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Analysis of *trans* Activation by Human Papillomavirus Type 16 E7 and Adenovirus 12S E1A Suggests a Common Mechanism

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The human papillomavirus E7 gene product is an oncoprotein with properties similar to those of the adenovirus E1A proteins. The human papillomavirus E7 proteins possess substantial amino acid sequence similarity to portions of conserved regions 1 and 2 of E1A, and the human papillomavirus type 16 E7 protein *trans*-activates the adenovirus E2 early promoter. Analysis of point mutations in the E2 promoter indicated that the E2F recognition sites were critical to E7 stimulation. In contrast to the activation of the E2 promoter, E7 could not *trans*-activate various other E1A-inducible promoters. Although the promoter specificity for E7 differs from that of 13S E1A *trans* activation, it is very similar to activation by the E1A 12S product. Moreover, analysis of the E7 protein has suggested that amino acid sequences critical for *trans* activation include those shared with E1A within conserved region 2. Biochemical studies demonstrate that the E7 protein, like the 12S E1A product, can alter the interaction of cellular factors with the E2F transcription factor. We therefore conclude that E7 *trans* activation is functionally related to that mediated by the 12S E1A product.

The major transforming proteins of the mucosal associated human papillomaviruses (HPVs) (e.g., HPV types 6, 11, 16, and 18) have been shown to be encoded by the E6 and E7 open reading frames. Integration of HPV16 or HPV18 sequences in human tumor tissue or in derived cell lines invariably results in the retention and continued expression of the E6-E7 region, suggesting that these proteins may be required for continued cell proliferation (4, 51, 52, 66). The E7 genes of HPV16 and HPV18 have been shown to transform a variety of established rodent cells (7, 23, 44, 59, 60), and E7 is capable of cooperating with an activated *ras* oncogene in the transformation of primary rodent cells (30, 44, 58). More recently, it has been demonstrated that the E6 and E7 genes together are sufficient for the immortalization of primary human keratinocytes (19, 20, 33).

Like the adenovirus E1A gene products and simian virus 40 (SV40) T and t antigens, the E7 protein is a multifunctional protein with both immortalizing activity and transcriptional modulatory functions. Substantial amino acid sequence similarities also exist among these proteins within conserved regions 1 and 2 (CR1 and CR2) of E1A (43, 44). The HPV E7 proteins will, like E1A and large T antigen, associate with the product of the retinoblastoma susceptibility gene (pRB) through CR2 (11, 12, 62). These conserved amino acid sequences in the E7 protein required for pRB binding are also required for transformation (13, 35, 42, 61).

A critical issue, of course, is the biochemical basis for the transforming function of these viral oncoproteins. Previous experiments have shown that E7 can function as a *trans* activator of transcription, an activity shared by adenovirus E1A and SV40 T antigen. Moreover, the transcriptional activating function of E7 has been demonstrated by using the E1A-inducible adenovirus E2 promoter, which can also be *trans*-activated by the SV40 T antigen (28), suggesting a common functional relationship. The functional similarities among these viral oncoproteins are intriguing in that each displays cell transformation functions, each encodes a protein that binds to cellular proteins including the pRB product, and each can *trans*-activate the same target promoter. However, *trans*-activation functions of E1A have been generally considered to be separate from the oncogenic activity of E1A. Specifically, the major E1A *trans*-activation function depends on sequences within CR3 (15, 24, 26, 31, 32, 50) that are not shared with T antigen and E7. A potential resolution of this discrepancy has been provided by recent experiments which have established a mechanism for *trans* activation of the E2 promoter by the E1A 12S product (2). This activity is directed at the E2F factor, a key component for the activation of E2 transcription. Moreover, this activity is dependent on E1A sequences within CR1 and CR2, including sequences shared with HPV E7. In this study, we demonstrate that E7 *trans* activation is similar to that mediated by the E1A 12S product and that E7 also targets the E2F factor, resulting in alteration of the proportion of E2F found in heteromeric protein complexes. Thus, we conclude that E7 and 12S E1A are functionally related.

MATERIALS AND METHODS

Cells. African green monkey kidney CV-1 cells and mouse L cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Recombinant plasmid DNAs. The adenovirus type 5 E2CAT constructs −80/−70, −64/−60, −45/−36, −64/−60 + −45/−36, and −75/71 + −65/61 were kindly provided by Mary Loeken (29). The hsp70CAT plasmid has been previously described (54). The plasmid pE3CAT contains E3 promoter sequence between +30 (*Sac*I) and −237 (*Eco*RI) cloned into pBluescript-KS upstream of a *Bgl*II-*Bam*HI

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fragment from pCAT3M. The pE4CAT plasmid contains E4 promoter sequence between +32 (*Taq*I) and −224 (*Hph*I) cloned into the *Acc*I-*Sma*I sites of pUC13. Chloramphenicol acetyltransferase (CAT) coding sequence was inserted downstream at the *Hin*dIII site and was derived from the *Hin*dIII fragment of pCAT3M. The plasmid p1059 has been previously described (44) and consists of the entire early region of HPV16 (nucleotides 79 to 4468) placed downstream of the SV40 early promoter. Mutations were created in the E7 open reading frame by oligonucleotide reconstruction and were cloned back into the p1059 background (42) to create p1465(ΔEDE) and p1467(ΔDLYC).

