experiments. (C) Pronase treatment of lysates containing inactive E2 mutant proteins. The reaction mixtures were incubated first with 0.2 or 2 μg of pronase for 10 min at room temperature and then with labeled BS9 for an additional 10 min. Protein-DNA complexes were resolved on 8% PAAG in 0.25× Tris-borate-EDTA. DBD, DNA binding domain.

tein mutants E74A and D143A/R172C were crippled in the transcriptional activation function but were competent to support replication in the transient-replication assay. E2 protein mutants R37A and D122A were also defective in transcriptional activation, though this defect was less obvious in the case of the P2 promoter. These data may indicate that the replication and transcriptional transactivation functions of the BPV1 E2 protein are encoded at least in part by separate determinants.

The activities of mutant E2 proteins in sequence-specific DNA binding. Sequence-specific DNA binding is essential for the expression of most of the functional activities of the E2 protein. Therefore, we decided to study the activities of mutant E2 proteins in sequence-specific DNA binding assays, even though mutations were made in the N-terminal transactivation domain of this protein. The expression vector pCG allows high-level expression of the cloned cDNA in COS cells, which makes the quantitation of the concentrations of mutant E2

proteins in lysates by ELISA more accurate. The expression levels of the E2 protein and mutants in CHO cells were sufficient for the detection of specific gel shift; however, they were difficult to measure by quantitative ELISA using the standard curve obtained with bacterially expressed E2 protein. In addition, the expression levels of wild-type and mutant E2 proteins were sufficient for the separation of lysates in the glycerol gradient to establish the physical oligomerization status of E2 mutants. The lysates of transfected COS-7 cells were tested for the ability to form an E2-specific complex with double-stranded high-affinity BS9 (5′-ACAAAGTACCGTTGCCGG TCGAA-3′) of the BPV1 E2 responsive element (E2RE1) by quantitative gel shift assay. Lysates obtained by the sonication of cells or by the use of 0.35% nonionic detergent Nonidet P-40 gave essentially the same results in gel shift assays. The shifted double band was specific for E2, as it was supershifted by anti-E2 monoclonal antibody 3F12 (epitope, residues 199 to 207) and was inhibited by an excess of the nonlabeled specific oligonucleotide (data not shown) (23). The cloned E2 open reading frame carried a mutation from the initiating methionine (codon ATG) to isoleucine (codon ATC) in the E2 transcription repressor and was unable to express the repressor form of E2. Therefore, the appearance of the lower band is not caused by the shorter repressor form of the E2 protein. A similar gel shift pattern has been observed in several instances for baculovirus-, yeast-, or bacterium-expressed E2 protein and probably reflects a different conformation of the complex (23, 35, 50). Equal amounts of lysates of cells containing wild-type and mutant E2 proteins were used in the binding reaction mixtures, and complexes were separated in a standard gel shift assay. Our data indicate that most of the E2 mutants had approximately the same or, in some cases, even better ability to bind DNA (Fig. 4A); however, E2 mutants with point mutations K111A, K112A, and E176A, which were inactive for both replication and transcription, failed to form the specific protein-DNA complex able to enter the gel. These data indicate that mutations K111A, K112A, and E176A seem to abolish the DNA binding abilities of these proteins and therefore the functioning of the E2 protein in transcription and replication, or to induce the formation of E2 protein oligomers or aggregates unable to enter the gel. Mutants E74A and D143A/R172C showed reduced affinity in the DNA binding assay (50 and 30%, respectively, from the wild-type level). The carboxy-terminal part of the E2 protein is responsible for DNA binding and contains a protease-resistant core (14). In order to test whether introduced mutations K111A, K112A, and E176A destroyed the total folding of the E2 protein, including the DNA binding domain, induced a protein conformation inactive for interaction with DNA, or resulted in a protein with high-level oligomerization-aggregation properties, we subjected the lysates to pronase treatment. Pronase treatment of the lysates of cells transfected with K111A, K112A, or E176A revealed the E2-specific DNA binding activities of these mutants (Fig. 4C). The same results were obtained when the extracts were treated with protease either before or after the addition of DNA to the binding reaction mixture. These data indicate that the point mutations introduced into the amino-terminal part of the molecule did not destroy overall folding of the E2 protein, but some other mechanism prevented the formation of an E2-DNA complex of the usual size. We also observed that the trapping of a specific DNA probe in a gel well, especially in the case of K111A, K112A, and E176A mutants, disappeared upon treatment with pronase.

