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The Human Papillomavirus Type 16 E7 Protein Complements Adenovirus Type 5 E1A Amino-Terminus-Dependent Transactivation of Adenovirus Type 5 Early Genes and Increases ATF and Oct-1 DNA Binding Activity

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We have previously shown that conserved region 1 (CR1) of the adenovirus type 5 (Ad5) E1A protein synergizes with CR3 in the transactivation of Ad5 early genes (H. K. Wong and E. B. Ziff, J. Virol. 68:4910–4920, 1994). CR1 lies within the E1A amino terminus and binds host regulatory proteins such as the RB protein, p107, p130, and p300. Since simian virus 40 (SV40) large T antigen and human papillomavirus type 16 (HPV16) E7 protein also bind host regulatory factors, we investigated whether these viral proteins can complement E1A mutants which are defective in early gene activation. We show that the HPV16 E7 protein but not SV40 T antigen can complement mutations in the Ad5 E1A CR1 in the transactivation of viral early promoters. The inability of SV40 T antigen to complement suggests that RB binding on its own is not sufficient for early promoter transactivation by the E1A amino terminus. Nuclear runoff assays show that complementation by HPV16 E7 restores the ability of the E1A mutants to stimulate early gene expression at the level of transcription. Furthermore, nuclear extracts from the E7-transformed cells show increased binding activity of ATF and Oct-1, factors that can recognize the elements of Ad5 early genes, consistent with gene activation by E1A and E7 at the transcriptional level.

Studies of the early proteins of DNA tumor viruses, in particular the adenovirus type 5 (Ad5) E1A protein, have revealed cellular targets that are critical regulators of growth (reviewed in reference 14). The specific binding of E1A to host proteins is essential for E1A's biological properties (9, 29, 41, 90). One host target of E1A is the product of the retinoblastoma susceptibility gene, the RB protein, which forms stable, noncovalent complexes with the E1A protein (87–89). Other E1A-associated proteins include the RB-related proteins p107 (23) and p130 (39, 51) as well as p60 cyclin A, a cell cycle regulator that associates indirectly with E1A through binding to p107 (9, 30, 31). An additional E1A-binding protein, p300, has recently been shown to be closely related to CREB-binding protein, a transcriptional coactivator which binds the CREB transcription factor (4) and the TATA factor (2, 28, 74, 86, 93).

The E1A proteins are the first viral products synthesized after infection (reviewed in reference 10). The E1A gene directs the synthesis of multiply spliced messengers of which the 12S and 13S species are the major transcripts (11, 12, 45). These differ only by a differentially spliced internal segment and encode a 243R protein and a 289R protein, respectively. During early viral growth, E1A transactivates early genes and is responsible for initiating the early phase of infection (10, 37, 52, 65). Within the E1A coding sequences, multiple domains, including the amino-terminal region, conserved region 2 (CR2), and CR3, have been identified as functionally important for virus growth (57, 63, 92). These domains cooperatively regulate early viral gene expression (reviewed in reference 13).

The products encoded by the E1A transcription unit can transform primary cells in cooperation with a secondary oncogene (76, 90). The 289R E1A protein is also a transcriptional transactivator of cellular genes (66, 81). Because the amino terminus of E1A contributes to both transformation and transcription activation, the latter two activities may be interdependent.

We have previously shown that the E1A amino terminus provides two distinct genetically defined functions (79). One of these is required for viral induction of cellular DNA synthesis, and one is required for focus formation. We have further shown that functions of the amino terminus, apart from those provided by CR3, are directly required for induction of viral early promoters (92). These studies showed that deletions within the E1A amino terminus which disrupt the binding of both RB and the cellular p300 protein block the activation of all viral early promoters. Mutations which selectively disrupt the binding of RB or p300 did not impair viral promoter activation, indicating that binding of these two proteins in some manner provides a redundant function with respect to promoter activation.

Interaction of E1A with the RB protein and related family members can disrupt complexes between members of the RB family with the E2F transcription factor and release E2F to transactivate genes required for cellular S-phase entry (32, 44) and viral E2 gene transcription (5). This ability of E1A to induce the function of E2F provides a pathway for activation of Ad5 promoters with E2F sites in their regulatory regions, such as the Ad5 E2A promoter. However, the majority of viral early promoters lack E2F sites (reviewed in reference 46) and there-

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fore cannot be induced by the E1A mechanism which activates E2F through the RB protein. Despite this, early promoters for regions E2, E3, and E4 are dependent on amino-terminal functions of E1A for their activity (92). This finding supports the view that the E1A amino terminus provides additional mechanisms for promoter activation in addition to releasing E2F from RB control (48, 62, 63).

Transformation by E1A takes place through mechanisms similar to those employed by the other DNA tumor viruses. It appears that the papovavirus simian virus 40 (SV40) as well as Ad5 and human papillomavirus types 16 and 18 (HPV16 and HPV18) all encode early proteins that have similar immortalizing activities and which target the RB protein (reviewed in references 14, 61, and 89). In this report, we describe the complementation of functions of the Ad5 E1A amino terminus by the HPV16 E7 protein and characterize the role of the transforming domain of E7 and E1A in transcription regulation. We show that the HPV E7 protein, but not the SV40 T-antigen protein, can complement functions of E1A provided by the E1A amino terminus and necessary for stimulation of Ad5 early promoters. This mechanism differs from the well-characterized activation of E2F in that sequestration of RB is not sufficient and that the association of protein complexes containing c-Jun with ATF sites is stabilized. This evidence supports the existence of an additional pathway of E1A-dependent promoter activation which is shared with the E7 HPV protein but not with the SV40 T-antigen protein.

MATERIALS AND METHODS

Plasmids. Plasmids were generously provided as follows: pVA1 and pRSVNeo from B. Aufiero and B. Schneider; E2a, E3, E4, and VA1 in M13 single-stranded DNA, gift of B. Aufiero and B. Schneider; pSVK166 and pSVK, from G. Pecorra and V. Defendi; and SV40 expression vectors pSVlac0T, pSVlac0U19, and pSVlac0K1, from J. DeCaprio and D. Livingston (Dana Farber Cancer Center). Plasmids were grown in Luria broth, isolated by the alkaline lysis protocol as described elsewhere (77), and then centrifuged on a cesium gradient.