Mammalian cell transfections. DNA transfections for transient CAT assays were performed by calcium phosphate coprecipitation as previously described (40) using a total of 10 to 15 μg of DNA per 60-mm dish. At 4 h after DNA addition, the cells were treated with 15% glycerol for 1 min, the monolayer was washed twice with fresh medium, and the cells were incubated in complete medium containing 5 mM sodium butyrate (pH 7.0) for 48 h.

CAT assays. Assays of CAT were performed as previously described (57). Briefly, 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, and the products were separated by ascending thin-layer chromatography in chloroform-methanol (95:5). Fractionated products were localized by autoradiography, and acetylated and unacetylated spots were excised for quantitation by liquid scintillation (Ready-Safe; Beckman). Cell lysates were quantitated for total protein concentration by using the Bio-Rad protein assay (Bio-Rad Laboratories) so that each CAT reaction was conducted with equivalent amounts of protein. CAT assay reaction mixtures contained 7.5 to 100 μg of protein and were incubated for 0.5 to 2.5 h.

Expression and purification of glutathione *S*-transferase-E7 fusion proteins. Full-length wild-type and mutant HPV16 E7 genes were cloned in frame with the glutathione S-transferase gene in pGex-2T (Pharmacia) by using polymerase chain reaction. The primers used were GGGGATCCATGCATGGAGA TACACCTACA (sense primer) and CCCTGAATTCATCT TATGGTTTCTGAGAACAGATGGGG (antisense primer). Polymerase chain reaction products were gel isolated from 1% agarose gels, cleaved with *Eco*RI and *Bam*HI, and cloned into pGex-2T.

Glutathione *S*-transferase HPV16 E7 proteins were expressed in *Escherichia coli* and purified as described elsewhere (56). Briefly, overnight cultures of *E. coli* HB101 containing the recombinant glutathione *S*-transferase E7 plasmids were diluted 1:10 in 500 ml of fresh Luria-Bertani broth containing 100 μg of ampicillin per ml. After 1 h, 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added, and the cells were grown for an additional 3 h. Cells were pelleted and washed extensively with phosphate-buffered saline (PBS). The cells were then lysed by mild sonication on ice in PBS containing 1% Triton X-100 (Sigma). The lysate was cleared by centrifugation (10,000 × *g*, 10 min, 4°C), and the supernatant was mixed with 1 ml of 50% glutathione-Sepharose (Pharmacia) in 1% Triton X-100–PBS at room temperature for 2 min. The Sepharose beads were collected by centrifugation (1,000 × *g*, 5 min, 4°C) and washed several times with 1% Triton–PBS. Fusion proteins were eluted with three washes of 0.5 ml of freshly prepared 5 mM reduced glutathione (Sigma) in 50 mM Tris HCl, pH 8.0. Fractions were pooled and stored in aliquots at −80°C. Protein concentrations were determined by a BioRad protein assay. Typical preparations yielded 15 to 30 mg of purified fusion protein per liter. The purity of the proteins was estimated to be >90% according to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Coomassie staining.

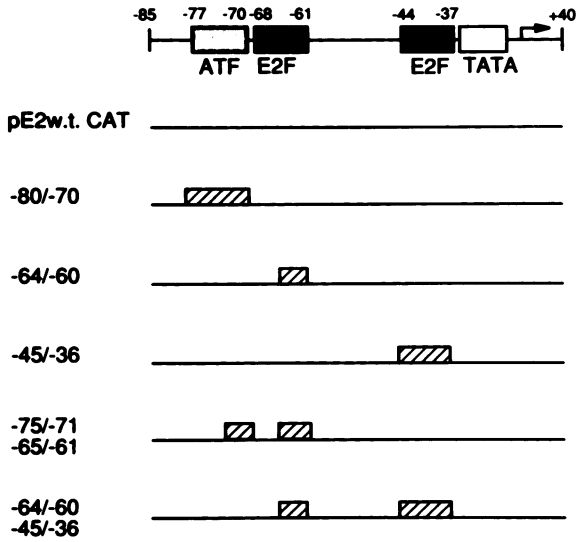
E2F binding assays. The isolation of the E2F-BF and E2F-I activities and the procedures for their assay with extracts containing E2F have been described elsewhere (47). The E2F binding activities, E2F-BF and E2F-1, were assayed on the basis of their abilities to alter the pattern of the E2F-DNA complex during gel retardation assays: E2F-BF interacts with E2F to yield a more slowly migrating DNA-protein complex and inhibits the formation of E2F-DNA complex, resulting in a loss of E2F-DNA complex in gel retardation assays. Typical assay mixtures (30 μl) for E2F-BF and E2F-I contained all the components required for E2F DNA binding as described before (2) and 5 μg of an F9 cell extract as the source of E2F. The incubations were started with the final addition of approximately 0.3 μg of hydroxylapatite- or 0.1 μg of glycerol gradient-purified E2F-BF or E2F-1 (47). After incubation for 30 min at room temperature, aliquots of the reaction mixtures were analyzed in gel retardation assays as described elsewhere (47).