One interesting group consisted of E2 mutants which were able to support DNA replication at the wild-type level in the transient-replication assay but had severely reduced transcriptional activities. E2 mutants with point mutations R37A and D122A bound DNA as well as wild-type E2 did; however, E74A and specifically D143A/R172C bound DNA at reduced levels (Fig. 4A). Again, considerable entrapment of the specific probe could be detected in wells.

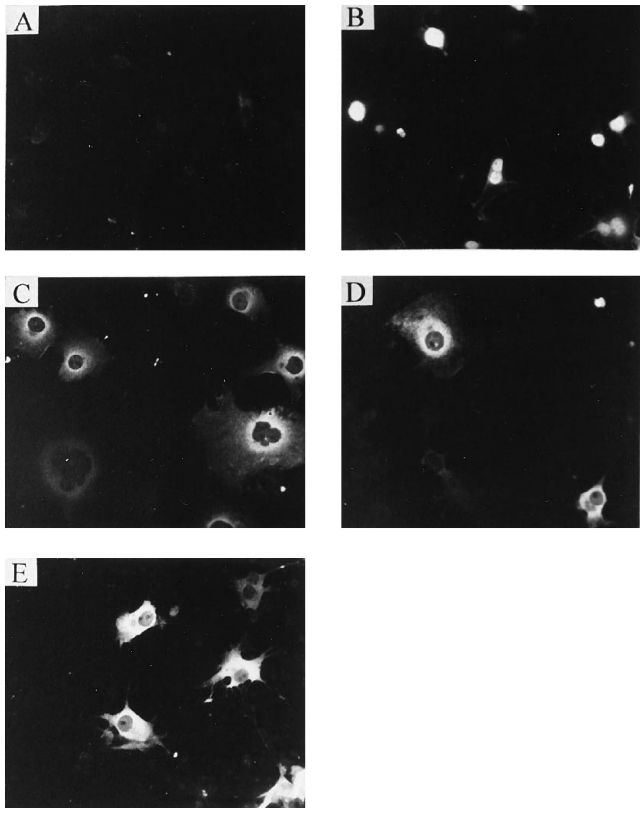

FIG. 5. The localization of E2 mutant proteins in COS-7 cells by immunofluorescence analysis. COS-7 cells were transfected with carrier DNA (A), pCGE2-wt (B), pCGE2 K111A (C), pCGE2 K112A (D), and pCGE2 E176A (E). Cells were probed with monoclonal antibody against the E2 protein and subsequently with fluorescein isothiocyanate-conjugated anti-mouse antibody as described in Materials and Methods.

DNA binding activities were quantitated with a PhosphorImager and normalized to the binding activity level of expressed wild-type E2 protein. The results, DNA binding together with the activities of mutant proteins to activate transcription from the two different reporter constructs and quantitation of the replication data, are shown in Fig. 4B. These results confirm earlier observations that the DNA binding ability of E2 is absolutely required for specific transcriptional activation. Furthermore, these data indicate that the transcription activation domain of the E2 protein has considerable impact on the regulation of the functioning of this protein.

Compartmentalization of mutant proteins in COS-7 cells. The E2 transactivator contains three regions which can constitute a potential nuclear localization signal for this protein, BR1 (residues 47 to 49), BR2 (residues 107 to 115), and BR3 (residues 340 to 353). It has been shown that only BR3 is able to mediate active transport of a hybrid protein (β-galactosidase) into the nucleus; however, BR2 has been suggested to work as the major nuclear localization signal of E2TA. Partial or complete deletion of this sequence or a proline-to-glycine mutation at residue 106 causes mutant proteins to accumulate in the cytoplasm (46). Two such mutations, K111A and K112A, were

