JOURNAL OF VIROLOGY, Apr. 1992, p. 2418–2427
0022-538X/92/042418-10$02.00/0

Vol. 66, No. 4

Structure-Function Analysis of the Human Papillomavirus Type 16 E7 Oncoprotein

WILLIAM C. PHELPS,[1]* KARL MÜNGER,[2] CAROLE L. YEE,[2] JULIE A. BARNES,[1] AND PETER M. HOWLEY[2]

Division of Virology, Burroughs Wellcome Co., Research Triangle Park, North Carolina 27709,[1] and Laboratory of Tumor Virus Biology, National Cancer Institute, Bethesda, Maryland 20892[2]

Received 2 October 1991/Accepted 8 January 1992

The E7 gene of human papillomavirus type 16 encodes a multifunctional nuclear phosphoprotein that is functionally and structurally similar to the adenovirus (Ad) E1A proteins and the T antigens of other papovaviruses. E7 can cooperate with an activated *ras* oncogene to transform primary rodent cells, *trans* activate the Ad E2 promoter, and abrogate transforming growth factor β-mediated repression of c-*myc*. Recent studies suggest that these functions may in part be a consequence of the ability of E7 to associate with the product of the retinoblastoma tumor suppressor gene (pRB). In this study, a series of site-specific mutations of the human papillomavirus type 16 E7 gene product were constructed and assessed for their effects on intracellular protein stability, *ras* cooperativity, transcriptional *trans* activation, pRB association, and phosphorylation. The results of these studies indicate that the transforming and *trans*-activating domains extensively overlap within a region of the protein analogous to conserved region 2 of Ad E1A, suggesting that pRB binding is necessary for both activities. Deletion of sequences in conserved region 1 abrogates cellular transformation but has only a marginal effect on *trans* activation. These data suggest that E7 *trans* activation and cellular transformation are interrelated but separable functions.

Both epidemiological and biological data support the division of the mucosa-associated human papillomaviruses (HPVs) into two groups: those associated with benign lesions, including condyloma acuminata, which are at low risk for malignant progression (HPV type 6 [HPV6] and HPV11), and those represented by HPV16 and HPV18, which are considered high risk because of their association with intraepithelial neoplasia, a precursor to cervical cancer (57). The E7 proteins of the high-risk HPVs, which are consistently expressed in HPV-positive cervical carcinomas and derived cell lines (3, 42, 43), can induce morphological transformation of established rodent cells in culture (7, 22, 37, 49, 50, 52). E7 also can cooperate with an activated *ras* or *fos* oncogene to transform primary rodent cells (37, 48). The expression of the high-risk HPV E6 and E7 proteins together can efficiently induce immortalization of primary human epithelial cells (19, 20, 29).

The HPV16 E7 protein is an acidic 21-kDa nuclear phosphoprotein with no known enzymatic activity. The amino terminus of the HPV E7 protein shares substantial amino acid sequence similarity with two noncontiguous portions of the adenovirus (Ad) E1A proteins (37). In addition, E7 is functionally related to E1A and simian virus 40 (SV40) large T antigen in that it can cooperate with *ras*, *trans* activate the Ad E2 promoter (37), and abrogate the transforming growth factor β-induced transcriptional repression of the c-*myc* promoter in keratinocytes (38). The amino acid sequences in conserved region 2 (CR2) of each of these oncoproteins are required for their association with pRB, the product of the retinoblastoma tumor susceptibility gene (5, 6, 13, 14, 30, 53, 54).

Previous genetic studies indicated that CR1 and CR2 are required for E1A cellular transformation functions, including stimulation of host DNA synthesis, cooperation with *ras*, pRB association, and transcriptional repression of certain genes. In contrast, CR3 of E1A, which is present in the product of the Ad E1A 13S RNA but not in the product of the 12S RNA, is responsible for the major E1A-mediated transcriptional *trans*-activation function (9, 10, 32). Amino acid sequences that are similar to that of Ad E1A CR3 are not present in SV40 T antigen or in the E7 proteins of the HPVs, suggesting that this transcriptional regulatory region is unique to E1A.

A recent analysis of HPV16 E7 *trans* activation indicated that the binding sites for the cellular transcription factor E2F are necessary for activation of the Ad E2 promoter by both E7 and E1A. Recent studies have indicated that the E2F transcription factor is a cellular target for the pRB protein (2, 11, 12). The E7 protein is functionally similar to the 12S E1A product in that it can perturb the association of E2F with cellular proteins, including cyclin A and pRB (34).

In this study, a series of HPV16 E7 mutants were analyzed to map the domains involved in the transcriptional *trans* activation and cellular transformation functions of the E7 gene product. These data suggest that for HPV E7, cellular transformation and *trans* activation of the Ad E2 promoter are related but separable functions. Cellular transformation required two separate regions analogous to the Ad E1A CR1 and CR2 domains, and transcriptional *trans* activation mapped to an overlapping region of the CR2 domain.

MATERIALS AND METHODS

Cell culture. African green monkey kidney CV-1 cells and COS-7 cells (ATCC CRL 1651) were maintained in Dulbecco modified Eagle medium with 10% fetal bovine serum supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). Primary baby rat kidney cells were prepared from 6-day-old Fischer rats as previously described (40). The kidneys were finely minced and digested with collagenase and dispase. Approximately 3×10^5 to 5×10^5 cells were plated in 60-mm dishes and grown in the medium described above.

* Corresponding author.

Recombinant plasmid DNAs. Mutations in the HPV16 E7 gene resulting in amino acid substitutions and deletions were generated by oligonucleotide reconstruction essentially as described previously (28, 45). A schematic diagram of this process is shown in Fig. 2. Briefly, overlapping synthetic oligonucleotides of specified sequence were treated with polynuc-leotide kinase and annealed between the *Nsi*I (nucleotide 565) and *Ban*II (nucleotide 711) sites in the amino half and the *Ban*II and *Nco*I (nucleotide 863) sites in the carboxyl half of the gene. Mutations were then verified by DNA sequence analysis, and the DNAs were recloned into the p1059 plasmid (37), which encodes the entire early region of the HPV16 genome downstream of the SV40 early promoter. In addition, mutated E7 genes were cloned into the SV40 late promoter vector pSVL (Pharmacia, Inc.) by polymerase chain reaction amplification and incorporation of new restriction enzyme sites for *Xho*I and *Bam*HI. The integrity of these clones was again verified by DNA sequence analysis. Ad type 5 pE2CAT (21) and pEJRAS expressing the activated *ras* oncogene (33) have been previously described.

Mammalian cell transfections. DNA transfections for transient chloramphenicol acetyltransferase (CAT) assays were performed by calcium phosphate coprecipitation as previously described (35) by using a total of 10 to 15 μg of DNA per 60-mm dish (normally 5 μg of CAT plasmid plus 5 to 10 μg of E7 expression plasmid). At 4 h after addition of the DNA precipitate, the cells were treated with 15% glycerol for 1 min, washed twice with fresh medium, and incubated in complete medium containing 5 mM sodium butyrate (pH 7.0) for 48 h. For transformation assays, primary baby rat kidney cells were transfected 2 days after plating with 5 μg of each DNA for a total of 10 μg/60-mm dish as previously described (37). Precipitates were allowed to sit on the cells overnight, and the plates were refed the next day. For analysis of protein stability, COS-7 cells were transfected with 60 μg of Lipofectin (GIBCO BRL) and 6 μg of DNA (10:1) per 100-mm dish overnight in Opti-Mem I (GIBCO BRL) and refed with Dulbecco modified Eagle medium with 10% fetal bovine serum.