RESULTS

Previous studies have established a functional and structural relationship between the HPV E7 and adenovirus E1A genes. Specifically, each displays oncogenic activity in the form of *ras* cooperativity, each encodes proteins that can *trans*-activate transcription as measured by activation of the adenovirus E2 promoter, and the two genes share amino acid sequence. Despite these general similarities, inconsistencies exist with respect to *trans* activation in that there is no extended sequence similarity within E7 to CR3 of adenovirus E1A, which has been previously defined as the principal *trans*-activating region of E1A (15, 26, 31, 32, 50) or, indeed, as sufficient in some assays for E1A *trans* activation (16, 27). In this study, we have further examined the relationship between *trans*-activation functions of the adenovirus E1A and HPV16 E7 proteins.

Requirements for E7 *trans* activation of the adenovirus E2 promoter. The HPV16 E7 protein is a transcriptional *trans* activator as defined in previous cotransfection assays which used the adenovirus E2 promoter (44). Analysis of a series of *Bal*31 deletion mutants of the adenovirus E2 promoter had previously indicated that the nucleotide sequences in the E2 promoter which were required for activation by HPV16 E7 and adenovirus E1A were similar (44) and included sequences within 79 nucleotides upstream of the major RNA start site. These studies, however, did not precisely identify the critical sequences involved in E7-mediated *trans* activation. More-recent experiments have defined four functional promoter elements that are required for E1A-induced transcription (29). These include an ATF (or CREB) site, two E2F recognition sites, and a TATA element (Fig. 1).

To assess the relative contributions of the individual transcription factor binding sites, a series of adenovirus E2CAT promoter mutants (29) were assayed for activation by HPV16 E7. Each of these promoter mutations consisted of nucleotide base transversions built into an E2 promoter containing 85 nucleotides of 5′ flanking sequence that has been previously shown to be fully responsive to *trans* activation by both adenovirus E1A (21, 22, 29) and HPV16 E7 (44). *trans* activation of the various mutated promoters was measured by cotransfection into CV-1 monkey cells and



	FOLD ACTIVATION			
	E1A		E7	
	L-Cells	CV-1	L-Cells	CV-1
pE2w.t. CAT	145.3	36.1	20.9	5.1
-80/-70	52.0	9.3	7.4	2.1
-64/-60	24.7	13.9	4.7	3.5
-45/-36	24.5	9.4	5.3	3.3
-75/-71 -65/-61	3.7	2.0	1.7	1.6
-64/-60 -45/-36	3.8	1.5	1.9	1.6

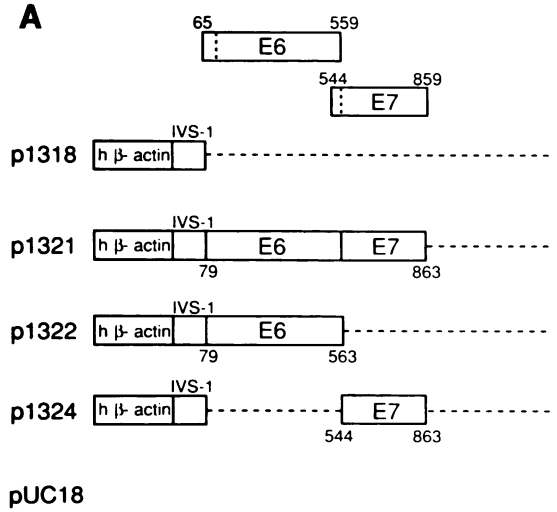
FIG. 1. E2 promoter elements essential for E7 activation. The E2CAT promoter mutations were provided by Mary Loeken (29) and consist of nucleotide base transversions built into an E2 promoter containing 85 nucleotides of 5′ flanking sequence. The ATF and E2F transcription factor recognition sites as well as the TATA element are shown at the top of the figure, and the regions of mutation are indicated both numerically (to the left) and schematically. Transfection of plasmids into both mouse L cells and CV-1 monkey cells utilized calcium phosphate coprecipitation. The numbers to the right express fold activation over the basal activity obtained by cotransfection with pUC18. The uninduced levels of acetylation were 0.2 to 0.45% in L cells and 0.35 to 0.9% in CV-1 cells. The values represent the averages of duplicate plates from at least two separate experiments.

mouse L cells with an E7-expressing or an E1A-expressing plasmid. Similar results were obtained in both cell lines, although the extent of *trans* activation was significantly higher in L cells. These cell lines have recently been shown to contain substantial levels of E2F in a multimeric complex which is important to transcriptional stimulation by E1A (2).