Plasmid construction. pSVKE7 was constructed from the parent vector pSVK. The vector was cut with *Nhe*I and *Hin*dIII to release an internal fragment. Phosphatase treatment of the vector was followed by sticky-end ligation of the insert, an E7 gene amplified from plasmid pSVK166. The fidelity of the PCR product was confirmed by sequencing using primers employed for PCR.

PCR was performed with a GeneAmp kit (Perkin-Elmer). The HPV16 E7 primers for PCR are as follows: 5′ primer, 5′-AAGCTAGCCGCCACCATGCA TGGAGATACACCTACATTGC-3′; and 3′ primer, 5′-GCAAGCTTGGTAGA TTATGGTTTCTGAGAA-3′. The 5′ primer has an *Nhe*I site and a Kozak consensus sequence at the ATG to maximize translation. The 3′ primer has a stop codon followed by a *Hin*dIII site. The PCR parameters for a total of 35 cycles were as follows: denaturing phase for 1 min at 94°C, annealing at 42°C for 2 min, and extension at 72°C for 1 min.

Tissue culture. Cells were cultured in Dulbecco minimal essential medium (DMEM; Gibco) supplemented with antibiotics (100 μg of streptomycin and 100 μg of penicillin [Sigma] per ml) and 10% fetal calf serum (Gibco) for BALB/c3T3 cells, Y-79 retinoblastoma cells (ATCC HTB 18), and H219 cells. HeLa cells and CaSki cells were grown in DMEM with 10% calf serum (Gibco). For selection of cells that express a stably transfected neomycin resistance (*neo*) gene, G418 (Gibco) was added to DMEM at 400 μg/ml.

Virus stocks. Adenovirus stocks were prepared following infection of human 293 cells as described previously (92).

Antibodies. M73 monoclonal antibodies against the carboxyl terminus of E1A were prepared from hybridoma cells (gift of Ed Harlow, Massachusetts General Cancer Center) (40). H219 monoclonal antibodies against the adenovirus E2 72-kDa DNA-binding protein were prepared from tissue culture supernatant (gift of A. Levine, Princeton University). Monoclonal antibodies to HPV16 were purchased from Triton Biosciences, Inc., Alameda, Calif. Anti-c-Jun was isolated from rabbits immunized to Trp-Jun fusion protein as described previously (3).

Modified Hirt isolation of viral DNA. Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then lysed in 1 ml of neutral lysis buffer (10 mM Tris [pH 7.9], 5 mM EDTA, 100 mM NaCl, 0.5% sodium dodecyl sulfate, 0.1 mg of proteinase K). The gelatinous solution was incubated for at least 6 h at 37°C. The NaCl concentration was adjusted to 1 M, and the mixture was chilled for 16 h at 4°C to precipitate high-molecular-weight DNA. The precipitated DNA was pelleted by centrifugation in an Eppendorf tube at 10,000 × *g*. The supernatant was recovered and extracted with phenol, phenol-chloroform, and chloroform. The DNA was then ethanol precipitated. Viral DNA was restricted with *Sal*I and separated in an 0.8% agarose gel.

Southern analysis. DNA to be analyzed was electrophoresed in 0.9% agarose gel and transferred to nitrocellulose as described elsewhere (77). In dot blot analyses, DNA was denatured in 0.1 M NaOH for 30 min, neutralized, and transferred to nitrocellulose with a dot blot manifold (Schleicher & Schuell). DNA on the nitrocellulose filters was UV cross-linked by a UV Stratalinker 2400 (Stratagene) at 1,200 μJ. Filters were analyzed with ^{32}P-labeled DNA probes prepared by random-oligonucleotide labeling as described previously (33).

Transfection. Calcium phosphate-mediated DNA transfection was performed essentially as described previously (84). Cells were grown to a density of 0.5 × 10^6/10-cm-diameter dish. Four hours prior to transfection, fresh medium was added. For formation of stable cell lines, 1 μg of pRSVNeo was precipitated with 20 μg of T-antigen expression vector with 5 μg of salmon sperm carrier DNA. The E7 expression vector had the *neo* gene colinear with the E7 gene. Therefore, 20 μg of pSVKE7 was precipitated with 5 μg of carrier DNA. The precipitate was added dropwise to the dish.

For selection of stably transformed cell lines, medium containing 400 μg of G418 per ml was added 48 h after transfection, and cells were subsequently passaged in medium containing G418.

Nuclear transcription runoff assay. Nuclei were prepared from at least two 15-cm-diameter dishes as described previously (6). For the run-on reaction, nuclei were incubated in transcription buffer containing 150 μCi of [^{32}P]UTP (Dupont, NEN) in 25 mM Tris-HCl (pH 8)–12.5 mM $MgCl_2$–325 mM KCl–1.25 mM remaining deoxynucleoside triphosphates–0.5 μM dithiothreitol–200 U of RNasin per ml. The reaction was processed as described previously (6) except that the nuclear RNA was precipitated with 7.5 M ammonium acetate in ethanol. The ^{32}P-labeled RNA was resuspended in distilled water, and unincorporated nucleotides were removed by passage through a G-50 spin column. Equivalent trichloroacetic acid-precipitatable labeled RNA was hybridized to nitrocellulose filters that had single-stranded M13 DNA probes for the early regions and plasmid DNA for the tumor antigens and negative control.

Preparation of nuclear extracts. Nuclear extracts were prepared as described previously (18). Cells grown on 5 to 10 dishes (15-cm diameter) were infected with viruses at 50 PFU per cell. At 21 h after infection, cells were scraped, washed with PBS, and washed with isotonic buffer (25 mM) *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.5], 5 mM KCl, 200 mM sucrose, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). Cells were centrifuged and resuspended in hypotonic buffer (isotonic buffer without sucrose) and swelled for 10 min at 4°C. Cells were then lysed with a Dounce homogenizer (10 strokes). Nuclei were pelleted, resuspended in 1 ml of 50 mM HEPES (pH 7.5)–10% sucrose–200 mM NaCl for 1 h, and then centrifuged for 1 h at 100,000 × *g*. Nuclear extract was quantitated and frozen at −70°C.