Metabolic labeling of cells. Approximately 72 h after transfection, cells were starved for either cysteine or phosphate for 1 h and labeled for 1.5 h with either [^{35}S]cysteine (0.5 mCi/100-mm plate) or [^{32}P]P_i (1 mCi/100-mm plate). Cells were washed twice with cold phosphate-buffered saline (PBS) and lysed in either radioimmunoprecipitation assay (RIPA) buffer (20 mM morpholinepropanesulfonic acid [MOPS], pH 7.0; 150 mM NaCl; 1% [vol/vol] Nonidet P-40; 1% [vol/vol] deoxycholate; 0.1% [wt/vol] sodium dodecyl sulfate (SDS); 1 mM EDTA; 100 μg of aprotinin per ml; 100 μg of phenylmethylsulfonyl fluoride per ml; 100 μg of *Nα-p*-tosyl-L-lysine chloromethyl ketone per ml; 100 μg of tolylsulfonyl phenylalanyl chloromethyl ketone [TPCK] or E1A extraction buffer (50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], pH 7.0; 0.1% Nonidet P-40; 250 mM NaCl; protease inhibitors [Sigma Chemical Co.]) per ml as described above.

Antibodies. Polyclonal rabbit antisera (1:500) to the HPV16 E7 protein expressed in *Escherichia coli* (32a) was used to immunoprecipitate the E7 protein, and mouse monoclonal antibody no. 100201 to HPV16 E7 (Triton Diagnostics, Alameda, Calif.) was used for Western blot (immunoblot) analysis of protein stability in COS-7 cells. For Western blot analysis of pRB protein, a mouse monoclonal antibody, PMG3-245.11 (PharMingen), was used at 10 μg/ml.

Immunoprecipitation. Immunoprecipitations from ^{35}S- or ^{32}P-labeled cell lysates were performed by incubation with antisera overnight (0°C). Immune complexes were collected by incubation (4°C) with protein A-Sepharose CL-4B for 30 min with rocking. The immune complexes were then washed twice with cold RIPA buffer plus protease inhibitors, once with a high-salt buffer (125 mM Tris-HCl, pH 7.5; 12.5 mM dithiothreitol; 6.25 mM $CaCl_2$; 500 mM NaCl) at 37°C for 30 min and once with TE (10 mM Tris-HCl, pH 7.5; 1 mM EDTA). Immune complexes were then resuspended in sample buffer and analyzed by polyacrylamide gel electrophoresis (PAGE).

Western blot analysis. Immunoprecipitated protein separated on polyacrylamide gels was electroblotted to nitrocellulose at 40 V for 2 h in 12.5 mM Trizma–100 mM glycine, pH 8.3. The membranes were blocked (5% nonfat dry milk; 10 mM Tris-HCl, pH 7.5; 2.5 mM EDTA; 50 mM NaCl; 0.1% Tween 20) for 1 h, incubated in fresh blocking buffer plus monoclonal antibody (no. 100201; Triton Diagnostics) for >1.5 h, and washed five times with PBS–0.1% Tween 20. Detection of mouse monoclonal antibodies was with ^{125}I-labeled goat anti-mouse antiserum (Amersham Corp.).

CAT assays. CAT assays were performed as previously described (46). Cell extracts were incubated with [^{14}C]chloramphenicol (50 mCi/mmol; Amersham Corp.) and 4 mM acetyl coenzyme A (Pharmacia, Inc.) in 250 mM Tris-HCl (pH 7.8) at 37°C for 0.5 to 2.0 h. The products of the acetylation reaction were separated by ascending thin-layer chromatography, localized by autoradiography, and quantitated by liquid scintillation after excision of the spots. Cell lysates were assayed for total protein concentration with the Bio-Rad protein assay (Bio-Rad Laboratories) so that each CAT reaction was conducted with equivalent amounts of protein (20 μg).

RESULTS

The amino-terminal 45 residues of the E7 proteins of the genital tract-associated HPVs possess a striking sequence similarity to portions of CR1 and CR2 of Ad E1A (Fig. 1, top) (37). The integrity of these domains is required for most of the transformation functions of E1A (32). Although the sequences in the carboxyl half of E7 are well conserved among the HPVs, this region possesses no substantial amino acid sequence similarity to any part of E1A with the exception of repeated Cys-X-X-Cys motifs, which are also found in CR3 of E1A (Fig. 1, bottom). In this study, mutations in the amino terminus of HPV16 E7 were constructed on the basis of the homology to E1A; mutations in the carboxyl terminus were based on conserved amino acids among the genital tract-associated HPVs. The E7 regions corresponding to Ad E1A CR1 and CR2 are referred to in this study as domains 1 and 2; the carboxyl portion of E7 (amino acids 38 to 98) is referred to as domain 3.

Construction of HPV16 E7 mutations. A series of specific mutations were created in the HPV16 E7 gene as depicted in Fig. 2. Mutations, including amino acid substitutions, deletions, and the introduction of premature termination codons, were created by oligonucleotide reconstruction between the *Nsi*I, *Ban*II, and *Nco*I sites. In most cases, the amino acid substitutions were chosen to introduce relatively conservative changes that might not be expected to severely disrupt the overall structure of the protein.

The effect of amino acid changes on the intracellular stability of the E7 protein was assessed by expression of the altered proteins from the SV40 late promoter in COS-7 cells. Transfected cells were metabolically labeled with [^{35}S]cys-

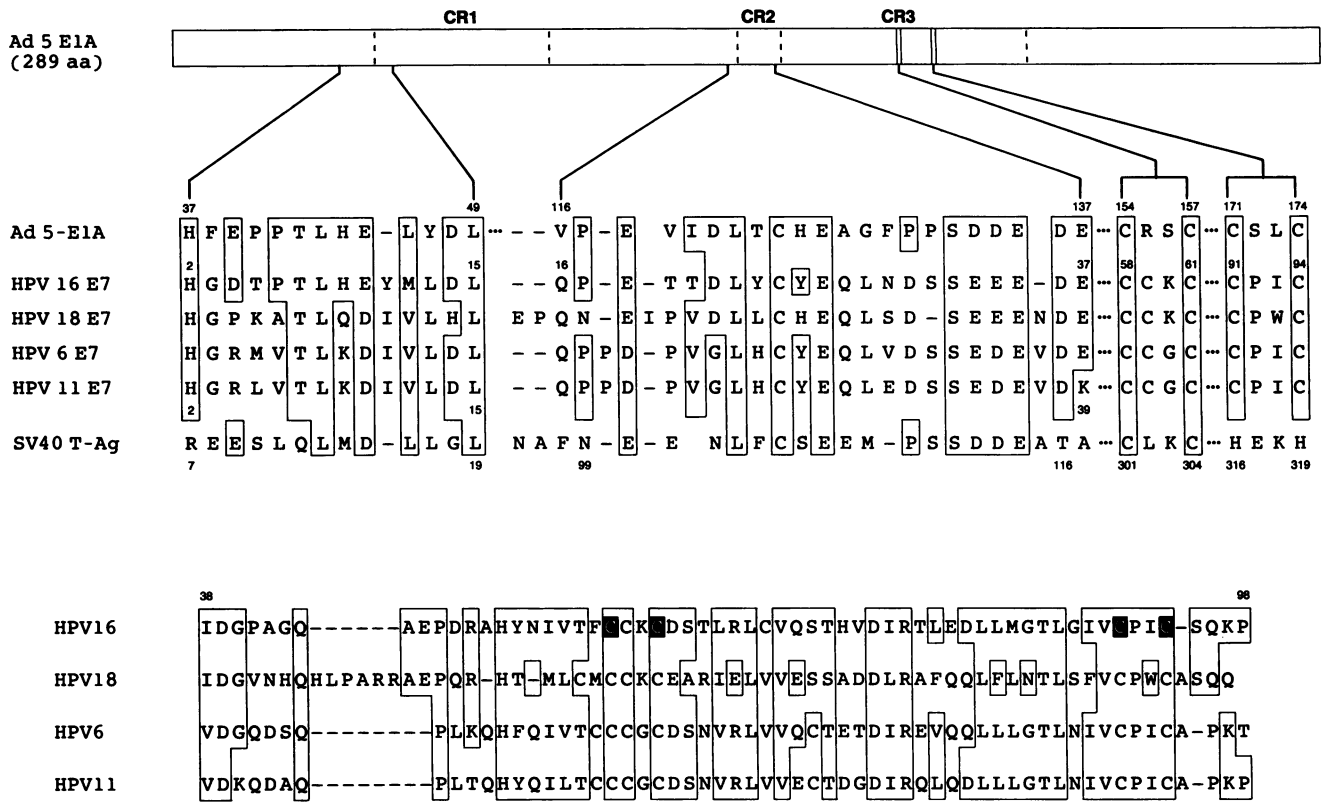

FIG. 1. (Top) Amino acid sequence homology between Ad E1A, HPV16 E7, and SV40 T antigen (T-Ag). Amino acids identical to those in Ad type 5 E1A are boxed. (Bottom) Amino acid sequence conservation in the carboxyl terminus of HPV E7. The carboxyl half of the E7 proteins from HPV types 16, 18, 6, and 11 are compared. Amino acids conserved with respect to the HPV16 E7 protein are boxed. The Cys-X-X-Cys motifs which are also found in CR3 of E1A are highlighted in black.

teine, and the E7 protein was immunoprecipitated with a polyclonal rabbit antiserum. In addition, Western blot analysis of unlabeled, immunoprecipitated E7 protein was used to confirm the relative levels of stable E7 protein that resulted from introduction of the various amino acid alterations. Under these conditions, [^{35}S]cysteine labeling was a more sensitive method for detection of transiently expressed E7 protein. Data from representative protein gels are shown for each domain, and although somewhat different gel conditions and immunoprecipitation washes were employed for some experiments, a reconstructed wild-type E7 was always included as an internal control.