As observed previously (44) and as shown in Fig. 1, cotransfection of a plasmid expressing the HPV16 E7 gene product *trans*-activates the adenovirus E2 promoter, although the extent of the activation is considerably less than that achieved with the genomic E1A, which encodes both the 12S and the 13S products. As shown in Fig. 1, alteration of sequences within the ATF binding site (−80/−70) reduced the levels of *trans* activation by both E1A and HPV16 E7 by a factor of approximately 3. Thus, a promoter containing only the E2F binding sites clearly retained the ability to respond to E1A or E7. In contrast, *trans* activation was essentially abolished when the two E2F sites were eliminated, leaving only the ATF site intact. A promoter containing a single E2F site was also responsive to both E1A and E7, although only when coupled to the ATF site, suggesting that ATF binding can contribute to *trans* activation. Taken together, these results indicate that an ATF site alone is unable to mediate transcriptional activation of the E2 promoter, whereas a pair of E2F sites are sufficient. Moreover, the results indicate that the requirements for activation by E7 are the same as for activation by E1A. Additional assays described below provide further evidence that the E2F sites and the E2F factor are important targets for E7 *trans* activation.

Promoter specificity for E7 *trans* activation. The results presented in Fig. 1 confirm that E7 shares properties with E1A with respect to transcriptional *trans* activation. However, *trans* activation by E7 has been demonstrated only with the adenovirus E2 promoter, which is only one of a number of promoters known to be E1A inducible. Therefore, a study was initiated to determine whether other promoters which can be stimulated by E1A could be *trans*-activated by the HPV16 E7 gene product. Whereas cotransfection of plasmids encoding HPV16 E7 led to a 5- to 10-fold activation of the adenovirus E2 promoter, there was no activation of the adenovirus E3CAT, adenovirus E4CAT, or hsp70CAT by E7 (Fig. 2A). Thus, since each of these promoters is inducible by E1A, it is apparent that HPV16 E7 exhibits only a portion of the transcriptional activating potential of adenovirus E1A.

At least two explanations could account for the limited promoter specificity of activation by E7 compared with that by E1A. First, the E1A and E7 *trans* activators may have different mechanisms of action, which are mediated through distinct but overlapping targets in the adenovirus E2 promoter. Alternatively, E1A may possess multiple and distinct transcriptional modulatory functions, of which only a subset is shared by E7. Indeed, this latter possibility appears most likely, since the E1A gene encodes multiple proteins that are produced as a result of alternative RNA splicing (8, 39). The two major proteins of 289 and 243 amino acids produced in a lytic infection and in transformed cells are products of a 13S mRNA and a 12S mRNA, respectively. A number of experiments have demonstrated that *trans* activation of early viral transcription in a lytic infection is primarily dependent on the 13S E1A product. Indeed, as shown in Fig. 2B, the E1A 13S product could *trans*-activate each of the target promoters. Despite the fact that the 13S product is critical for activation of early viral transcription, various studies have shown that the 12S E1A product is also a *trans* activator (14, 25, 55, 64, 67). Moreover, recent experiments have shown that the 12S product can activate transcription dependent on E2F (2). As depicted in Fig. 2B, the 12S E1A product can indeed activate the E2 promoter. In contrast, the 12S E1A product was unable to stimulate the adenovirus E3 and E4 promoters. Although E7 *trans* activation does not follow the



B

	FOLD ACTIVATION			
	AdE2CAT	AdE3CAT	AdE4CAT	HSP70CAT
E1A 13S cDNA	17.1	10.6	100.3	15.0
E1A 12S cDNA	7.6	0.6	1.5	5.9

FOLD ACTIVATION			
AdE2CAT	AdE3CAT	AdE4CAT	HSP70CAT
0.9	1.1	1.1	0.6
8.4	0.5	1.3	0.7
1.4	0.8	0.7	0.7
5.2	0.8	0.8	0.6
1.0	1.0	1.0	1.0