Oligonucleotide. The following oligonucleotides were synthesized on an Applied Biosystems Oligo Nucleotide Synthesizer by Bernie Goldsmidt (New York University) and purified on an acrylamide gel: Oct-1/NFIII, 5′-GATCCAATAT GATAATGAGGGGGTGGAGTA-3′ and 5′-AGCTTACTCCACCCCCTCA TTATCATATTGG-3′; E4-ATF, GATCCGGAAAATGACGTAACGGTTCC A-3′ and 5′-AGCTTGGCCAAACTACGTCATCTCCAG-3′; E2-ATF, 5′-GA TCCTGGAGATGACGTAGTTTGGCCA-3′ and 5′-AGCTTGGCCAAACTA CGTCATCTCCAG-3′; and DSE, 5′-GATCCAGGATGTCCATATTAGGAC ATCTGCG-3′ and 5′-CGCAGATGTCCTAATATGGACATCCTGGATC-3′. For gel mobility shift assays, 500 ng of each oligonucleotide was phosphorylated by [γ-^{32}P]ATP with T4 polynucleotide kinase, and oligonucleotides were annealed by slow cooling over 30 min after boiling.

Gel mobility shift assay. Detection of DNA-binding proteins was done essentially as described previously (6), with the following modification: 5 μg of nuclear extracts was incubated with 1 μg of poly(dI-dC) (Pharmacia), and 10,000 cpm of oligonucleotide probes was incubated with or without 100-fold excess of specific competitor in binding buffer (25 mM HEPES [pH 7.5], 50 mM NaCl, 10% glycerol, 0.05% Nonidet P-40, 5 μg of bovine serum albumin per ml, 1 mM dithiothreitol, 1 mM EDTA). The binding reaction mixture was incubated 20 min at room temperature and resolved by nondenaturing high-ionic-strength (50 mM Tris [pH 8.5], 0.38 M glycine, 1 mM EDTA) gel electrophoresis (4% acrylamide).

RESULTS

Schematic structures of strains of Ad5 which encode mutant forms of the 289R E1A protein are shown in Fig. 1. These viruses contain deletions in CR1, a region of E1A which we have shown is required for the activation of Ad5 early genes (92). In an assay for the function of E1A in BALB/c3T3 and human diploid fibroblasts, viruses with large deletions in the E1A amino terminus, *sub*/*dl*1004-13, *sub*/*dl*1006-13, *sub*/*dl*1008-13, and *sub*/*dl*1015-13, show greatly reduced ability to replicate or to transactivate early genes relative to wild-type viruses, such as *dl*309, relative to viruses encoding wild-type 289R protein, such as *dl*348, or relative to viruses with small

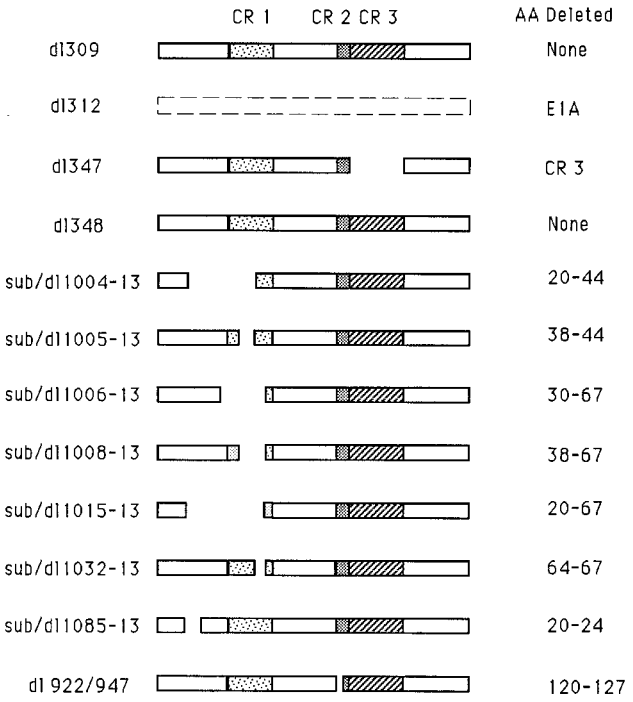

FIG. 1. Structures of mutant Ad5 strains which encode the larger 289R E1A protein. The first and last residues deleted from *sub*/*dl* mutants are indicated, and the mutant viral proteins have a two- to three-amino-acid (AA) insertion encoded by linkers. These viruses have amino-terminus deletions as depicted, and all viruses encode the wild-type CR3 domain. Also shown are *dl*347, which encodes only the wild-type 243R protein, *dl*348, which encodes only the 289R protein, and *dl*922/947, which encodes both the smaller and larger E1A proteins. *dl*312 does not express E1A.

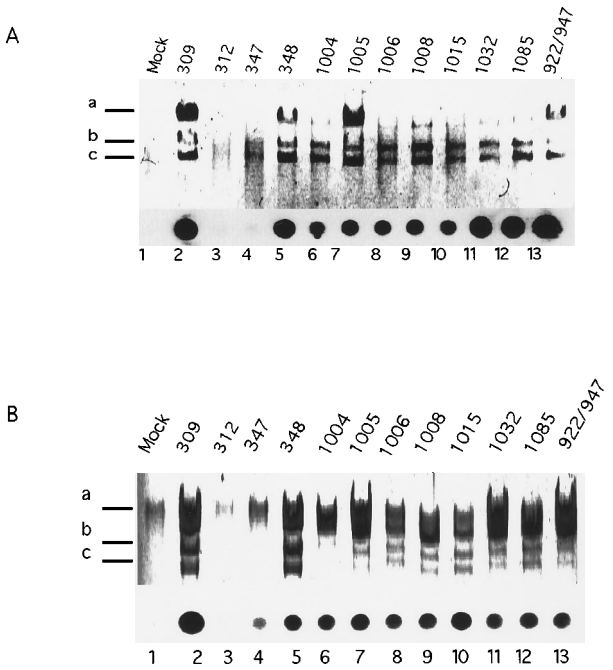

FIG. 2. Replication of amino-terminal mutant adenoviruses. Cells were infected at 10 PFU per cell, and viral DNA was prepared as described in Materials and Methods. Viral DNA was restricted with *Sal*I, and DNA fragments were resolved on an agarose gel. The arrows denote the expected specific DNA restriction products (fragments a, b, and c are 19.4, 9.4, and 7.2 kb in size, respectively). Nonspecific high-molecular-weight fragments of 20 kb in size can be seen on the gels. (A) HeLa cells analyzed at 24 h after infection. The ethidium bromide-stained gel of *Sal*I-restricted DNA product is shown, with the corresponding Southern dot blot shown below. (B) CaSki cells analyzed at 48 h after infection. Hirt analysis of viral replication on an ethidium bromide-stained agarose gel is shown. The corresponding dot blot analysis performed as described in Materials and Methods is also shown.

deletions in the amino terminus, including *sub*/*dl*1005-13, *sub*/*dl*1032-13, and *sub*/*dl*1085-13. Table 1 summarizes the phenotypes of these mutant viruses, their transactivation and replication potentials, and the association of these mutant E1A proteins with cellular proteins (79, 92).