made in the BR2 region, therefore raising the possibility that the inactivity of these mutants in biological assays is caused by the failure of this protein to enter the nucleus. We studied the nuclear localization of all mutant proteins in COS-7 and C127 cells. The localization of E2 mutants in COS-7 cells is presented in Fig. 5. We used mouse monoclonal antibody 3F12, directed against the epitope within residues 199 to 207, for the detection of the E2 protein. Essentially, the same results were obtained when another monoclonal antibody, 3C1 (residues 280 to 310), or polyclonal rabbit immunoglobulin G against the E2 protein was used in these experiments (data not shown). Figure 5A and B show the immunostaining of COS-7 cells transfected with a mock and wild-type E2 expression construct, respectively. We detected specific exclusive nuclear immunostaining in cells expressing wild-type E2 protein compared with mock-transfected cells. Contrary to wild-type E2, mutant proteins K111A (Fig. 5C), K112A (Fig. 5D), and E176A (Fig. 5E) are preferentially localized in the cytoplasm, with very little, if any, detectable specific immunostaining in the nucleus. All the remaining mutant proteins were localized in the cell nucleus (data not shown).

Oligomerization of mutant proteins. The expression levels of all mutant proteins in CHO (Fig. 1B) and COS-7 cells were comparable, as estimated by Western blotting of the total cell extract and soluble cell extract used for gel shift analysis. In the case of mutants K111A, K112A, and E176A, we were unable to detect the formation of a specific E2-DNA complex with mobility similar to that of wild-type E2 protein; however, we observed considerable retention of the probe in the wells of the gel, indicating that mutant E2 proteins may form larger oligomers which are unable to enter the gel. Pronase digestion of the lysate revealed protease-resistant cores within the mutant E2 proteins (Fig. 4C) as well as the disappearance of the signal in wells, supporting the possibility that these mutant proteins exist as oligomers or larger aggregates which are still able to bind DNA but are unable to enter the gel or nucleus. We studied the oligomerization status of wild-type and mutant E2 proteins in COS-7 lysates by using the sedimentation of this protein in 10 to 30% glycerol gradients. Five-milliliter gradients were dripped into 14 fractions after centrifugation at 45,000 rpm for 15 h at 4°C with a Beckman SW55 rotor. The fractions were analyzed for E2 protein content by a specific monoclonal antibody 3F12-based sensitive ELISA and for E2 protein sequence-specific DNA binding. We used PhosphorImager quantitation of the gel shifts in the region of the unit-sized E2-DNA complex and in the region near wells for the detection of the larger protein-DNA complex, which has difficulties in entering the gel. The results are presented in Fig. 6. The distribution of the E2 protein from the lysate of COS-7 cells transfected with the wild-type E2 expression construct is shown in Fig. 6A. The E2 protein moves as a single peak in the gradient and could be found mostly in fractions 7 and 8. These fractions also show the maximal levels of E2 protein DNA binding activity. We did not detect any considerable entrapment of the probe in the wells of this gel. For marker proteins, alcohol dehydrogenase (150 kDa) peaked in fraction 2 and BSA (66 kDa) peaked in fraction 9. We also did not detect any wild-type protein precipitated onto the bottom of the centrifuge tube (Fig. 6B). From these results, we concluded that the wild-type protein exists in COS-7 lysates as a soluble dimeric protein. Completely different results were obtained with inactive E2 protein mutants K111A, K112A, and E176A. Very little, if any, E2 protein was detected in the fractions in which the dimeric wild-type protein was found. Instead, we detected the E2 protein by ELISA in the near-bottom fractions, fractions 1 to 5 (Fig. 6A), and in the precipitated form (Fig. 6B). An inspection of the gel shifts indicated that there was very little specific E2-DNA complex with the mobility of the wild-type protein detected in this gel; however, considerable amounts of signal could be detected in the regions of this gel near wells. An analysis of mutants E74A and D143A/R172C, which display weaker DNA binding (Fig. 4A), showed that fractions of these mutants exist in the dimeric form, which allows some of the specific complex to enter the gel, as well as in the oligomeric form, which is capable of binding DNA (Fig. 6). Some of the oligomers which are stable enough to be separated by the glycerol gradient from the dimeric form of E2 in the case of E74A, were dissociated under the conditions for the formation of the E2-DNA complex, and an E2-DNA complex of the size of the wild-type could be detected in the bottom fractions of the gradient (Fig. 6A). From these results, we concluded that the inactivity of mutants K111A, K112A, and E176A may be caused by the oligomerization of the protein, which inactivates the transport of mutant proteins to the nucleus. Mutants E74A and D143A/R172C create proteins with oligomerization capabilities greater than that of the wild-type protein; however, this process is reversible and a considerable fraction of the protein still exists in the dimeric and active form.