FIG. 2. E7 does not activate other E1A-inducible promoters. (A) Analysis of CAT activity for transcriptional induction of promoter elements fused to the CAT gene. Plasmids encoding promoter sequences from the adenovirus E2, E3, and E4 and the human hsp70 genes (described in Materials and Methods) were cotransfected into CV-1 monkey cells together with the HPV16 E6 and/or E7 genes expressed from the human (h) β-actin promoter (34). The average uninduced levels of acetylation obtained by cotransfection with equivalent amounts of pUC18, which are indicative of the basal promoter activity, were variable: E2CAT, 1.0%; E3CAT, 9.0%; E4CAT, 5.0%; and hsp70CAT, 5.0%. (B) CV-1 cells were transfected by calcium phosphate coprecipitation with E2CAT (10 μg), E3CAT (2.5 μg), E4CAT (1 μg), and hsp70CAT (5 μg) together with 0.5 μg of 12S or 13S E1A cDNA. Values represent the averages of duplicate plates from at least two separate experiments.

pattern of activation mediated by the E1A 13S product, it does appear similar to the activation displayed by the 12S E1A product with regard to promoter specificity.

Analysis of the human heat shock promoter, hsp70, for stimulation by both E1A and HPV16 E7 suggests that although E1A 12S and E7 are similar, they are not identical. The hsp70 promoter is activated by the 12S E1A product in a viral infection (55) and, as shown here, by cotransfection with the E1A 12S-expressing plasmid (Fig. 2B). In contrast, the HPV16 E7 protein was unable to *trans*-activate the heat shock promoter. Therefore, if indeed E7 *trans* activation is functionally similar to that encoded by the 12S gene product, E7 does not appear to possess the full spectrum of activities exhibited by E1A 12S.

Sequences homologous to E1A CR2 are involved in E7 *trans* activation. Recent studies have shown that *trans* activation by the E1A 12S product is dependent on CR2 sequence as well as CR1 sequence. The finding that the HPV E7 protein exhibits a *trans*-activating function similar in nature to that of the adenovirus 12S E1A product suggests that sequences in E7 that are homologous to those in E1A may be involved in transcriptional stimulation. As shown in Fig. 3, deletion of amino acids in the carboxyl half of CR2 (ΔEDE) reduced *trans* activation by 30 to 50% in CV-1 cells and by about 70% in mouse L cells. Deletion of sequences in CR2 involved in binding pRB(ΔDLYC), however, completely abolished E7 activation of the E2 promoter, reducing CAT activity to background levels in both cell lines. Expression of these E7 mutant proteins in Cos-7 cells indicated that the proteins were stable (data not shown). Moreover, cotransfection of plasmids encoding the E7 mutants with the adenovirus E2CAT promoter mutations (depicted in Fig. 1) failed to reveal any additional specificity or complexity between the E7 polypeptide domains and the sequences in the E2 promoter which are critical for *trans* activation (data not shown). These results are in agreement with those of other studies which have indicated that amino acid sequences within CR2 are required for activation of the adenovirus E2 promoter (13, 42, 61). Thus, E7 *trans* activation appears to be a function of sequences related to those of the 12S E1A product.

E7 protein alters the interaction of E2F with cellular factors. In most cell types, the E2F transcription factor is found to be complexed with cellular proteins (2). Recent experiments have shown that the E1A 12S protein, which is dependent on sequences in CR2, is capable of dissociating these E2F protein complexes and releasing free E2F that can interact with a 19-kDa product of the E4 gene of adenovirus (2). The E2F-E4 complex binds to the E2 promoter with a high degree of cooperativity, resulting in the formation of a stable complex (17, 18, 48) and a stimulation of transcription (1, 38, 49, 65). Moreover, the E2F in these cellular complexes may be nonfunctional, since E2 transcription can be stimulated in the absence of E4 by conditions resulting in the release of E2F from these heteromeric complexes (2). Recent experiments have shown that the major complex detected in L cells or in S-phase NIH 3T3 cells contains the cyclin A protein (36). It is believed that the stable association observed between E1A and cyclin A (45) may result from the dissociation of the E2F-cyclin A complex. Since HPV E7 *trans* activation of the E2 promoter involves the E2F sites (Fig. 1) and since E7 shares substantial amino acid sequence similarity with E1A, the possibility that the E7 protein perturbs the association of E2F with cellular factors such as cyclin A was examined.

Initial experiments utilizing procedures similar to those previously used for E1A failed to provide evidence that the HPV16 E7 protein could dissociate the cellular E2F com-