HPV E7 complements E1A amino-terminus function. Although mutants with large deletions in the E1A amino terminus are defective in viral transcription and replication in primary human fibroblasts and BALB/c3T3 cells, these mutant E1A viruses complete the viral life cycle and form infectious virus particles in HeLa cells with efficiencies comparable to that of *dl*309 wild-type virus (79). Figure 2A shows an analysis of viral DNA extracted from infected HeLa cells at 24 h after infection during the linear replicative phase. Measurement of the levels of Hirt-extracted viral DNA by restriction with *Sal*I, fractionation by agarose gel electrophoresis, and visualization by ethidium staining shows that these mutant adenoviruses with large deletions in E1A can replicate like the wild type in HeLa cells (Fig. 2A; compare lanes 6, 8, 9, and 10 with lane 2). This finding was confirmed by Southern dot blot analysis of viral DNA. Viruses *dl*312 and *dl*347, which have a deletion of the E1A gene and express the 243R form of E1A which lacks CR3, respectively, do not replicate their DNA in HeLa cells at 24 h after infection. We examined whether the permissiveness of HeLa cells results from expression of an endogenous E1A complementing activity. HeLa cells are derived from human cervical carcinoma cells and express the E7 transforming gene of HPV18 (78). HPV18 and the related HPV16 belong to a class of papillomaviruses whose infections are highly correlated with the genesis of cervical malignancies (95). The E7 protein of HPV16 provides transactivation, transformation, and host protein binding activities similar to those of E1A (17, 22, 60, 61, 71, 72). Like E1A, the HPV E7 protein binds the RB protein (22, 26, 27). The similarities between the two DNA tumor virus proteins extend to the level of amino acid sequence homology in the regions of the two proteins that are required for RB protein binding (14, 70).

TABLE 1. Viral replication and cellular protein binding by E1A proteins encoded by mutant adenoviruses[a]

Virus	Mutation[b]	Binding			Early gene activation	Cell DNA synthesis	CaSki or HeLa cell infection
		RB	p107	p300			
*dl*309	None	+	+	+	+	ND	+
*dl*312	−E1a	−	−	−	−	−	−
*dl*347	243R only	+	+	+	−	+	−
*dl*348	289R only	+	+	+	+	ND	+
sub/*dl*1005	38–44	−	+	+	+	+	+
sub/*dl*1032	64–67	+	+	−	+	+	+
sub/*dl*1085	20–24	+	+	−	+	+	+
sub/*dl*1004	20–44	−	+	−	−	−	+
sub/*dl*1006	30–67	−	+	−	−	−	+
sub/*dl*1008	38–67	−	+	−	−	−	+
sub/*dl*1015	20–67	−	+	−	−	−	+
*dl*922/947	120–127	+	−	+	+	ND	+

[a] The assay of cell DNA synthesis is described in reference 79. Early gene activation was analyzed as described in reference 92. ND, not determined.
[b] Numbers indicate amino acids deleted.

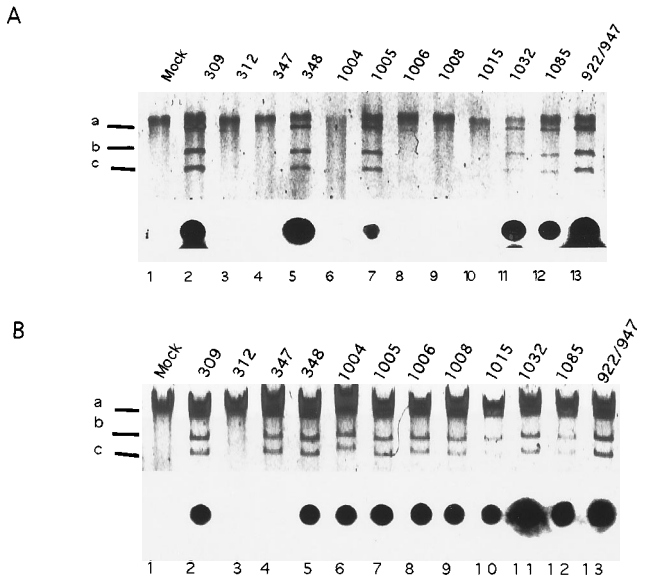

FIG. 3. Replication of mutant adenoviruses. (A) BALB/c3T3 cells were infected at 50 PFU per cell. Mutant adenovirus replication in parental BALB/c3T3 cells at 48 h after infection was assayed by using Hirt-extracted virus DNA. An ethidium bromide-stained gel of replicated adenoviral DNA is shown along with dot blot analysis. (B) Replication of mutant adenovirus in E7-transformed BALB/c3T3 cells at 48 h after infection. The ethidium bromide-stained gel and corresponding dot blot are shown.

To determine whether the HPV E7 protein indeed complements the deficiencies of the large-deletion mutant E1A proteins, cells known to express HPV E7 were analyzed for permissiveness for Ad5 E1A mutant growth. CaSki cells, which are derived from a cervical carcinoma, were chosen because they express the HPV16 E7 gene (8, 80). Analysis of Hirt-extracted viral DNA showed that in CaSki cells, viral DNA from large-deletion mutant adenoviruses was replicated at levels comparable to those of the small-deletion E1A mutant viruses, *dl*348, and the wild-type virus (Fig. 2B). Thus, endogenous CaSki cell function(s) can restore the ability of mutants with large amino-terminal deletions in E1A to replicate (Fig. 2B, *sub*/*dl*1004-13, *sub*/*dl*1006-13, *sub*/*dl*1008-13, and *sub*/*dl*1015-13). Replication is still dependent on CR3, in as much as replication of *dl*347, which lacks CR3, is defective in CaSki cells, as shown in Fig. 2. In CaSki cells, of the E1A regions analyzed, only E1A CR3 is required for replication. Other epithelial cells transformed by HPV E7, including SiHa, and primary epithelial cells immortalized with the early region of HPV16 were also permissive for growth of the large-deletion mutant Ad5 strains (data not shown).

