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Complementary Functions of E1a Conserved Region 1 Cooperate with Conserved Region 3 To Activate Adenovirus Serotype 5 Early Promoters

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The amino-terminal region of the adenovirus type 5 E1a protein including conserved regions (CRs) 1 and 2 binds the 105-kDa retinoblastoma protein and a second, 300-kDa, cellular protein. We show that mutant viruses with deletions of CR1 which release the binding of either p105 or p300 still activate early promoters and infect cells productively. However, mutations which disrupt binding of both proteins disrupt early promoter activity and block the viral life cycle. E1a CR3, which has an established role in early promoter activation, can act in *trans* to the amino-terminal functions. This suggests that the amino terminus provides distinct, redundant functions related to p300 and Rb binding that synergize with CR3 to transactivate early genes.

The adenovirus type 5 (Ad5) E1a proteins belong to a group of polypeptides encoded by DNA tumor viruses which, in addition to acting on viral targets, modify host cell proliferative programs and thereby potentiate viral infection. These viral polypeptides include early gene products encoded by the adenoviruses, the papovaviruses, and the papillomaviruses (12). The E1a proteins can induce host DNA synthesis (58, 64). When the E1a gene is integrated into the genomes of primary cells, E1a proteins can immortalize cells (40, 62, 90). In cooperation with a second oncogene such as *ras* (75), E1a can induce full morphological transformation.

The E1a proteins are the major virus-encoded regulators of viral transcription (46, 81). During infection, E1a gene expression is required for the activation of the five Ad5 early promoters (7, 45). E1a also induces the activity of specific cellular promoters as well as various synthetic promoters (29, 48, 67, 84, 103). Differential splicing of E1a transcripts produces 12S and 13S E1a mRNA species that encode, respectively, the small (243-amino-acid [aa]) and large (289-aa) E1a proteins, which differ by an internal peptide specific to the 289-aa species (8, 20, 69). Previous studies have demonstrated a role for the larger protein in early promoter activation (53, 63, 87).

Sequence comparison of E1a proteins encoded by different adenovirus serotypes revealed three highly conserved regions (65). Conserved region 1 (CR1) and CR2 span residues 41 to 80 and 120 to 139, respectively, and are present in both E1a proteins. CR3, which extends from aa 140 to 188, is unique to the 289-aa protein and contains the internal peptide retained by differential splicing. CR3 is required for the efficient transactivation of early viral gene promoters; these include the E1b, E2, E3, and E4 promoters (6, 7, 45, 66, 81, 87). Viruses that encode only the 243-aa protein, which lacks CR3, poorly transactivate viral promoters. Nevertheless, the 243-aa protein has been reported to possess a transactivation function (11, 14, 52, 56, 97).

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E1a associates with a group of cellular proteins (34, 101) which were characterized initially by their molecular weights and later by their sites of interaction with E1a. Interaction of cellular proteins with molecular masses of 60, 105, 107, and 130 kDa is dependent on CR2, with p105 binding also dependent on CR1 (24, 27, 96), while a 300-kDa factor is dependent on sequences of the amino terminus including CR1 (24, 44, 85, 91, 96, 99). The 105-kDa protein is the product of the retinoblastoma locus (Rb) (95), the 60-kDa peptide is cyclin A (72), and p107 is a factor related to the Rb protein (25). p130 is structurally related to Rb, but its function remains to be characterized (21, 33, 57, 61).

Studies of pRb and p107 reveal that they are regulators of transcription. The transcription factor E2F (51) is found in a physical complex with Rb protein (4, 5, 17, 19), and E1a binding to Rb can disrupt this complex (3), as can binding by other DNA virus tumor antigens (18). The p107 protein also forms complexes with E2F (13, 22, 55, 68). Association of Rb with E2F inhibits E2F-dependent transcription (37, 94). Because E2F is required for the activity of a number of genes whose products function in DNA synthesis, transcriptional regulation by Rb can control progression of the cell into S phase (9, 36, 88). In agreement, induction of cellular DNA synthesis and cell immortalization by E1a are dependent on CR1 and CR2 in stably transformed cells (32, 64, 77, 83, 95, 102). However, the individual contributions of CR1 and CR2 functions to productive infection have been less extensively investigated. The finding that mutant viruses with E1a proteins that have deletions within the amino-terminal region, at sites overlapping or neighboring CR1, grow in HeLa cells with kinetics similar to those of the wild type (83) suggests that these E1a regions are dispensible in other virally transformed host cells.