Stability of HPV16 E7 mutants. The amino acid mutations in domains 1, 2, and 3 are depicted in Fig. 3, 4, and 5, respectively. Amino acids which are conserved with respect to Ad E1A are indicated by solid circles. The two point mutants in Fig. 3, p1460 and p1461, make comparable amounts of stable E7 protein compared with a reconstructed wild type, indicating that any biological effect is not a result of gross alteration of the intracellular stability of the mutated protein. By contrast, substitution of a single valine residue for D-14 and L-15 in p1468 which is analogous to that described for E1A (23) resulted in an unstable protein which was undetectable by either ^{35}S labeling or Western blot analysis. The deletion mutant (P-6 T-7 L-8 H-9 E-10), p1469, which has been previously described (30), also was found to encode a stable polypeptide.

The introduction of various mutations into domain 2 of the E7 protein (Fig. 4) led to a substantial reduction in protein stability in only one case, that of p1455 (S-32 to C). Each of the other amino acid substitutions and deletions did not significantly reduce the intracellular stability of the protein.

Amino acid changes in the carboxyl terminus of the E7 polypeptide (Fig. 5) in many instances resulted in unstable proteins. Substitution or deletion of cysteine residues (p1650, p1651, p1656, and p1660) resulted in an unstable protein that could not be detected by either ^{35}S labeling or Western blotting (data not shown). Deletions of six to eight amino acids in p1657, p1658, and p1659 each resulted in a partial reduction in protein stability; however, the deletion of the carboxyl-terminal Cys-X-X-Cys motif, p1660, had the most profound effect on the level of stable protein, since little or no E7 could be detected. The amino acid substitutions in p1647, p1648, and p1649 were not assessed for protein stability, although, as will be discussed below, they were fully wild type in their biological characteristics, suggesting that they made stable E7 proteins. The introduction of premature termination codons resulting in the deletion of as few as nine amino acids (I-89 to stop) resulted in biologically inactive mutant proteins presumably because of reduced polypeptide stability (data not shown). These mutations were not analyzed further.

Biological activity of the E7 mutant proteins. Each of the stable, mutated proteins was then assessed for biological activity (Tables 1 to 3). The two point mutations in domain 1, p1460 and p1461, demonstrated a wild-type capacity for cooperation with an activated *ras* oncogene in the transformation of BRK cells in three separate experiments. Further-

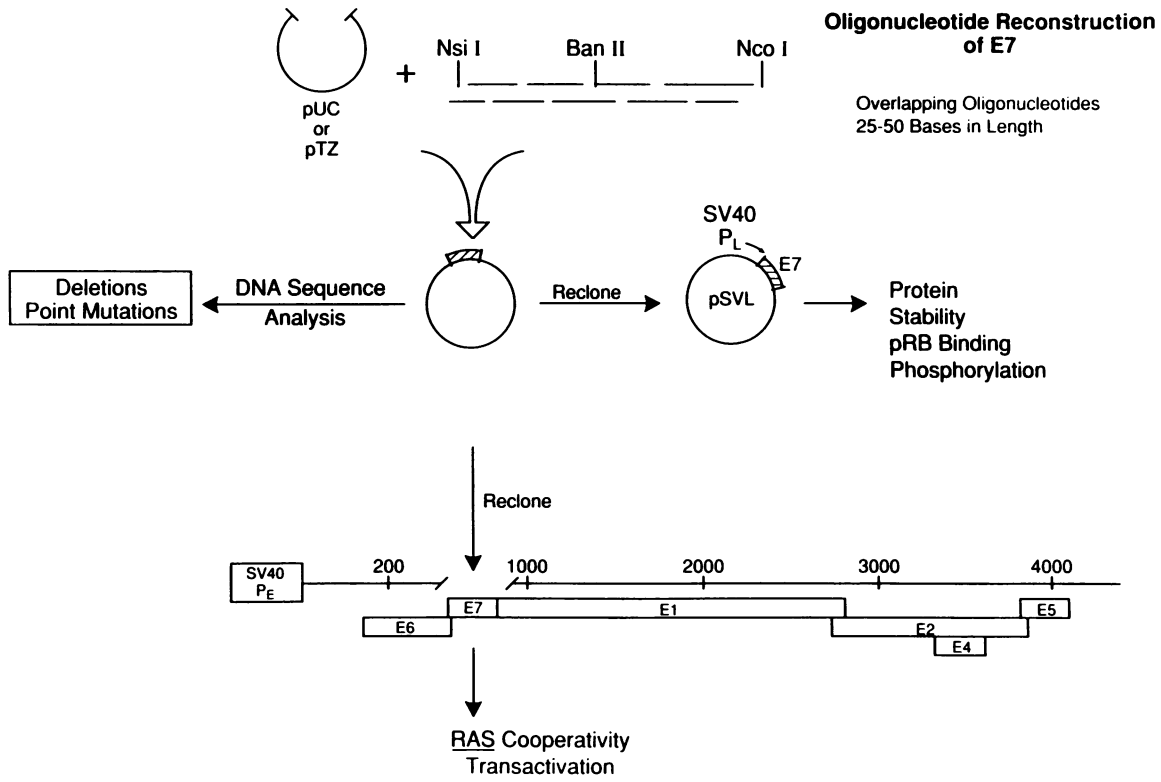

FIG. 2. A schematic diagram of the mutagenesis. Site-specific mutations were created in HPV16 E7 by oligonucleotide reconstruction between the *Nsi*I and *Ban*II sites in the N terminus and between the *Ban*II and *Nco*I sites in the C terminus. Annealed oligonucleotides were cloned into pUC19 or pTZ19, and mutations were verified by DNA sequence analysis. Selected mutant E7 genes were subcloned into the entire early region of HPV16 positioned downstream of the SV40 early promoter or in the SV40 late promoter vector pSVL.

more, these amino acid changes had little or no effect on the *trans*-activation function of E7, showing 100 and 79% of wild-type activity, respectively. The deletion mutant p1469 possessed interesting biological properties in that it was completely inactive in *ras* cooperativity but retained substantial *trans*-activating capacity (70% of wild type). These data indicate that the *trans*-activation and transformation functions can be separated because *ras* cooperativity, but not the *trans*-activation property, requires an intact domain 1.

Examination of the biological activity of the domain 2 mutations (Table 2) revealed a good correlation between *trans* activation and *ras* cooperativity in this domain. Mutations that did not severely impair the *trans*-activating function of E7, including p1454 (E-35 to Q), p1646 (S-31 S-32 deletion), and p1465 (E-35 D-36 E-37 deletion), had no effect on *ras* cooperativity. In contrast, alterations in the NH_2-terminal half of domain 2 eliminated both the *trans*-activating and transforming functions: p1457 (C-24 to S), p1458 (E-26 to Q), and p1467 (D-21 L-22 Y-23 C-24 deletion). These data indicate that amino acid sequences critical for both the *trans*-activating and transforming functions of E7 essentially overlap in the NH_2-terminal portion of domain 2. This region was previously identified as critical to the ability of E7 to interact with the pRB (5, 6, 30). Alterations in the C terminus of domain 2 selectively reduced the levels of *trans* activation, suggesting that these sequences play some indirect role in transcriptional activation of the Ad E2 promoter.