To assay directly for complementation of E1A functions by E7, BALB/c3T3 cells, which are nonpermissive for growth of large-deletion E1A mutant viruses but are permissive for growth of wild-type virus (Fig. 3A), were stably transformed with an HPV16 E7 expression plasmid, pRSVE7. In addition to the E7 gene, this plasmid contains a neomycin resistance gene which confers growth in the presence of the neomycin analog G418. In assays of E1A function, cells transformed by the E7 gene were permissive for replication of E1A large-deletion mutant viruses, as shown by restriction in analysis of viral DNA in Hirt extracts and by dot blot analysis (Fig. 3B). This result is consistent with the results for CaSki cells and HeLa cells and confirms that it is the HPV E7 protein which complements the mutations in the E1A amino terminus.

RB binding function of the E1A amino terminus is dispensable for virus growth. A critical role of the E1A amino terminus is to bind the RB protein (90, 91). To test the sufficiency of sequestering RB as a step in E1A function in transactivation, Y-79 cells (73), which have homozygous deletions of the RB locus, were assayed for permissiveness for E1A large-deletion mutant Ad5 growth. A Southern blotting assay of Ad5 mutant replication in Y-79 cells demonstrated that E1A large-deletion mutant viruses *sub*/*dl*-1004, *sub*/*dl*-1006, *sub*/*dl*-1008, and *sub*/*dl*-1015, which are defective for growth in BALB/c3T3 cells, were also defective in Y-79 cells (Fig. 4A). Viruses that exhibited wild-type growth in BALB/c3T3 cells grew in Y-79 cells (Fig. 4A). These results suggest that the ability of the E1A amino terminus to transactivate is not dependent solely on sequestration and inactivation of the RB protein and that additional steps or factors must be required for E1A's ability to transactivate.

It is clear that E1A binds to a family of RB-related factors (16, 39, 56), and on its own, an absence of RB from the Y-79 cells may be insufficient to permit the growth of the mutant viruses. E1A and E7 also share CR2 homology with another DNA tumor virus transforming protein, the SV40 large T antigen, which also binds the RB protein (14, 24, 85). If binding the RB protein or another cellular target protein is a necessary and sufficient property for complementation of the mutant E1A functions, then the SV40 large T-antigen proteins should be capable of complementing the mutant E1A functions. To test the capacity of this viral antigen to complement the E1A mutants, BALB/c3T3 cells were stably transformed with plasmids that express either SV40 T antigen (pSVlac0T) or a mutant T antigen that fails to bind RB (pSVlac0U19). The expression of T antigen was confirmed by immunofluorescence

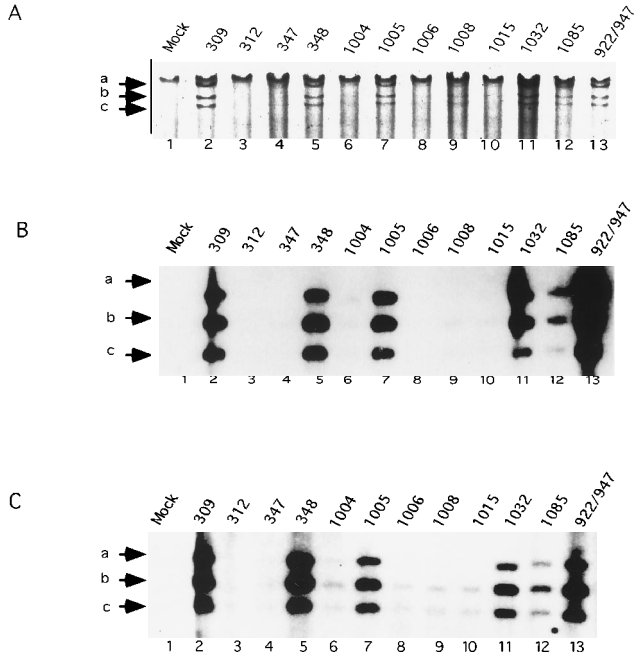

FIG. 4. Replication of mutant adenoviruses in Y-79 cells and T-antigen-transformed cells. (A) Y-79 cells were infected at 20 PFU per cell. Shown is Southern analysis of Hirt-extracted viral DNA isolated from infected Y-79 cells at 48 h after infection. (B) Replication of mutant adenoviruses in wild-type T-antigen (pSVlac0T)-transformed BALB/c3T3 cells. Shown is Southern analysis of *Sal*I-restricted virus DNA at 48 h after infection. (C) Replication of mutant adenoviruses in RB-binding mutant T-antigen (pSVlac0U19)-transformed cells. Shown is Southern analysis of *Sal*I-restricted viral DNA at 48 h after infection.

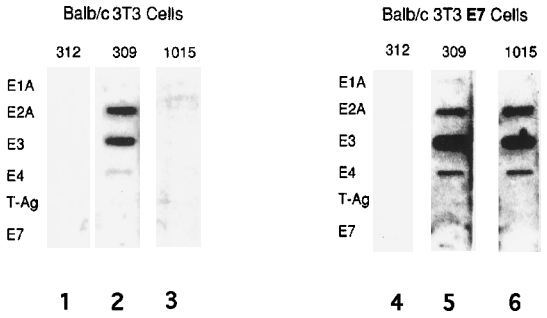

FIG. 5. Nuclear run-on assay of transcription of viral early genes. Nuclear transcription analyses of parental BALB/c3T3 cells (lanes 1 to 3) and E7-transformed BALB/c3T3 cells (lane 4 to 6) are shown. Cells were infected at 50 PFU, and nuclei were prepared at 24 h after infection. The transcription run-on assay was performed as described in Materials and Methods (6). T-Ag, T antigen.

assay (not shown). A Southern assay of Hirt-extracted viral DNA showed that mutant Ad5 replicated in wild-type T-antigen-transformed cells and in mutant T-antigen-transformed cells with a pattern similar to that seen in BALB/c3T3 cells (Fig. 4B and C). Large-deletion mutant viruses failed to replicate (Fig. 4B and C, lanes 6 and 8 to 10), indicating that T antigen cannot complement the function provided by the E1A amino terminus. The complementation analysis demonstrated a difference between the tumor virus proteins in that HPV E7 can complement the E1A amino-terminus defects but the SV40 large T antigen cannot. The difference in the complementation afforded by these two RB-binding viral proteins suggests that additional functions of E1A and E7 which contribute to viral growth are not provided in SV40-transformed cells and that the necessary functions are not provided simply by sequestration and inactivation of RB, p107, and p130.