DISCUSSION

We constructed a set of BPV1 E2 protein mutants by a scheme called clustered charged-to-alanine scan. This approach allows the identification of functionally important side chains involved in interaction with other proteins. Alanine is the most abundant amino acid in proteins and was selected as the replacement residue because it is found in all types of secondary structure and because this substitution does not impose new structural effects related to hydrogen bonding, unusual hydrophobicity, or steric bulk. Charged-to-alanine substitution generally does not interfere with the packing of buried residues nor disrupt the structural integrity or expression of the protein (11). It is highly likely that clustered charged residues are exposed on the protein surface. It is reasonable to assume that the functioning of the protein in the regulation of different biological activities takes place through direct interactions and is mediated through the surfaces of components of the system (2). Therefore, clustered charged-to-alanine scan is a good approach for identifying the structural determinants of E2 interacting with factors of transcription and replication machinery. Our initial analysis of the clustered charged residues of the BPV1 E2 protein identified about 30 epitopes (determinants) which were located presumably on the surface of the protein and therefore could be identified as potential candidates for interactions with basal transcription and replication machinery. In the current study, we decided to include only the conserved charged residues of the BPV1 E2 protein. Altogether, 14 E2 mutants with alanine substituted for charged residues were constructed by PCR-mediated mutagenesis. In the process of construction of the 13th mutation, Asp-143→Ala, we picked up a double mutation, Asp-143→Ala and Arg-172→Cys, which was also included in the analysis. Altogether, we tested 15 E2 mutants in transient-replication, transcriptional activation, and sequence-specific DNA binding assays. We found that all E2 mutants, except for K111A, K112A, and E176A, were able to activate the replication of the BPV1 origin. In addition to these three mutants, four E2 mutants, E37A, E74A, D122A, and D143A/R172C, were crippled for the activation of transcription. We concluded that only determinants containing residues E-37, E-74, D-122, and R-172 are involved in the activation of tran-