In domain 3, there was generally a very good correlation between *ras* cooperativity and the ability of E7 to *trans* activate the Ad E2 promoter (Table 3). E7 mutants that had wild-type levels of *trans* activation (p1647, p1648, p1649, p1653, and p1654) possessed essentially wild-type transformation activities. In contrast, those amino acid changes that resulted in a substantial reduction in *trans*-activation levels (p1652, p1657, p1658, and p1659) had a diminished capacity to cooperate with an activated *ras* oncogene. In each case, as shown in Fig. 3, these latter mutations which diminished the biological activity of the E7 protein also substantially reduced the intracellular protein stability. One exception in domain 3, p1655, (L-82 L-83 to R R) can be noted. This mutant E7 polypeptide was stable and possessed good transformation capacity but was reduced in its *trans*-activating capacity (approximately 39% of wild type). This suggests that these leucine residues, which are well conserved in the E7 proteins of other genital tract-associated HPVs, play some direct or indirect structural role in the *trans*-activating function that is not important to the transformation functions.

In vivo pRB binding. In a previous study, domain 2 was found to be responsible for the ability of HPV16 E7 to associate with the pRB protein (30). That work also demonstrated that the E7 proteins from the genital tract-associated HPVs exhibit various capacities for the direct association with pRB depending on whether the E7 protein is encoded by a high-risk virus (such as HPV16 or HPV18) or a low-risk virus (such as HPV6 or HPV11) (18, 30). To further evaluate the role of this domain in pRB association, the domain 2 mutants of HPV16 E7 were examined for the capacity to associate with pRB in vivo.

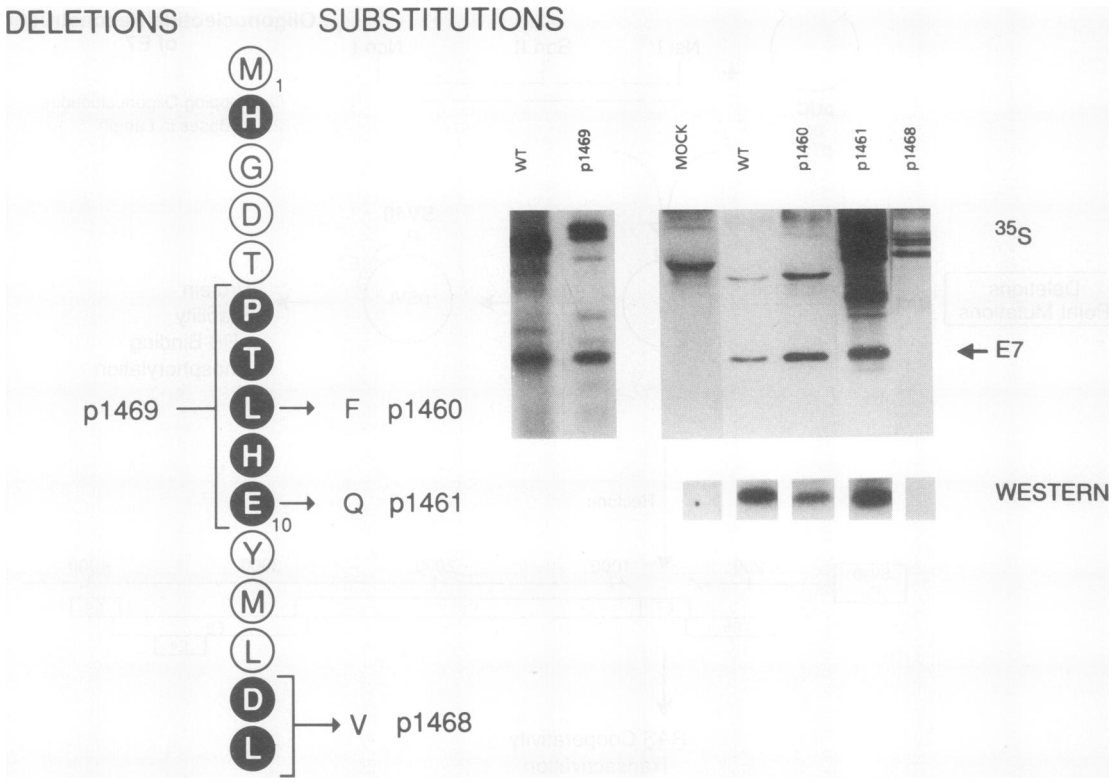

FIG. 3. Mutations in CR1. The amino acid sequence of the HPV16 E7 gene from M-1 to L-15 is shown. Amino acid residues which are also found in Ad E1A are indicated by shading and boldface type. Deletions are indicated on the left and amino acid substitutions are indicated on the right. An assessment of the intracellular stability of the mutant proteins is provided by the autoradiographs on the far right. COS-7 cells were transfected with plasmids expressing the indicated mutations from the SV40 late promoter. In the top panel, cells were metabolically labeled with [^{35}S]cysteine and immunoprecipitated. Representative lanes were excised and aligned horizontally according to the position of the E7 protein as indicated by the arrow. In the bottom panel, transfected COS-7 cells (unlabeled) were immunoprecipitated with an HPV16 E7 rabbit polyclonal antiserum, Western blotted with an HPV16 E7 monoclonal antibody (no. 100201; Triton Diagnostics), and detected with ^{125}I-labeled goat anti-mouse antiserum. Protein was separated in SDS-PAGE (12% polyacrylamide) gels, and autoradiography was for 1 to 4 days. The polypeptide derived from the deletion mutant, p1469, was immunoprecipitated with a mouse monoclonal antibody (7F3) specific to the carboxyl terminus of HPV16 E7, which was provided by Triton Diagnostics.

COS-7 cells were transfected with plasmids that expressed the CR2 mutations from the SV40 late promoter. The cells were lysed under conditions that would be expected to preserve the pRB-E7 intracellular protein complex. The protein lysates were then immunoprecipitated with a rabbit polyclonal antibody to HPV16 E7, washed, and subjected to SDS-PAGE. After electrophoretic transfer to nitrocellulose, coprecipitated pRB was detected by immunoblot analysis as shown in Fig. 6. On the far left is a positive control in which pRB was immunoprecipitated with an antibody to pRB from actively growing COS-7 cells. Transfection with a plasmid expressing wild-type E7 (p1453) is shown on the far right, and, as can be seen, a substantial amount of pRB was immunoprecipitated by the E7 antibody, presumably reflective of the stable in vivo association between E7 and pRB. Mutations in the carboxyl end of CR2 within the casein kinase II (CKII) domain (p1454, p1646, and p1465) had no apparent effect on the ability of E7 to associate with pRB. In contrast, mutations in the previously described pRB binding domain (p1467, p1457, and p1458) impaired the ability of E7 to associate with pRB in vivo. It had been previously shown that the mutations within p1467 (D-21 L-22 Y-23 C-24 deletion) and p1458 (E-26 to Q) virtually eliminated the in vitro association with pRB while the mutation within p1457 (C-24 to S) reduced but did not abolish pRB binding. These data are entirely consistent with those from the in vitro mixing experiments (6, 30) and further support the concept that these are stable, biologically relevant heteromeric protein complexes.

The biological data presented in Table 2 confirm that both *ras* cooperativity and Ad E2 promoter *trans* activation require overlapping sequences within domain 2 for activity. Mutations in p1458 and p1467 completely abrogated both biological activities, while the mutation in p1457, which still retained some pRB binding capacity, exhibited a residual (35% of wild type) capacity for *trans* activation but no apparent transforming activity. These data suggest that the pRB binding region, and likely the association with pRB itself, is critical to both transformation and *trans*-activating functions of E7.