E7 activates Ad5 early genes in cooperation with E1A CR3. We next examined whether the complementation observed in E7-transformed cells takes place at the level of transcription. E7-transformed BALB/c3T3 cells were infected with E1A mutant viruses, and the nuclei were isolated at 24 h after infection. Nascent viral early RNA transcripts were synthesized in vitro in the presence of $^{32}PO_4$-labeled UTP, and the relative levels of nascent RNA from specific viral genes were measured by hybridization of an equivalent quantity of trichloroacetic acid-precipitated [$^{32}PO_4$]UTP-incorporating RNA to cDNA immobilized on nitrocellulose filters (6). In control cells transformed by the neomycin resistance plasmid alone and lacking E7, mutant *sub*/*dl*1015-13 transcribed early regions E2, E3, and E4 at levels significantly lower than that of the wild-type virus, *dl*309 (Fig. 5, lanes 1 to 3). In contrast, in infections of E7-transformed cells, *sub*/*dl*1015-13 expressed early gene transcripts at a rate comparable to that of *dl*309 (Fig. 5, lanes 4 to 6). This finding indicated that the E7 protein, like the E1A amino terminus, can stimulate viral transcription.

E7 induces activities of ATF and Oct-1/NFIII. The induction of early gene transcription by E1A and E7 suggests that these viral proteins may increase the levels or activities of specific transcription factors. To characterize such changes conferred by E7 and E1A, transcription factors which were previously shown to be dependent on E1A and which are essential for induction of virus transcription or replication were studied. Extensively characterized factors which act at viral early promoters include E4-ATF and Oct-1/NFIII (20, 50, 69, 75). Nuclear extracts prepared from the E7-transformed cells and control *neo*-transformed cells were assayed for protein binding to E4-ATF and Oct-1 sites (Fig. 6A). When equal quantities of extracts were used for gel mobility assay with the respective $^{32}PO_4$-labeled oligonucleotide probes, extracts prepared from E7-transformed cells had greater DNA binding activity as assayed with both transcription factor binding sites (Fig. 6A, lanes 3 and 9). This finding suggests that the ability of E7 to complement E1A mutants may result from increases in the DNA binding affinity or the level of these transcription factors. SV40-transformed cells did not exhibit an increase in transcription factor DNA binding activity (Fig. 6A, lane 5 and 11) when assayed with either the ATF probe or the Oct-1/NFIII probe, in agreement with the finding that the complementing of virus replication is not provided by T antigen.

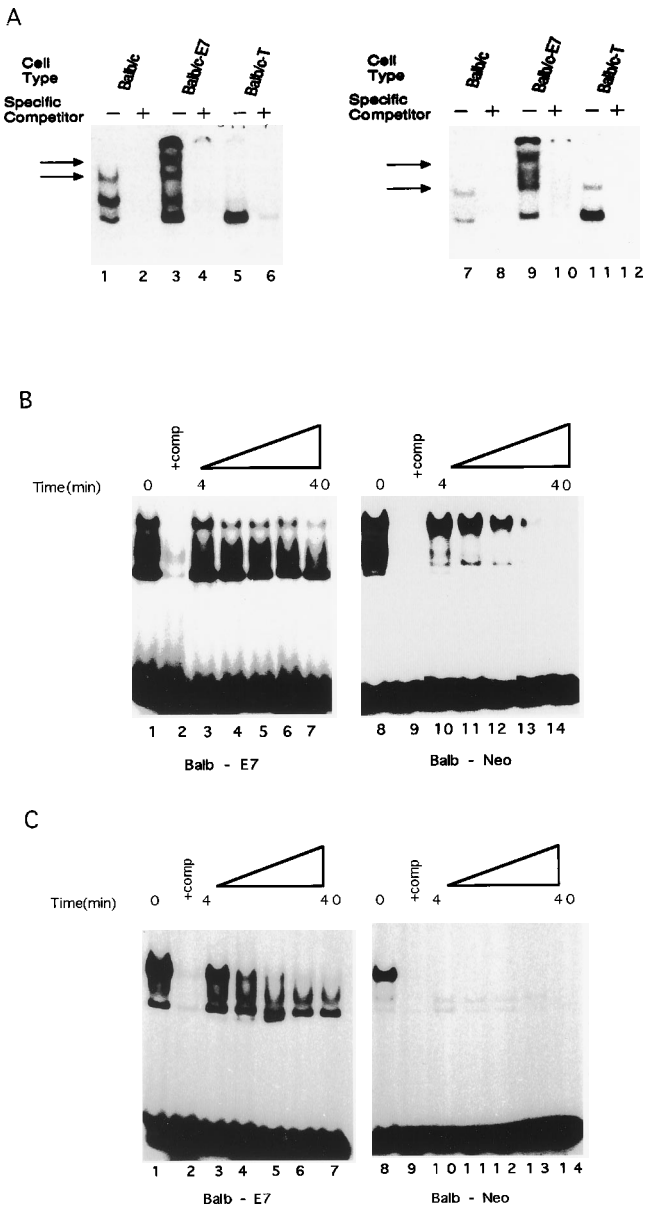

FIG. 6. Off-rate analysis of DNA binding activity in extracts of E7-transformed cells and in parental cells. (A) Binding to Oct-1/NFIII (lanes 1 to 6) and E4-ATF (lanes 7 to 12) recognition site oligonucleotides labeled with ^{32}P. The arrows denoted the specific complexes expected. (B) Off-rate analysis of DNA binding in extracts from E7-transformed cells (lanes 1 to 7) and untransformed cells (lanes 8 to 14) to labeled E4- ATF probe. comp, competitor. (C) Off-rate analysis of DNA binding in extracts from E7-transformed (lanes 1 to 7) and *neo*-transformed (lanes 8 to 14) BALB/c3T3 cells to labeled Oct-1/NFIII.