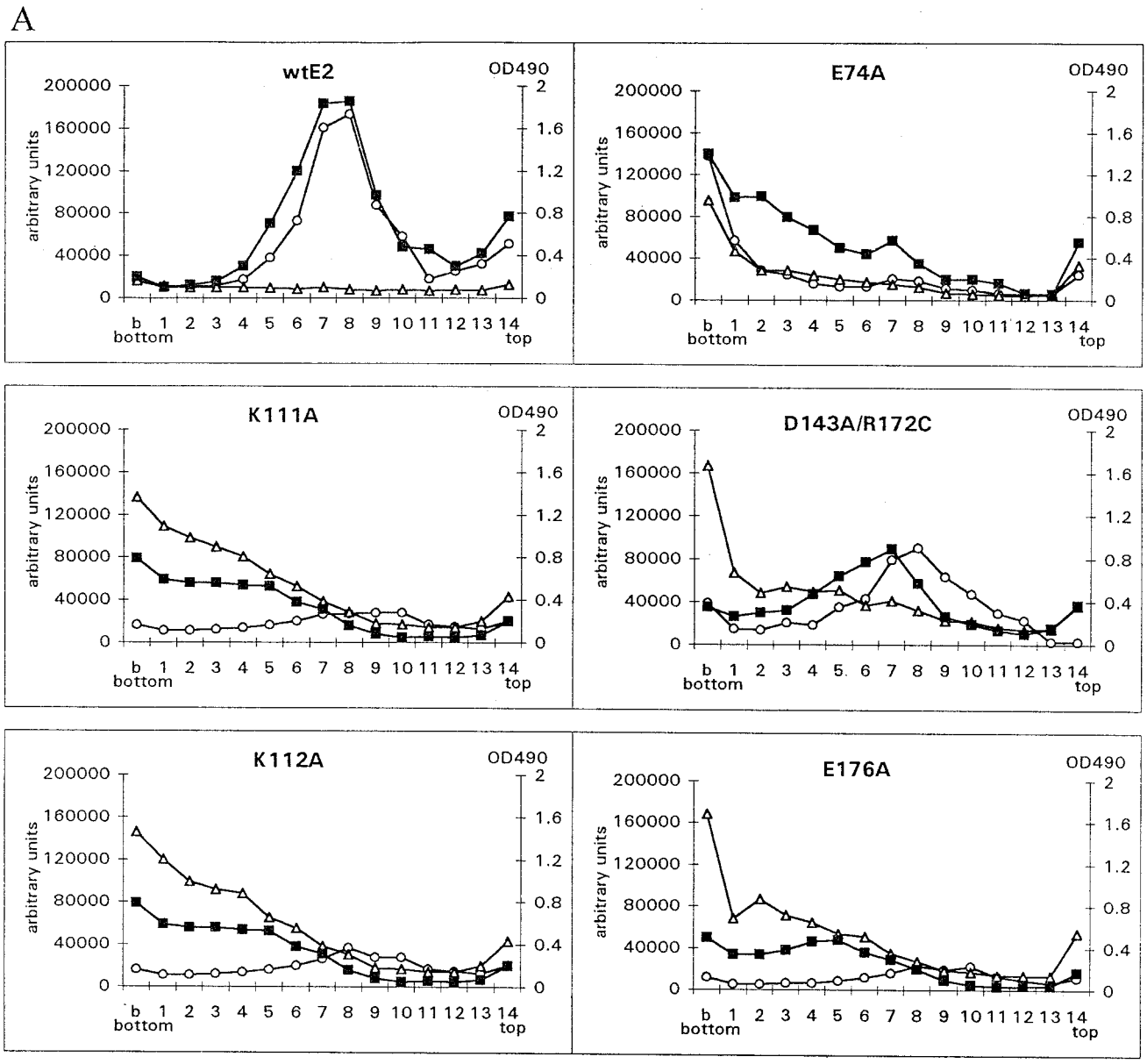

FIG. 6. (A) Sedimentation analysis of wild-type and mutant E2 proteins expressed in COS-7 cells. COS-7 cells were transfected with the respective expression vectors for wild-type and mutant E2 proteins, and extracts of transfected cells were loaded onto the top and sedimented through a 10 to 30% glycerol gradient. Fourteen fractions were collected, the bottom of the tube was washed with 250 μl of buffer, and all fractions were subjected to ELISA analysis (optical density at 490 nm [OD490]) (■) and tested for a sequence-specific gel shift of the size (in arbitrary units) of the wild type (○) and for a signal (in arbitrary units) near wells (△). DNA binding activities were quantitated with a PhosphorImager, and radioactivity values are expressed in arbitrary units. Marker proteins, alcohol dehydrogenase (150 kDa) and BSA (66 kDa), were centrifuged in the parallel gradient and peaked in fractions 2 and 9, respectively. b, bottom fraction. (B) Immunoblot analysis of wild-type and mutant E2 proteins precipitated upon centrifugation in 10 to 30% glycerol gradients. The precipitated protein at the bottom of the tube was solubilized with 50 μl of SDS sample buffer and subjected to Western blot analysis using an E2-specific monoclonal antibody. The purified E2 protein expressed in bacteria was used as a control. wtE2, wild-type E2.

scription and therefore presumably interact with components of the transcription machinery. It has been shown that the E2 protein is able to interact with transcription factor Sp1 in solution and cooperate with Sp1 in the transcriptional activation of promoters (17, 26). In addition, E2 has a capacity to interact with TBP and with TFIIB (39). Although biochemical experiments have demonstrated that major determinants of interaction between E2 and TBP and TFIIB lie within the carboxy-terminal domain of the E2 protein (39, 50), these data do not exclude the involvement of certain determinants within the transactivation domain in interaction with TBP (39, 50). We believe that mutants R37A, E74A, D122A, and D143A/R172C are inefficient in transcription activation because of weakened interaction with Sp1, TBP, TFIIB, or other general transcription factors. Further biochemical studies are in progress to identify the interactions of E2 with these factors.