E7 phosphorylation. HPV E7 is a phosphoprotein (44) and is a substrate for CKII phosphorylation (6, 16) at one or more serine residues (47). The consensus recognition sequence of the HPV E7 proteins, which is also found in Ad E1A and SV40 large T antigen, is conserved within the carboxyl end of CR2, and in HPV16 E7, the predicted CKII site is between D-30 and E-37 (D S S E E E D E). Therefore, a selected set of E7 mutants were analyzed for the ability to be phosphorylated in vivo in COS-7 cells. Duplicate plates were transfected, and one was labeled with [^{35}S]cysteine and

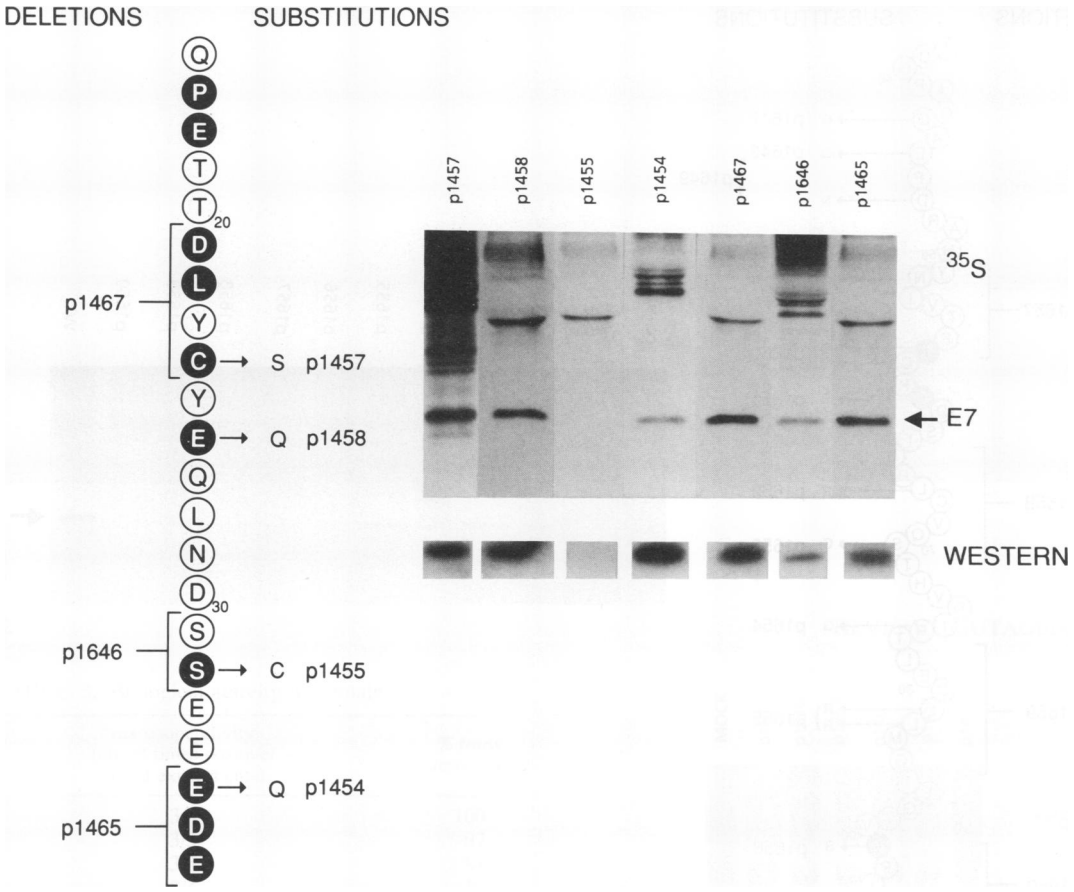

FIG. 4. Mutations in CR2. The amino acid sequence between Q-16 and E-37 is shown. Amino acid residues that also are found in Ad E1A are indicated by shading and boldface type. Analyses of intracellular protein stability are shown on the right, including both metabolically labeled cells ([^{35}S]cysteine) and Western blots.

one was labeled with $^{32}P_i$. The ^{35}S-labeled profiles demonstrate that roughly equivalent amounts of each of the proteins were expressed in vivo after transfection (Fig. 7, top). The bottom panel of Fig. 7 is an autoradiograph of ^{32}P-labeled proteins, and the position of the E7 protein is indicated by the arrow. A wild-type control (p1453) is shown on the far right, and a mock-transfected negative control is shown on the far left. As expected, alteration in the CKII recognition sequence, p1454 (E-35 to Q) or p1465 (E-35 D-36 E-37 deletion), significantly reduced the ability of the protein to be phosphorylated in vivo. In addition, deletion of the putative target serine residues (S-31 S-32) in p1646 completely eliminated E7 phosphorylation in vivo, confirming that one or both of these serines are the major sites of phosphorylation. In contrast, deletion of the pRB binding sequences in p1467 had no effect on the ability of E7 to be phosphorylated, and substitution of S-71 (S-71 to C), a conserved serine in the carboxyl portion of E7, also had no effect on phosphorylation, indicating that it is not a site for serine phosphorylation.

Examination of the biological activities of the CKII mutations (Table 2) indicated that phosphorylation-negative mutants were unaffected in their transformation function assayed by *ras* cooperativity; however, *trans* activation was reduced somewhat in p1465 (56% of wild-type) and p1646 (65% of wild type). Therefore, phosphorylation of the serine residues in CR2 by CKII is not essential for either the transformation or the *trans*-activation functions of E7.

DISCUSSION

In this study, a series of mutations in the HPV16 E7 protein were generated and assayed for their effects on intracellular protein stability, the ability to cooperate with *ras* in transformation of BRK cells, the ability to activate the Ad E2 promoter, pRB association, and phosphorylation. The goal of this work was to provide a better definition of the functional domains of the HPV16 E7 oncoprotein involved in transformation and transcriptional *trans* activation.

Previous genetic studies indicated that two conserved regions of Ad E1A, CR1 and CR2, are necessary for the functions involved in cellular transformation, including the stimulation of host cellular DNA synthesis, cellular immortalization, *ras* cooperativity, pRB association, and transcriptional repression (9, 24, 32, 56). These functional domains are physically distinct from CR3 of E1A, which has been shown to be the primary determinant of E1A-mediated *trans* activation (25, 27, 55).

Although the major *trans*-activating domain of E1A has been identified as CR3, a number of studies have indicated that the 243-amino acid product of the 12S E1A mRNA, which contains the transforming domains (CR1 and CR2) but not CR3, has *trans*-activation properties (10, 32). The 12S E1A product will activate a subset of the promoters that are responsive to E1A (12S plus 13S), including the Ad E2 promoter. Activation of the Ad E2 promoter by 12S E1A is at least in part mediated by alteration of heteromeric protein