In this report we show that residues 20 to 68, within the amino-terminal 70 aa of E1a which encompass CR1, provide complementary functions, related to the binding of Rb and p300, either of which is sufficient for viral growth in nontransformed human cells. Thus, we show that loss of binding of either Rb or p300 as a consequence of deletions within or adjacent to CR1 does not block early promoter activation or the viral life cycle. However, larger deletions which lead to the failure to bind both Rb and p300 inactivate early transcription,

in agreement with previous reports. The work further shows that CR1 functions are required for all viral early transcription units, not just E2, the transcription unit which binds the pRb-regulated factor E2F. These results, together with the finding that mutants which bind either p300 or pRb are active, indicate functional redundancy in this region. We also show that CR3 can act in *trans* to CR1 and present a model based on these findings.

MATERIALS AND METHODS

Plasmids. Plasmids were generously provided as follows: pVA1 and the clones of E2a, E3, E4, and VA1 in M13 single-stranded DNA were from B. Aufiero and R. Schneider. pE2CAT was from N. Jones (93). Plasmids were grown in Luria broth and isolated by the alkaline lysis protocol (76) and further purified by a CsCl gradient centrifugation.

Tissue culture. Cells were cultured in Dulbecco minimal essential medium (DMEM; GIBCO) supplemented with antibiotic (100 μg of streptomycin per ml and 100 μg of penicillin per ml [Sigma]) and 10% fetal calf serum (GIBCO) for BALB/c3T3 cells, AG1523 cells, and E1a monoclonal antibody M73 cells, and 10% bovine calf serum (Hazelton) for Chang liver cells. For selection of cells that stably expressed the transfected neomycin resistance gene, G418 (GIBCO) was added to DMEM at 400 μg/ml.

Virus stocks. Adenovirus stocks were prepared by infection of human 293 cells, harvested at 48 h postinfection, by a wash with phosphate-buffered saline PBS, and by sonication in 5 ml of 100 mM Tris-HCl, pH 8. After clearing cell debris by centrifugation at 1,000 × *g* for 5 min, the supernatant was fractionated on a step gradient of 5 ml of CsCl solution in PBS consisting of 2.5 ml of 1.25-g/ml CsCl over 1.4 g of CsCl per ml by centrifugation at 35,000 rpm for 1 h in an SW41 rotor. The viral band, collected from drips from bottom-punctured tubes, was rebanded by equilibrium centrifugation in 5 ml (total) of 1.35-g/ml CsCl in PBS at 45,000 rpm in an SW50.1 rotor for at least 5 h. The viral band was dialyzed extensively against 10 mM Tris-HCl (pH 8)–80 mM NaCl–2 mM $MgCl_2$ and diluted with 4 volumes of 0.1% bovine serum albumin (BSA)–10 mM Tris-HCl (pH 8)–80 mM NaCl–2 mM $MgCl_2$–50% glycerol. The concentration of virus particles was estimated by determining A_{260}. The concentration of PFU was determined by titration of plaques formed on confluent 293 cells immobilized by an overlay of DMEM in Noble agar.

Antibodies. M73 monoclonal antibodies against the carboxy terminus of E1A were prepared from hybridoma cells (gift of Ed Harlow, Massachusetts General Hospital Cancer Center) (34). In brief, 2×10^6 M73 cells were injected into mice that had been primed with Pristane (2,6,10,14-tetramethylpentadecanoic acid; Aldrich Chemical Company, Inc.) for 2 weeks. The hybridoma cells were grown for 2 to 3 weeks until the mouse abdomens became large and round. Ascites fluid was collected with an 18-gauge needle from the enlarged abdomens. H219 monoclonal antibodies against the adenovirus E2 72-kDa DNA-binding protein were prepared from a tissue culture supernatant (gift of A. Levine, Princeton University).