The amino-terminal domain of the E2 protein has remarkable structural integrity (7, 15, 19, 23), and very little is known about the structural and functional determinants located in this part of the E2 molecule. The computer prediction of the structure of the N-terminal domain of the BPV1 E2 protein was made by the PredictProtein method (41) and is presented in Fig. 1A. In addition to our results, two recently published studies have analyzed E2 protein functional and structural determinants in the N-terminal domain. Human papillomavirus type 16 (HPV16) E2 protein was analyzed by alanine substitution of conserved residues (42), as in our study, and BPV1 E2 protein was analyzed by using highly conservative amino acid substitutions (7) in the activation of replication and transcription, as well as in interaction with the E1 protein. A comparison of these studies allows us to reach interesting conclusions about the structural and functional determinants of the E2 protein. The replacement of conserved residues in the first alpha-helix (Fig. 1A) with alanine or a conservative amino acid does not change the biological or biochemical properties of the HPV16 or BPV1 E2 protein. Thus, as in all three studies good care in design and characterization of the mutant protein was made to avoid gross changes of the structural organization of the protein, we may conclude that mutations in positions Q-12, E-13, and E-20 of the first alpha-helix have a phenotype essentially similar to that of the wild-type protein. At the same time, deletion of the first alpha-helix from the BPV1 E2 protein inactivates E2 completely in all biological assays and reveals denatured protein-specific epitopes within the N-terminal part of E2 (23). This allows us to speculate that the first alpha-helix is an important structural organizer of the N-terminal part of the E2 protein and determines the integrity of the structure of the transactivation domain. This may be achieved through electrostatic interaction of the negatively charged first alpha helix-surface, consisting of glutamic acid residues.

In the second alpha-helix of the E2 protein (Fig. 1A), substitutions for four conserved residues, W-33, R-37, E-39, and R-47, have been studied. Conservative changes at two positions, W-33 and E-39, of BPV1 E2 rendered the protein inactive in replication and transcription (7), while alanine substitutions for W-33 and R-47 in HPV16 E2 had no inactivating effect and that for E-39 abolished the replicational activation ability of E2 (42). In BPV1 E2, the replacement of E-39 and R-47 with alanine had little effect on protein activity. The replacement of arginine by alanine at position 37 impaired HPV16 E2 (42) and BPV1 E2 for transcriptional activation, leaving the replication function of the protein intact. The replacement of this residue in BPV1 E2 by a conservative amino acid, lysine, produced a mutant with an essentially wild-type phenotype (7). Although the E2 proteins of different papillomaviruses have the same functions, activation of transcription and replication, they are not readily interchangeable in replication assays (9). For example, HPV11 E2 and BPV1 E2 are very inefficient in the activation of replication with heterologous E1 protein and in interaction with heterologous E1 protein (9, 44). This incompatibility of functionally identical proteins in replication assays may be a reflection of differences in the structural determinants responsible for the activation of replication and may explain the differences in replicational activation by E39A mutants of HPV16 and BPV1 E2 proteins.

In the third alpha-helix (Fig. 1A), the effects of mutations at three positions, R-68, I-73, and E-74, can be compared. Mutation R68A did not induce very large changes in the properties of E2 proteins (42), while E74A was not studied by others. In our study, we found that this protein was transcriptionally inactive but moderately functioned in the activation of replication. Interestingly, mutation I73A in HPV16 E2 induced a protein with essentially the same phenotype, while conservative change I73L produced a BPV1 E2 protein with reduced transcriptional activation ability (7). In addition to being impaired in transcriptional activation, the same protein with mutation E74A showed another biochemical property. Although localized in the nucleus, this mutant protein exhibited an increased ability to form oligomers. These oligomers were still able to dissociate into the dimeric form and were able to bind DNA and form an E2-DNA complex of normal size. From these three studies, we may conclude that the third alpha-helix carries the capacity to interact, presumably through the conserved determinant between residues 72 and 74 (alanine-isoleucine-glutamic acid), with the transcription machinery of the cell. Hydrophobic and aromatic residues may be critical for and participate directly in the interaction of the transcription activation domain with components of the basal transcription complex (16, 24). We may speculate that the relatively hydrophobic isoleucine at position 73 is probably critical for this interaction, while a charged residue, E-74, is responsible for the presentation of this determinant for interaction.