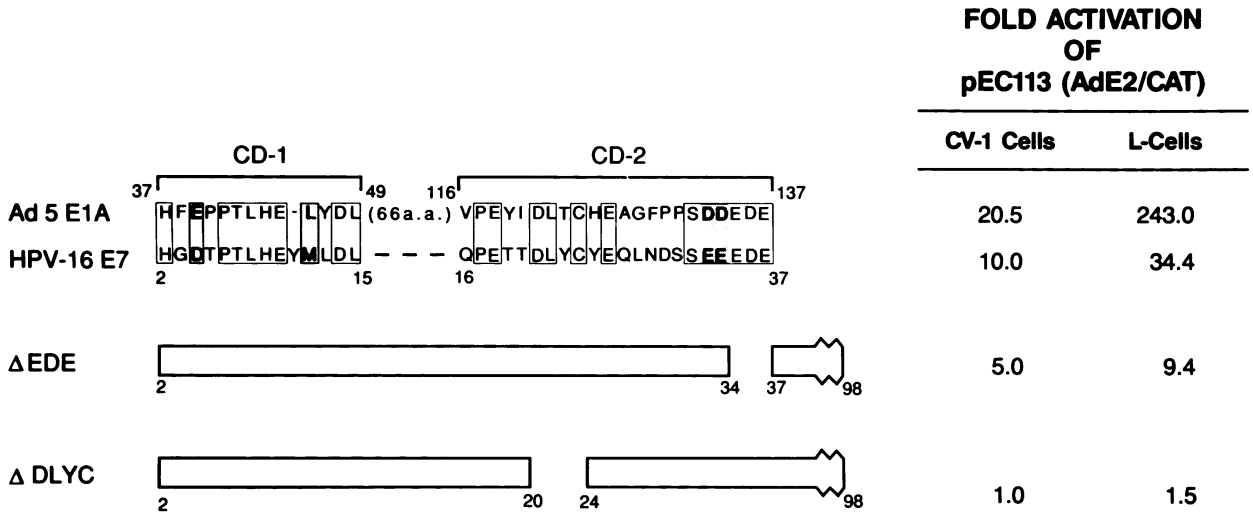


FIG. 3. E7 sequences homologous to E1A CR2 are required for *trans* activation. Specific deletions were created in E7 by oligonucleotide reconstruction and were then built back into the p1059 plasmid expressing the entire early region of HPV16 (44). These plasmids were cotransfected together with E2CAT into both CV-1 monkey cells and mouse L cells by the calcium phosphate coprecipitation technique and harvested 48 h posttransfection. The regions of amino acid sequence similarity between HPV E7 and E1A CR1 and CR2 are indicated at the top, and the positions of the amino acid deletions in E7 are schematically depicted below. The basal promoter activity obtained by cotransfection with pUC18 averaged about 1% for CV-1 cells and about 0.3% for mouse L cells. The values represent the averages of duplicate plates from at least two separate experiments. a. a., amino acids; Ad, adenovirus.

plexes. Dissociation of the E2F complex was not detected after incubation with HPV16 E7 synthesized in a reticulocyte lysate (data not shown). Although this result might suggest that the E7 protein did not function like E1A, it was also possible that there was less synthesis of active E7 in these extracts than of E1A or possibly that the E7 protein was functionally less active than E1A. Two additional approaches were therefore taken to increase the amount of the E7 protein in the in vitro assay and to improve the sensitivity of the assay. The first approach utilized E7 proteins produced in *E. coli* as glutathione *S*-transferase fusion proteins, thus permitting the introduction of considerably higher concentrations of protein into the assay.

Recent studies have led to the isolation of an E2F binding factor (E2F-BF) that can complex with E2F to produce the same apparent E2F-cyclin A complex as detected in crude extracts (47). Addition of the E1A protein during incubation of the two components prevents the formation of the complex. This assay appeared to be considerably more sensitive than the dissociation reaction. When this assay was used, it was apparent that the E7 protein can readily block E2F complex formation. As shown in Fig. 4, the addition of the E2F binding protein (E2F-BF) to E2F generates the typical E2F complex (E2Fc) as measured by gel retardation analysis. As has previously been observed for E1A, addition of the wild-type HPV16 E7 fusion protein to the incubation mix completely prevented the formation of E2Fc, whereas addition of a control glutathione *S*-transferase protein had no effect. Therefore, the E7 protein, like the E1A protein, does indeed possess the ability to prevent formation of the E2F complex.

Also shown in Fig. 4 are the results obtained when two E7 fusion proteins deleted of amino acid sequences which are homologous to portions of E1A CR2 were used. Deletion of sequences at the N terminus of the CR2-homologous region (ΔDLYC), which was shown to eliminate *trans* activation (Fig. 3), also abolishes the capacity of E7 to block the formation of the E2F-containing complex (E2F-BF). In contrast, deletion of sequences at the C terminus of CR2 (ΔEDE), which retained substantial *trans*-activation function (30 to 50%), had little effect on the activity of E7 in this assay. Thus, the HPV16 E7 protein can disrupt E2F complex formation, which is dependent on amino acid sequences that

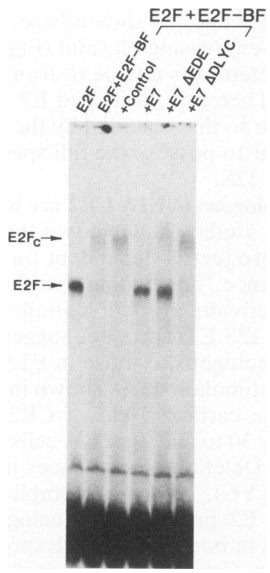


FIG. 4. HPV E7 protein can block the formation of the E2F complex. E2F DNA binding was assayed by gel retardation either alone (lane 1) or after incubation with 0.1 μg of the glycerol gradient-purified binding factor E2F-BF (lane 2) as described in Materials and Methods. Alternatively, the incubations included either 0.5 μg of the control glutathione transferase protein (lane 3) or 0.5 μg of the fusions with wild-type E7 protein (lane 4) or E7 mutants (lanes 5 and 6). The migration positions of the E2F complex and free E2F are indicated.