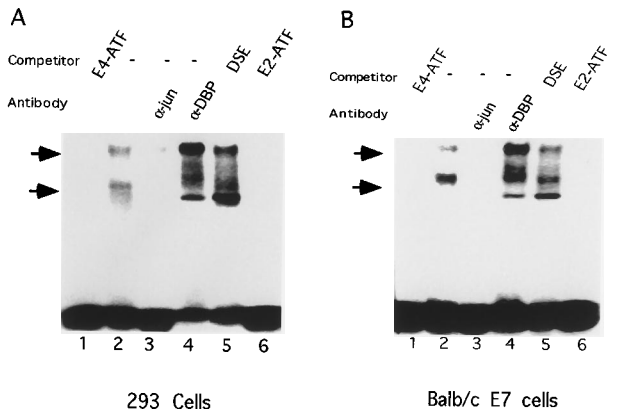

FIG. 7. Antibody disruption assay of protein complexes to ATF sites. Gel shift assays were performed with a ^{32}P-labeled E4-ATF nucleotide probe. Nuclear extracts were incubated with an antibody specific for Jun (α-Jun) or the E2 72-kDa DNA-binding protein (α-DBP). Competitors were E2-ATF and dyad symmetry element (DSE) oligonucleotides. (A) Anti-Jun antibody disruption of complex of protein in 293 cell nuclear extracts with an ATF binding site probe. Specific complexes are denoted by the arrows. (B) Anti-Jun antibody disruption of binding of protein in E7-transformed cell extracts to a labeled ATF site probe.

To characterize the increased DNA binding activity in extracts from the E7-transformed cells, DNA binding off-rate analyses were performed. Nuclear extracts were first incubated with each specific $^{32}PO_4$-labeled probe until binding reached equilibrium. Excess unlabeled oligonucleotide competitor was added, and at specific times, an aliquot was removed for gel electrophoretic assay of the amount of labeled probe which remained in protein complex. If transcription factors in the E7-transformed cells had an increased DNA binding affinity relative to factors in the control cells, they would dissociate from DNA less readily and have a lower DNA exchange rate. This would lead to an increased level of protein-[$^{32}PO_4$]DNA complex seen with the extracts from E7-transformed cells relative to extracts from the control cells at late times. To compare levels of DNA binding by E4-ATF from E7-transformed and control cells, the nuclear extracts were incubated with the respective labeled probe such that the initial levels of DNA binding were the same. Measurements of the rate of dissociation of protein from the E4-ATF oligonucleotide (Fig. 6B) and the Oct-1/NFIII oligonucleotide (Fig. 6C) show that in the presence of unlabeled competitor oligonucleotide, higher levels of protein complexes are seen with E7-transformed cells extracts than with control cell extracts. This difference is not simply a result of a quantitative change in the level of protein-DNA complexes but arises from the formation of more stable complex between the E7-transformed cell DNA-binding proteins and their cognate sites (Fig. 6A and C, lanes 1 to 7).

c-Jun contributes to increased ATF binding activity. It has previously been shown that E1A can induce levels of c-Jun as well as of members of both the E4F and ATF families (34, 47, 59). Extensive studies of ATF demonstrated that ATF forms homodimers, but ATF-2 heterodimerizes with c-Jun to form a complex that can bind the E4-ATF site in vitro (82, 83). To assess the contribution of c-Jun to Ad5 gene expression, we assayed whether c-Jun was present in the protein complexes that formed with the ATF site oligonucleotides. In analyses of nuclear extracts prepared from 293 cells, a cell line which constitutively expresses E1A, antibodies to c-Jun disrupted protein complexes with the ATF binding site oligonucleotide (Fig. 7A). Incubation of extracts from E7-transformed cells with antibodies against c-Jun likewise prevented the formation by extracts from E7-transformed BALB/c3T3 cell extracts of protein complexes with the ATF site oligonucleotide, indicating that these complexes also contain c-Jun (Fig. 7B). Control antibodies to the Ad5 72-kDa DNA-binding protein did not affect protein-DNA complex formation with nuclear extracts from either cell type. The ATF site from the adenovirus E2 promoter had a similar competitive potential to prevent formation of specific complexes (Fig. 7, lanes 6). The dyad symmetry element could not compete for specific binding for the E4-ATF probe (Fig. 7, lanes 5). This finding indicates that E7 proteins can increase the levels of transcription factor complexes which bind to ATF sites such as found in the promoters of Ad5 early genes and contain c-Jun and that such complexes are found in E1A transformed cells.

DISCUSSION

We have shown that the human papillomavirus E7 protein can complement transcription transactivation functions provided by the amino terminus of E1A. A region of identity between the amino acid sequences of E1A and E7 (14) may account for the functional similarities between E7 and E1A. These similarities are further supported by genetic complementation analyses, which provides a sensitive means for detection of in vivo interactions that would not otherwise be obvious from immunocoprecipitation studies that measure noncovalent protein-protein interactions in vitro. Complementation was observed both in assays of viral DNA replication and in nuclear run-on experiments which measured viral early promoter activity. The increase in Ad5 early promoter transcription provided by E7 is accompanied by increases in the DNA binding affinity of E1A-responsive transcription factors which act through E4-ATF and Oct-1/NFIII DNA binding sites. Antibody disruption demonstrated that c-Jun is a component of the complex which binds the E4-ATF site in E1A- and E7-transformed cells. Proteins in this complex have a decreased rate of DNA dissociation when isolated from E7-transformed cells relative to control cells. These results support a model of E1A function described previously whereby the amino terminus provides a distinct transcription transactivation function from CR3 (92). The findings are consistent with a two-step mechanism of transactivation by E1A whereby the E1A amino terminus increases the available pool of transcription factors needed for early gene transcription and subsequently CR3 is required for mediating local promoter-specific interaction to stabilize RNA polymerase binding for gene expression.

CR3 was the first E1A region demonstrated to provide a transcription transactivation function (10, 11, 45, 58). The discovery that interaction of the E1A CR2 domain with RB liberates E2F from an RB complex in which E2F is repressed demonstrated that E1A regions in addition to CR3 play a role in transcription control (43, 67). We have previously suggested that the E1A amino terminus regulates viral early gene transcription through RB-independent mechanisms (92) and further confirm this implication through studies in cells lacking RB or cells with T antigen. We show here that other transcription factors besides E2F which induce Ad5 early gene transcription can be regulated by the E1A amino terminus. This is consistent with findings from studies of HPV E7 that show that binding RB is not essential for inducing adenovirus E2 gene expression (17, 48). These results also indirectly support the hypothesis that additional factors besides RB, such as p107 and p130, whose binding is unaffected by the deletions outside CR2 in the E1A amino terminus, regulate E2F. Furthermore, the induction of the transformed state itself is not sufficient to

complement functions in the E1A amino terminus, since T-antigen-transformed cells are not permissive for early gene transcription by E1A amino-terminal mutant viruses.