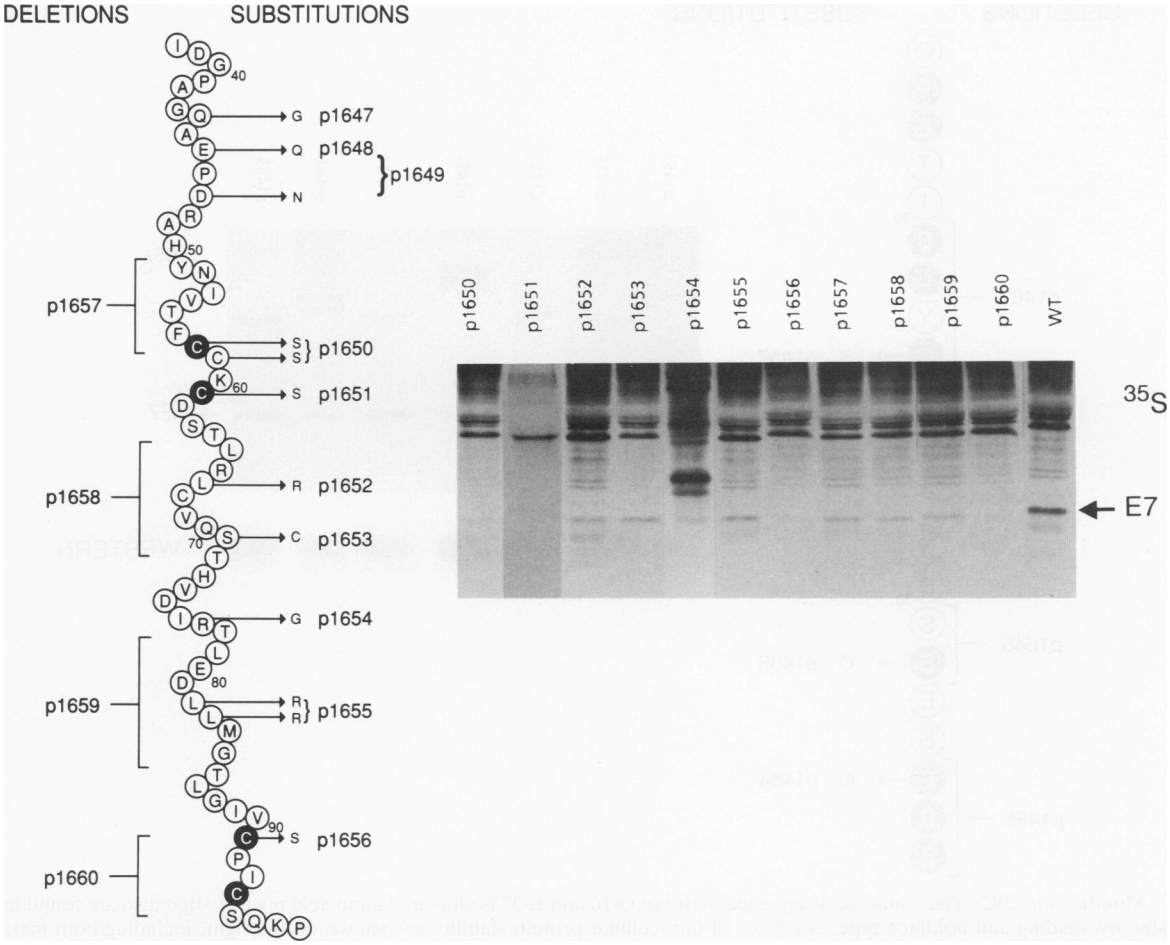

FIG. 5. Mutations in CR3. The amino acid sequence between I-38 and P-98 is shown. The conserved cysteines are indicated by shading. Analysis of protein stability by metabolical labeling of transfected COS-7 cells is shown as previously described. Western blots are not shown.

complexes containing the cellular transcription factor E2F (1). The *trans*-activating properties of the HPV16 E7 protein are quite similar to those described for 12S E1A. HPV16 E7 mediates transcriptional *trans* activation through the E2F transcription factor, and E7, like E1A, can disrupt cellular protein complexes containing E2F (34).

The point mutations in domain 1 of E7 assayed in this study had no effect on transformation (p1460 and p1461); however, deletion of five conserved amino acids, P T L H E (p1469), completely eliminated the ability of E7 to cooperate with *ras*. This deletion has no effect on the ability of E7 to associate with pRB in vitro (30). These results indicate that domain 1 plays a crucial role in cellular transformation independent of pRB binding and further indicate that although pRB binding is required for *ras* cooperativity, it alone is not sufficient. These data are in agreement with other analyses, indicating a critical role for CR1 sequences of E1A (54) and of HPV16 E7 (5) in transformation that do not affect pRB association.

Although previous analyses of the HPV16 E7 protein determined that domains 1 and 2 are required for transformation, the domains of the protein involved in *trans* activation have not been well delineated. Some mutational analyses indicated that the integrity of domain 2 is important for *trans* activation (15, 36); however, other studies concluded that either the entire polypeptide (51) or simply the carboxyl terminus (39) is required for *trans* activation. The results of the present study clearly indicate that it is the integrity of the CR2 sequences that is critical to *trans*-activation activity and

TABLE 1. Biological activity in domain 1

Plasmid	*ras* cooperativity (no. of times positive/ no. of experiments)	% *trans* activation
p1460	3/3	100
p1461	3/3	79
p1469	0/3	70

TABLE 2. Biological activity in domain 2

Plasmid	*ras* cooperativity (no. of times positive/ no. of experiments)	% *trans* activation
p1457	0/3	35
p1458	0/3	<5
p1454	3/3	97
p1467	0/3	<5
p1646	3/3	65
p1465	3/3	56

TABLE 3. Biological activity in domain 3

Plasmid	*ras* cooperativity (no. of times positive/ no. of experiments)	% *trans* activation
p1647	3/3	100
p1648	3/3	97
p1649	3/3	84
p1652	2/4	10
p1653	4/4	100
p1654	4/4	100
p1655	4/4	39
p1657	1/4	32
p1658	2/4	35
p1659	3/4	57

suggest that the primary effect of mutations in the carboxyl terminus result from structural perturbations that affect the intracellular stability of the protein.

It has been recently suggested that alterations of the CKII site preferentially affect *trans*-activation (6) and transformation functions (16). The role of serine phosphorylation or of the amino acids comprising the CKII site itself is not clear; however, the data presented in this study indicate that phosphorylation itself is not essential for transformation or transcriptional regulatory functions of the protein. However, the mutated E7 proteins that were poor substrates for phosphorylation (p1465 and p1646) were slightly impaired for *trans* activation of the Ad E2 promoter (56 and 65% of the wild type, respectively). The mutant p1454, which was wild-type in *trans* activation, demonstrated a much-reduced ability to be phosphorylated, suggesting that a high level of phosphorylation per se is not required. It is important that mutations in the CKII site did not alter the ability of E7 to interact with the pRB protein, indicating that substantial structural perturbations were not introduced by these mutations, and further, that in vivo phosphorylation plays no direct role in the association of E7 and pRB. Furthermore, mutations in the CKII domain described in this study do not substantially alter the acidic nature of this region (16). Thus, if maintenance of the negative charge in this domain is important, the deletions and amino acid substitutions described in this work would not have substantially altered this property.

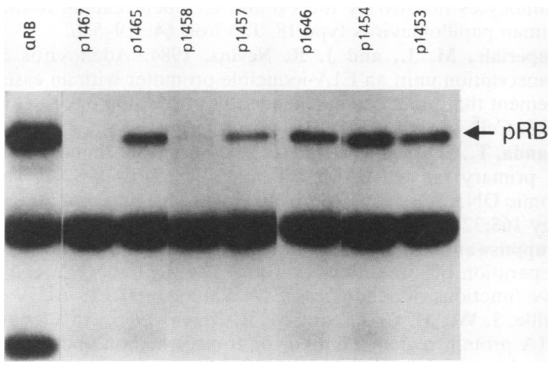

FIG. 6. pRB binding. Transfected COS-7 cells were lysed and immunoprecipitated with polyclonal antisera to HPV16 E7. Immune precipitates were run on SDS-PAGE (7.5% polyacrylamide) gels, electroblotted to nitrocellulose, and probed with a monoclonal antibody to human pRB. Detection was with ^{125}I-labeled goat anti-mouse antiserum. Autoradiography was for 2 to 4 days.

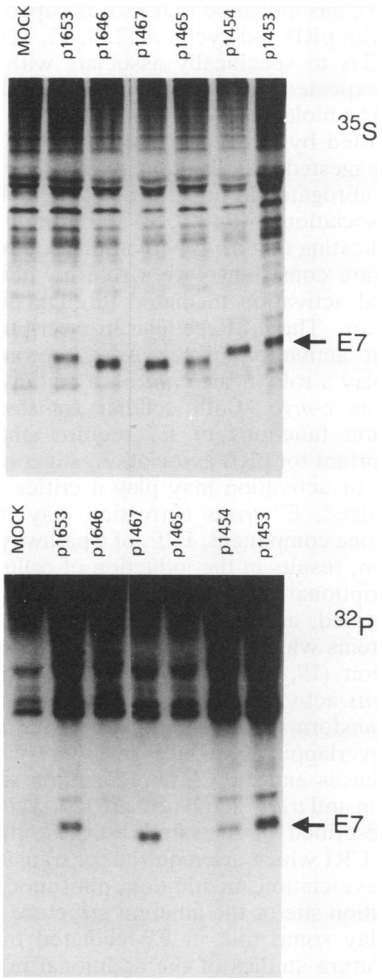

FIG. 7. Phosphorylation of E7 mutants. Transfected COS-7 cells were metabolically labeled with [^{35}S]cysteine or [^{32}P]P_i. Products of immunoprecipitations were separated on SDS-PAGE (12% polyacrylamide) gels.