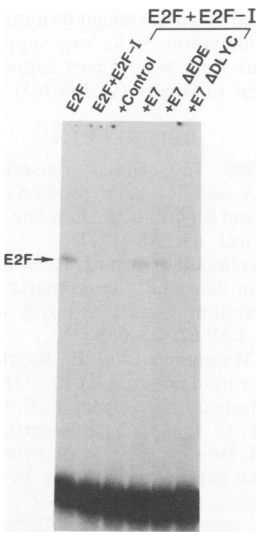


FIG. 5. HPV E7 protein can prevent the inhibition of E2F binding mediated by E2F-I activity. Assays were as described in the legend to Fig. 4 except that E2F was incubated with 0.1 μg of the glycerol gradient-purified E2F-I activity as described in Materials and Methods.

are homologous to E1A CR2 and essential for *trans* activation of the adenovirus E2 promoter.

Recent experiments have also demonstrated the presence of an activity, E2F-I, in extracts of mouse L cells which inhibits the binding of E2F to DNA (47). This inhibition may involve direct complex formation with E2F, since the inhibition can be reversed by treatment with deoxycholate and by incubation with E1A. Thus, inhibition of DNA binding appears to result from the formation of an E2F-I complex with E2F that blocks the ability of E2F to bind to DNA, perhaps in a fashion analogous to the NFκB-IκB interaction (5). The E7 protein, like E1A, can also block this inhibition. As shown in Fig. 5, the addition of the E2F-I factor abolished the binding of E2F to DNA, whereas the addition of the wild-type E7 fusion protein blocked this inhibition. Assays using E7 fusion proteins with deletions in CR2 produced a result similar to that observed for the prevention of the formation of the E2F complex. The EDE deletion mutation had no effect on the E7-mediated blocking of E2F binding by E2F-I, whereas the DLYC-deleted fusion protein was defective for this blocking function. These results suggest that the HPV16 E7 protein is functionally similar to adenovirus E1A with respect to its ability to alter E2F complexes. Furthermore, the region in the HPV16 E7 protein which is similar in amino acid sequence to CR2 of adenovirus E1A is required for these activities.

DISCUSSION

Previous experiments have demonstrated that the HPV E7 gene product can function as a transcriptional *trans* activator and specifically stimulate the adenovirus E2 promoter (44). HPV E7 and adenovirus E1A share primary amino acid sequences similarity, but the region of this similarity is not within CR3 of E1A, which has been shown to be critical for early viral transcription activation. Indeed, the region of E7 sequence similarity with E1A is within CR1 and CR2, and our analyses have shown that the region of homology to CR2 is important for E7 *trans* activation (13, 42, 61). Most analyses have focused on the role of the 12S E1A product in transcription repression and cellular transformation. Nevertheless, several reports have shown that the 12S product can also *trans*-activate, and a recent study has provided a mechanism for this activation. These experiments have shown that the E1A 12S product can *trans*-activate the E2 promoter, that this *trans* activation is dependent on sequences within CR1 and CR2, and that the E2F factor is targeted (2, 47). The promoter localization assays described in this paper demonstrate that the E2F binding sites are critical for E7-mediated *trans* activation, and biochemical assays demonstrate that E7 can block the formation of E2F complexes. On the basis of these results, we suggest that the mechanism for E7 activation likely involves an action similar to that of the 12S E1A product, that is, an ability to block the formation of the E2F complexes, which apparently represent an inactive state of the E2F factor, at least with respect to E2 transcription (2, 36). We suggest that the ability of E1A or E7 to alter these E2F interactions generates free E2F that can stimulate transcription from the E2 promoter.