We found that substitutions K111A, K112A, and E176A in the E2 transactivation domain completely abolished the ability of these expressed mutant proteins to form an E2-DNA complex of the usual size; however, considerable retention of the probe in wells was detected. The pronase treatment of extracts of COS-7 cells containing inactive mutant K111A, K112A, and E176A proteins showed that the DNA binding abilities of these mutants were readily present in the proteins; however, only large complexes were formed. The formation of oligomers and aggregates with these mutants was detected by glycerol gradient centrifugation. We also studied the cellular localization of mutant E2 proteins and observed that three mutants, K111A, K112A, and E176A, were exclusively localized in the cytoplasm. In a study by Skiadopolus and McBride, the BR2 region (residues 107 to 115) was identified as a potential nuclear localization signal for the E2 transactivator protein E2TA (46). Two of the mutations, K111A and K112A, fell in the same region. Conservative change K111R did not disrupt nuclear transport (7, 46); however, this protein was inactive in transcription but retained replicational activity at a very low level. This indicates that the lysine residue at position 111 is invariant and is important for the active conformation of this protein; the replacement of lysine by arginine creates an inactive nuclear E2 protein, while a replacement by alanine induces a conformation of this protein which is able to oligomerize and even to aggregate, as estimated by glycerol gradient centrifugation. In our study, the replacement of lysine by alanine at position 112 resulted in a mutant protein with the same phenotype as that of K111A. However, the same mutation in HPV16 E2 (42), as well as replacement K112R in BPV1 E2

(7), had little effect on the activity of the protein. This discrepancy may be a reflection of the differences in the structures of the HPV16 and BPV1 E2 proteins. The BR2 region of the BPV1 E2 protein can be responsible for directing E2TA into the nucleus (46); however, in our experiments, oligomerization and aggregation are probably responsible for the retention of mutants K111A, K112A, and E176A in the cytoplasm. The amino-terminal parts of E2 dimeric molecules mediate the cooperative binding of E2 on the clustered E2 binding sites in DNA (35). This interaction may also take place in solution and could serve as a mechanism for the regulation of E2 biological activity. Amino terminus-mediated oligomerization could explain the self-squelching of E2 described by Rank and Lambert (39) and by us here. Self-squelching is specific to the E2 transactivation domain, as its replacement with the p53 or VP16 transactivation domain abolished self-squelching, although the expression levels of E2 and hybrid proteins were comparable. We may conclude that introduced mutations K111A, K112A, and E176A remove certain constraints in the interactions of E2 molecules in solution and induce the formation of inactive configurations of E2 oligomers.

Several studies have presented data consistent with the finding that interaction between BPV1 E2 and E1 facilitates the binding of E1 to its cognate site (29, 44, 45, 48, 53, 55). This cooperative binding has been suggested to play an important role in the initiation of BPV1 DNA replication and is very specific for E1 and E2 proteins. We tested a number of transactivation domains, p53, VP16, c-jun, and Epstein-Barr virus BZLF-1, linked to the E2 DNA binding domain and found all of them to be unable to support BPV1 origin replication in vivo (1). The first 91 residues of E2 participate in interaction with the E1 protein, as shown in biochemical assays and in a two-hybrid system (3). Eight of our designed mutations fell in this region, and curiously none affected the activation of BPV1 origin replication, though two of them were inactive in transcription. These data indicate that the determinants for interaction with the E1 protein are not encoded by the clustered conserved charged residues, but some other sequences within this region may be involved in the specific interaction between E2 and E1. This conclusion is supported by the results of in vivo and in vitro replication assays using mixed and matched combinations of the E1 and E2 proteins from BPV1 and HPV11 (9, 44). Certain combinations of the E1 and E2 proteins of BPV1 and HPV11 were inactive for the activation of replication, indicating that the interaction between E1 and E2 is virus type specific. In contrast, BPV and HPV E2 proteins are able to transactivate in different mammalian cell lines and in yeasts (36, 49, 54). This indicates that the interactions with the transcription machinery are well conserved. Mutations at conserved positions lead to the disruption of interactions with the transcription machinery but not with E1.

As shown by in vitro replication experiments, transcription per se is not required for replication of the BPV1 origin (58). Our data also show that the ability of E2 to activate transcription is not required at all for the activation of BPV1 replication in vivo and that the determinants responsible for the activation of transcription and replication only partly overlap.

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