Mutations in the carboxyl terminus of E7 generally affected the intracellular stability of the polypeptide. Both E1A and the HPV E7 proteins contain Cys-X-X-Cys motifs that have been implicated in the coordination of Zn^{2+} atoms. At least two functions, DNA binding activity (8) and protein dimerization (17), have been ascribed to polypeptide domains containing zinc thiolate complexes. As yet, there is no evidence that E7 is capable of sequence-specific DNA binding, and the role of Zn^{2+} chelation in E7 dimerization has not yet been explored. With the extended distance (30 amino acids) between the Cys-X-X-Cys motifs in E7, this protein is unlikely to form a classical zinc finger structure as originally proposed for transcription factor IIIa (26). Nevertheless, the zinc thiolate complex may be important for the structural integrity of the E7 protein. Recent studies with chimeric HPV6 and HPV16 E7 proteins have also shown that the carboxyl-terminal regions of both high- and low-risk E7 proteins are interchangeable and thus functionally equivalent (31). Therefore, substitutions which disrupt this framework would be expected to severely alter the tertiary structure of the protein. In this work, the deletion or substitution of cysteine residues in the carboxyl terminus generally resulted in an unstable, biologically inactive protein.

Recent work has indicated that E2F is capable of directly associating with pRB and cyclin A (2, 4, 11, 12). The ability of E7 and E1A to specifically associate with pRB would therefore be expected to play a role in the modulation of E2F complexes. The biological relevance of the E7-pRB interaction is supported by the recent studies of Scheffner et al. (41), which suggested that the putative regulatory function of pRB may be abrogated in transformed cells either by mutation or by association with the HPV E7 protein. The data in this study indicating that *trans* activation requires sequences in domain 2 are consistent with a role for pRB binding in transcriptional activation mediated through the E2F transcription factor. The E2F cellular transcription factor is necessary for activation of the Ad E2 promoter and is believed to play a role in the control of cell cycle-regulated genes such as c-*myc*. Both cellular transformation and *trans*-activation functions of E7 require amino acid sequences important for pRB association, suggesting that E2F displacement or activation may play a critical role in both activities. Indeed, E7 *trans* activation may represent the activation of one component, E2F, of a pathway that, during virus infection, results in the induction of cellular proliferation. Transcriptional *trans* activation by HPV E7 may be more complicated, as recent studies indicate that low-risk HPV E7 proteins which are defective for transformation or immortalization (19, 29) and pRB binding (30) can still efficiently *trans* activate the Ad E2 promoter (31).

Cellular transformation and transcriptional *trans* activation require overlapping sequences in CR2. The precise roles of CR1 sequences and the CKII recognition site in CR2 in transformation and *trans* activation are less well understood. Mutations described in this study suggest that there are sequences in CR1 which are required for transformation but not for pRB association. In addition, phosphorylation at the CKII recognition site or the inherent structure of the region itself may play some role in E7-mediated transcriptional activation. Future studies of the additional proteins which interact with E7 during the cell cycle and further studies of the mechanism of E2F activation are likely to elucidate the roles of these regions in the function of E7.

ACKNOWLEDGMENTS

We thank Greg Pahel and Dave Lobe for production of the E7 polyclonal antisera and Triton Diagnostics for the gift to P.H. and K.M. of the monoclonal antisera used for Fig. 3.

K.M. was supported by an advanced training grant from the Swiss National Science Foundation.

REFERENCES

1. **Bagchi, S., P. Raychaudhuri, and J. R. Nevins.** 1990. Adenovirus E1A proteins can dissociate heteromeric complexes involving the E2F transcription factor: a novel mechanism for E1A trans-activation. Cell **62**:4352–4356.
2. **Bagchi, S., R. Weinmann, and P. Raychaudhuri.** 1991. The retinoblastoma protein copurifies with E2F-I, an E1A-regulated inhibitor of the transcription factor E2F. Cell **65**:1063–1072.
3. **Baker, C. C., W. C. Phelps, V. Lindgren, M. J. Braun, M. A. Gonda, and P. M. Howley.** 1987. Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. J. Virol. **61**:962–971.
4. **Bandara, L. R., and N. B. La Thangue.** 1991. Adenovirus E1A prevents the retinoblastoma gene product from complexing with a cellular transcription factor. Nature (London) **351**:494–497.
5. **Banks, L., C. Edmonds, and K. H. Vousden.** 1990. Ability of the HPV16 E7 protein to bind RB and induce DNA synthesis is not sufficient for efficient transforming activity in NIH3T3 cells. Oncogene **5**:1383–1389.
6. **Barbosa, M. S., C. Edmonds, C. Fisher, J. T. Schiller, D. R. Lowy, and K. H. Vousden.** 1990. The region of the HPV E7 oncoprotein homologous to adenovirus E1A and SV40 large T antigen contains separate domains for RB binding and casein kinase II phosphorylation. EMBO J. **9**:153–160.
7. **Bedell, M. A., K. H. Jones, and L. A. Laimins.** 1987. The E6-E7 region of human papillomavirus type 18 is sufficient for transformation of NIH 3T3 and Rat-1 cells. J. Virol. **61**:3635–3640.
8. **Berg, J. M.** 1988. Proposed structure for the zinc binding domains from transcription factor IIIA and related proteins. Proc. Natl. Acad. Sci. USA **85**:99–102.
9. **Berk, A. J.** 1986. Adenovirus promoters and E1A transactivation. Annu. Rev. Genet. **20**:45–79.
10. **Braithwaite, A. W., C. C. Nelson, and A. J. D. Bellett.** 1991. E1a revisited: the case for multiple cooperative trans-activation domains. New Biol. **3**:18–26.
11. **Chellappan, S. P., S. Heibert, M. Mudryj, J. M. Horowitz, and J. R. Nevins.** 1991. The E2F transcription factor is a cellular target for the RB protein. Cell **65**:1053–1061.
12. **Chittenden, T., D. M. Livingston, and W. G. Kaelin.** 1991. The T/E1A-binding domain of the retinoblastoma product can interact selectively with a sequence-specific DNA-binding protein. Cell **65**:1073–1082.
13. **DeCaprio, J. A., J. W. Ludlow, J. Figge, J.-Y. Shew, C.-M. Huang, W.-H. Lee, E. Marsilio, E. Paucha, and D. M. Livingston.** 1988. SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. Cell **54**:275–283.
14. **Dyson, N., P. M. Howley, K. Münger, and E. Harlow.** 1989. The human papillomavirus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science **243**:934–937.
15. **Edmonds, C., and K. H. Vousden.** 1989. A point mutational analysis of human papillomavirus type 16 E7 protein. J. Virol. **63**:2650–2656.
16. **Firzlaff, J. M., B. Luscher, and R. N. Eisenman.** 1991. Negative charge at the casein kinase II phosphorylation site is important for transformation but not for Rb protein binding by the E7 protein of human papillomavirus type 16. Proc. Natl. Acad. Sci. USA **88**:5187–5191.
17. **Frankel, A. D., D. S. Bredt, and C. O. Pabo.** 1988. Tat protein from human immunodeficiency virus forms a metal linked dimer. Science **240**:70–83.
18. **Gage, J. R., C. Meyers, and F. O. Wettstein.** 1990. The E7 proteins of the nononcogenic human papillomavirus type 6b (HPV-6b) and of the oncogenic HPV-16 differ in retinoblastoma protein binding and other properties. J. Virol. **64**:723–730.
19. **Hawley-Nelson, P., K. H. Vousden, N. L. Hubbert, D. R. Lowy, and J. T. Schiller.** 1989. HPV16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. EMBO J. **8**:3905–3910.
20. **Hudson, J. B., M. A. Bedell, D. J. McCance, and L. A. Laimins.** 1990. Immortalization and altered differentiation of human keratinocytes in vitro by the E6 and E7 open reading frames of human papillomavirus type 18. J. Virol. **64**:519–526.
21. **Imperiale, M. J., and J. R. Nevins.** 1984. Adenovirus 5 E2 transcription unit: an E1A-inducible promoter with an essential element that functions independently of position or orientation. Mol. Cell. Biol. **4**:875–882.
22. **Kanda, T., S. Watanabe, and K. Yoshiike.** 1988. Immortalization of primary rat cells by human papillomavirus type 16 subgenomic DNA fragments controlled by the SV40 promoter. Virology **165**:321–325.
23. **Kuppuswamy, M., T. Subramanian, and G. Chinnadurai.** 1988. Separation of immortalization and T24-ras oncogene cooperative functions of adenovirus E1A. Oncogene **2**:613–615.
24. **Lillie, J. W., M. Green, and M. R. Green.** 1986. An adenovirus E1A protein region required for transformation and transcriptional repression. Cell **46**:1043–1051.
25. **Lillie, J. W., P. M. Loewenstein, M. R. Green, and M. Green.** 1987. Functional domains of adenovirus type 5 E1A proteins. Cell **50**:1091–1100.
26. **Miller, J., A. D. McLachlan, and A. Klug.** 1985. Repetitive zinc-binding domains in the protein transcription factor IIIA from *Xenopus* oocytes. EMBO J. **4**:1609–1614.