Although many studies have shown that a variety of promoters can be stimulated by E1A, often leading to the characterization of E1A *trans* activation as promiscuous, this clearly pertains only to the 13S E1A product. This contrasts with HPV E7, which can *trans* activate the adenovirus E2 promoter but not the adenovirus E3 or E4 or the hsp70 promoter, as shown in this study. Furthermore, the SV40 early promoter, the Rous sarcoma virus and human immunodeficiency virus long terminal repeat promoters, and the HPV16 p97 promoter are not stimulated by E7 (41). These various enhancer-promoter sequences contain a number of both general and specific transcription factor binding sites but lack E2F recognition sites, supporting the conclusion that the E2F sites are critical to E7-mediated *trans* activation. The 12S E1A product shares this specificity with HPV E7 in that the E3 and E4 promoters are not activated. However, the specificities are not identical, since the hsp70 promoter can be *trans* activated by the 12S E1A product but not by E7. Previous experiments have shown that the hsp70 promoter was activated by the 12S E1A product in a virus infection (55), and cotransfection of a 12S E1A-expressing plasmid, as shown in Fig. 2, clearly demonstrates that the 12S product alone can *trans* activate the hsp70 promoter. The target in the hsp70 promoter for E1A activation is the TATAA element (54), suggesting that the TFIID factor, or a related factor, is involved in this activation. While it appears that the 12S E1A product can target at least two cellular transcription factors, E2F and TFIID, the E7 protein may interact only with E2F. This additional functional complexity of 12S E1A compared with E7 may be a reflection of amino acid sequences in the 12S product that are absent in E7. For example, although E7 shares sequence homology with portions of CR1 and CR2, it lacks homology to the N terminus of E1A, a region that is important for transformation and for interaction with the 300-kDa cellular protein (63).

There may also be cell type specificity in this activation process, since the E1A 12S product does not *trans*-activate the E2 promoter in a HeLa cell, whereas the 13S product does (2). The basis for this difference appears to lie in the fact that E2F is not complexed to cellular factors in a HeLa cell and thus the activity mediated by the 12S E1A product is not necessary. Of course, this finding is of interest with respect to the function of the E7 protein to target E2F, since HeLa cells contain transcriptionally active HPV18 sequences and express the E7 protein (52, 53). Moreover, the

12S E1A does *trans* activate the hsp70 promoter in HeLa cells (55), which is consistent with the finding that the E7 product appears to lack the ability to target the TATAA-specific factor necessary for activation of hsp70 by E1A.

In a recent study, Rawls et al. (46) found that chemically synthesized HPV16 E7 protein microinjected into HeLa cells *trans*-activated the adenovirus E2 promoter but not the adenovirus E3, E4, or MLP promoter. Using truncated peptides, they localized *trans*-activation function to the carboxyl half of E7. The physiological importance of these results is difficult to interpret, however, since this was done with HeLa cells, which have been shown to express the HPV18 E7 protein and are incapable of supporting 12S activation of the adenovirus E2 promoter (2).

Although E7 and the 12S E1A product appear to function in a similar manner to block the formation of complexes containing the E2F transcription factor, quantitative and/or qualitative differences between these viral proteins are apparent. The E1A protein appears to be considerably more active than E7 in these assays. E1A protein produced in vitro in reticulocyte lysates can readily function in the various E2F dissociation assays described here, whereas significant activity for E7 required the use of large amounts of protein produced in *E. coli*. In addition, the E7 protein exhibited only marginal activity to actually dissociate the E2F complexes; the E7 protein could inhibit complex formation but could not easily dissociate the complex once it was formed. It should also be noted that E7 is less active than E1A in its other biological parameters, including cooperativity with *ras* and *trans* activation of the E2 promoter. Furthermore, whereas both CR1 and CR2 of E1A appear to be required for dissociation of E2F complexes, an intact CR2 region does not seem to be necessary for E1A to inhibit formation of the E2F complex in vitro (47). The observation that the CR2-homologous sequences in the HPV E7 polypeptide are essential for inhibition of E2F complex formation suggests the possibility of subtle biochemical differences within the conserved regions or simply that CR2 improves the efficiency of the protein and is thus critical for any function of E7.

Finally, recent studies have demonstrated that E2Fc, as formed with the E2F-BF fraction, contains the cyclin A protein (36) and that the ability of E1A to dissociate E2F from this complex coincides with the ability of E1A to specifically interact with cyclin A. Although a similar interaction between E7 and cyclin A has yet to be demonstrated, the observation that E7 does disrupt the E2F-cyclin A complex suggests that E7 may indeed recognize cyclin A, although possibly with less affinity than E1A. It has also been shown that E2F is found associated with pRB (6, 9, 10) and that E1A can disrupt this complex and release free E2F (6, 9). Moreover, the E2F-I activity, which is shown to be blocked through the action of E7, has recently been found to contain the Rb protein (3). Thus, the requirement for the *trans* activation of the E2 promoter of amino acid sequences in E7 that include the previously defined pRB binding domain (35) suggests that the ability of HPV E7 to interact with pRB contributes to *trans* activation via E2F. These results suggest a common mechanism of action for two viral oncoproteins, E1A and E7, that involves the targeting of regulatory proteins (37) such as cyclin A and pRB and leads to the release and utilization of the E2F transcription factor.

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