E7 complementation of E1A amino-terminus functions suggests that the E7 and the E1A amino termini regulate transcription factor activity in similar manners so as to activate early promoter activity in adenovirus infection. This finding demonstrates that E7 encodes functions, in addition to RB binding, that can cooperate with the E1A CR3 to activate viral early genes E3 and E4. Consistent with our model of E1A and E7 complementation analysis is the finding that E7 alone cannot activate E3 and E4 transcription (70, 71). E7 may be able to activate E2 transcription without the adenovirus CR3 because of the strong contribution of the E2F sites, which are targets for E7 transactivation through E7 binding of the RB protein. In our experience, the E1A and E7 viral transforming proteins both activate a specific subset of transcription factors, since DNA binding by USF, a factor that associates with the Ad5 major late promoter, is unchanged by E1A (not shown). We find that the E7 protein leads to a qualitative change in the binding properties of transcription factors (Fig. 6) and that E1A likely induces a similar change in transcription factors through the amino terminus. It was previously shown that Oct-1/NFIII activity is significantly increased by the 243R E1A (20). The results in this report suggest that E7, like E1A, controls Oct-1/NFIII DNA binding. The increased stability of the Oct-1/NFIII DNA complexes shown by the DNA off-rate analysis is consistent with a posttranslational modification which alters the factor's DNA binding affinity. Oct-1/NFIII is phosphorylated in the presence of E1A (20), suggesting that the E1A amino-terminus complementing activity of E7 either directly or indirectly regulates an Oct-1/NFIII kinase activity. The E1A amino-terminal domain interacts with cyclins and cdc2 kinase via RB or p130 (15, 21), and through these interactions, E1A may control the phosphorylation of transcription factors.

ATF sites are similar to the cyclic AMP (cAMP) regulatory element in that both participate in E1A-dependent regulation of Ad5 early gene transcription (reviewed in references 46 and 94). In addition to the initial factors characterized members of the ATF family (49, 53, 54), the Jun family of factors is also stimulated by E1A (34, 47, 83). The specific roles of the members of the two families in Ad5 early gene transcription are unclear, but the results presented here are consistent with a model in which heterodimerization between members of the two families contributes to activation of early promoters. The finding that an anti-c-Jun antibody can disrupt complexes with an ATF site is consistent with the induction of a heterodimeric complex which contains c-Jun and an ATF family member. Indeed, ATF-2 can form heterodimeric complexes with c-Jun at an ATF site (38). In addition, the smaller, 243R E1A protein can induce transcription complexes binding to AP-1 sites through an increase in c-Jun/ATF-2 heterodimers, suggesting that heterodimer formation by members of the ATF and c-Jun families of factors indeed plays a role in the pleiotropic functions of the amino terminus of E1A (82). The complex is not likely to consist of cAMP-responsive factors that bind ATF sites, since we did not observe complementation of viral growth with forskolin (not shown). However, phosphorylation may play a role in E4-ATF activation, since regulatory hyperphosphorylation of ATF protein has been described (1) and E1A can be found associated with kinase activity (7, 36).

E1A-cellular protein interaction. Sites of binding of cellular proteins RB, p130, and p300 have been mapped to a region extending from residues 1 to 70 within the E1A amino terminus (29, 41, 91). It is presumed that these interactions occur in vivo and contribute to the mechanism of transformation of cells by E1A. With respect to adenovirus early gene transcription, the requirement for a specific cellular protein to bind to the amino terminus of E1A is not strictly observed. Small-deletion mutants viruses can activate transcription as long as the E1A protein can bind either the RB or the p300 protein (Table 1). Furthermore, we show that disruption of RB function alone through E1A binding is not likely to be a sufficient activity of the E1A amino-terminal domain for viral early gene activation. Specifically, cells which have homozygous deletions of RB or which express T antigen, that is, cells in which RB function is inactivated, are not permissive for mutant virus growth. Possibly E1A also acts through another E1A-bound factor. The existence of such a host protein is consistent with our findings, but no evidence for such a protein is provided by our studies.

The failure of E1A mutant Ad5 strains to replicate in T-antigen-transformed BALB/c3T3 T cells shows that T antigen, although it contains a CR2-homologous sequence, cannot functionally complement mutations in the E1A amino terminus. Since T antigen also binds to the RB protein, p107, and p60 (25), the fact that an intact E1A amino terminus is still required for early gene activation in T-antigen-transformed cells suggests that viral protein interactions with RB protein and its family members are not sufficient for transactivation. This interpretation is consistent with the results of mutant viral infection of Y-79 cells, which do not express RB and in which the mutant viruses are also defective for growth. This finding suggests that another factor besides RB, either previously described or not yet detected, which interacts with the amino terminus or some other property of the amino terminus is important for the transactivation function.

Although the small-deletion mutant viruses *sub*/*dl*1032-13 and *sub*/*dl*1085-13, mutants that fail to bind high levels of p300, grow efficiently in cells in which RB and its related members are inactivated, the necessity of binding p300 remains unclear. It remains possible that the small-deletion E1A proteins can bind to threshold levels of p300 which are sufficient in vivo to mediate the biological activity of E1A. p300 is related to CREB-binding protein, a coactivator protein which associates with the cAMP-regulated factor CREB, and it is also possibly related to a family of p300-like proteins (4). Possibly the E1A amino terminus regulates ATF via p300 interaction.

An alternative possibility is that the E1A amino terminus interacts directly with transcription factors and TATA factors and acts as a bridging factor. In vitro studies have demonstrated that an E1A column retains proteins which are capable of binding to cAMP-responsive element and AP-1 sites (55). Coimmunoprecipitation studies have shown that E1A can interact directly with ATF family members and that this interaction is not dependent on CR3 of E1A (19). Indeed, direct interaction between E1A and the TATA factors has been characterized (35, 42).

The ability of E7 to complement E1A functions is not likely a coincidence and may represent a parasitic requirement for virus growth to be coordinately regulated with cellular proliferation. Indeed, HPV has evolved requirements for transcription transactivation similar to those of adenovirus. In the HPV16 and HPV18 enhancers, DNA-binding elements that are similar to adenovirus transcription control elements, including NF-1, AP-1/ATF and E2F sites (64, 68), are found.

The fact that the viral E1A and E7 proteins are both transforming proteins that regulate transcription reinforces the significance of transcription regulation in the maintenance of the transformed phenotype. This fact suggests that pivotal cellular transcription pathways must be altered to deregulate growth

control. It is therefore not entirely surprising that these two viruses, which are unrelated in evolution over generations, target the same critical regulators of normal cellular growth. These are regulators whose activity, when altered by mutation or by interaction with viral transforming proteins, can lead to cellular immortalization.

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