27. **Moran, E., B. Zerler, T. M. Harrison, and M. B. Mathews.** 1986. Identification of separate domains in the adenovirus E1A gene for immortalization activity and the activation of virus early genes. Mol. Cell. Biol. **6**:3470–3480.
28. **Moser, R., S. Frey, K. Münger, T. Hehlgans, S. Klauser, H. Langen, E.-L. Winnacker, R. Mertz, and B. Gutte.** 1987. Expression of the synthetic gene of an artificial DDT binding polypeptide in *E. coli*. Protein Eng. **1**:339–343.
29. **Münger, K., W. C. Phelps, V. Bubb, P. M. Howley, and R. Schlegel.** 1989. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. J. Virol. **63**:4417–4421.
30. **Münger, K., B. A. Werness, N. Dyson, W. C. Phelps, E. Harlow, and P. M. Howley.** 1989. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. EMBO J. **8**:4099–4105.
31. **Münger, K., C. L. Yee, W. C. Phelps, J. A. Pietenpol, H. L. Moses, and P. M. Howley.** 1991. Biochemical and biological differences between E7 oncoproteins of the high- and low-risk human papillomavirus types are determined by amino-terminal sequences. J. Virol. **65**:3943–3948.
32. **Nevins, J. R.** 1989. Mechanisms of viral-mediated trans-activation of transcription. Adv. Virus Res. **37**:35–83.
32a. **Pahel, G., and D. Lobe.** Unpublished data.
33. **Parada, L. F., C. J. Tabin, C. Shih, and R. A. Weinberg.** 1982. Human EJ bladder carcinoma oncogene is a homologue of Harvey sarcoma virus *ras* gene. Nature (London) **297**:474–478.
34. **Phelps, W. C., S. Bagchi, J. A. Barnes, P. Raychaudhuri, V. Kraus, K. Münger, P. M. Howley, and J. R. Nevins.** 1991. Analysis of *trans* activation by human papillomavirus type 16 E7 and adenovirus 12S E1A suggests a common mechanism. J. Virol. **65**:6922–6930.
35. **Phelps, W. C., and P. M. Howley.** 1987. Transcriptional *trans*-activation by the human papillomavirus type 16 E2 gene product. J. Virol. **61**:1630–1638.
36. **Phelps, W. C., K. Münger, C. L. Yee, and P. M. Howley.** 1990. Site directed mutagenesis of the HPV16 E7 gene. UCLA Symp. Mol. Cell. Biol. **124**:305–311.
37. **Phelps, W. C., C. L. Yee, K. Münger, and P. M. Howley.** 1988. The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1A. Cell **53**:539–547.
38. **Pietenpol, J. A., R. W. Stein, E. Moran, P. Yaciuk, R. Schlegel, R. M. Lyons, M. R. Pittelkow, K. Münger, P. M. Howley, and H. L. Moses.** 1990. TGF-β1 inhibition of c-*myc* transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains. Cell **61**:777–785.
39. **Rawls, J. A., R. Pusztai, and M. Green.** 1990. Chemical synthesis of human papillomavirus type 16 E7 oncoprotein: autonomous protein domains for induction of cellular DNA synthesis and for *trans* activation. J. Virol. **64**:6121–6129.
40. **Ruley, H. E.** 1983. Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature (London) **304**:602–606.
41. **Scheffner, M., K. Münger, J. C. Byrne, and P. M. Howley.** 1991. The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. Proc. Natl. Acad. Sci. USA **88**:5523–5527.
42. **Schwarz, E., U. K. Freese, L. Gissmann, W. Mayer, B. Roggenbuck, A. Stremlau, and H. zur Hausen.** 1985. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature (London) **314**:111–114.
43. **Smotkin, D., and F. O. Wettstein.** 1986. Transcription of human papillomavirus type 16 early genes in cervical cancer and a cervical cancer-derived cell line and identification of the E7 protein. Proc. Natl. Acad. Sci. USA **83**:4680–4684.
44. **Smotkin, D., and F. O. Wettstein.** 1987. The major human papillomavirus protein in cervical cancers is a cytoplasmic phosphoprotein. J. Virol. **61**:1686–1689.
45. **Spalholz, B. A., J. C. Byrne, and P. M. Howley.** 1988. Evidence for cooperativity between E2 binding sites in E2 *trans*-regulation of bovine papillomavirus type 1. J. Virol. **62**:3143–3150.
46. **Spalholz, B. A., Y.-C. Yang, and P. M. Howley.** 1985. Transactivation of a bovine papillomavirus transcriptional regulatory element by the E2 gene product. Cell **42**:183–191.
47. **Storey, A., N. Almond, K. Osborn, and L. Crawford.** 1990. Mutations of the human papillomavirus type 16 E7 gene that affect transformation, transactivation, and phosphorylation by the E7 protein. J. Gen. Virol. **71**:965–970.
48. **Storey, A., D. Pim, A. Murray, K. Osborn, L. Banks, and L. Crawford.** 1988. Comparison of the *in vitro* transforming activities of human papillomavirus types. EMBO J. **7**:1815–1820.
49. **Tanaka, A., T. Noda, H. Yajima, M. Hatanaka, and Y. Ito.** 1989. Identification of a transforming gene of human papillomavirus type 16. J. Virol. **63**:1465–1469.
50. **Vousden, K. H., J. Doniger, J. A. DiPaolo, and D. R. Lowy.** 1988. The E7 open reading frame of human papillomavirus type 16 encodes a transforming gene. Oncogene Res. **3**:167–175.
51. **Watanabe, S., T. Kanda, H. Sato, A. Furuno, and K. Yoshiike.** 1990. Mutational analysis of human papillomavirus type 16 E7 functions. J. Virol. **64**:207–214.
52. **Watanabe, S., and K. Yoshiike.** 1988. Transformation of rat 3Y1 cells by human papillomavirus type-18 DNA. Int. J. Cancer **41**:896–900.
53. **Whyte, P., K. J. Buchkovich, J. M. Horowitz, S. H. Friend, M. Raybuck, R. A. Weinberg, and E. Harlow.** 1988. Association between an oncogene and an anti-oncogene: the adenovirus E1a proteins bind to the retinoblastoma gene product. Nature (London) **334**:124–129.
54. **Whyte, P., H. E. Ruley, and E. Harlow.** 1988. Two regions of the adenovirus early region 1a proteins are required for transformation. J. Virol. **62**:257–265.
55. **Winberg, G., and T. Shenk.** 1984. Dissection of overlapping functions within the adenovirus type 5 E1A gene. EMBO J. **3**:1907–1912.
56. **Zerler, B., R. J. Roberts, M. B. Mathews, and E. Moran.** 1987. Different functional domains of the adenovirus E1A gene are involved in regulation of host cell cycle products. Mol. Cell. Biol. **7**:821–829.
57. **zur Hausen, H., and A. Schneider.** 1987. The role of papillomaviruses in human anogenital cancers, p. 245–263. *In* N. Salzman and P. M. Howley (ed.), The *Papoviridae* 2: the papillomaviruses. Plenum Press, New York.