Modified Hirt isolation of viral DNA. Cells were washed twice with ice-cold 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 [SDS], and 0.1 mg of proteinase K). The gelatinous solution was incubated for at least 6 h. The NaCl concentration was adjusted to 1 M, and the solution 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 extracted with phenol-chloroform and ethanol precipitated. Viral DNA was restricted with *SalI* and separated in 0.8% agarose gels.

Southern analysis. DNA was electrophoresed in a 0.9% agarose gel and transferred to nitrocellulose as previously described (76). DNA was UV cross-linked by treatment with a UV Stratalinker 2400 (Stratagene) at 1200 μJ. Filters were hybridized to ^{32}P-labeled DNA probes prepared by random oligonucleotide labeling (26).

Immunoprecipitation. E1a-associated proteins were analyzed essentially as previously described (35). Cells were grown in 6-cm-diameter culture dishes and were labeled with 250 to 500 μCi of [^{35}S]methionine for 4 h. The cells were then washed twice with ice-cold PBS and lysed for 30 min with E1a lysis buffer (250 mM NaCl, 0.1% Nonidet P-40, and 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.0]) while chilled on ice. Cell debris was removed by centrifugation at 10,000 × *g* for 2 min. The cell lysate was precleared with rabbit serum and protein A-Sepharose for 1 h. Specific antibodies were added and incubated for 1 h, and then protein A-Sepharose was added and incubated on a rocker at 4°C for 1 h. The protein A-Sepharose beads were washed three times, resuspended in Laemmli buffer, and released-protein fractionated by SDS-polyacrylamide gel electrophoresis (PAGE).

Western immunoblot analysis. For immunoblotting, 20 μg of cell lysate was diluted with 2× Laemmli buffer (80 mM Tris-HCl [pH 6.8], 2% SDS, 5% β-mercaptoethanol, 15% glycerol, 0.01% bromophenol blue) and loaded onto a 5% stacking gel over an 8% separation gel. Proteins were electrophoretically transferred to nitrocellulose at 200 mA with a Bio-Rad Mini-Protein transfer apparatus. Filters were blocked with 3% BSA in PBS for 1 h and then incubated with primary antibody. The filter was then washed six times with PBS, reblocked for 30 min, and incubated with ^{125}I-protein A (NEN, Dupont) for 1 h. The filter was washed with six changes of PBS, and specific proteins were visualized by autoradiography.

Transfection. Calcium phosphate-mediated DNA transfection was performed essentially as previously described (2). Cells were grown to a density of 0.5×10^6 per 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 pE2CAT vector with 5 μg of salmon sperm carrier DNA, and 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.

Isolation of RNA and S1 nuclease analysis. RNA was isolated as described by M. Greenberg (2). Briefly, cells were lysed in Nonidet P-40 buffer, nuclei were separated for nuclear run-on analysis, and cytoplasmic RNA was isolated from the cytoplasmic fraction by treatment with proteinase K (Boehringer) and subsequent extraction with phenol. S1 nuclease analysis was performed with 10 μg of total cytoplasmic RNA as previously described (2).

Nuclear transcription run-off assay. Nuclei were prepared from at least two 15-cm-diameter dishes as described by Ausubel et al. (2). For the run-off reaction, nuclei were incubated in transcription buffer containing 150 μCi of [^{32}P]UTP (Dupont, NEN) in a solution of 25 mM Tris-HCl (pH 8); 12.5 mM $MgCl_2$; 325 mM KCl; a 1.25 mM concentration (each) of ATP, GTP, and CTP; 0.5 μM dithiothreitol; and 200 U of RNasin per ml. The reaction mixture was processed as described with the exception 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 Sephadex G-50 microspin column. The labeled RNA was hybridized to nitrocellulose filters that had single-stranded M13 DNA clones as probes for the Ad5 early regions and plasmid DNA for the negative control.

CAT assay. Cells grown in 10-cm-diameter dishes were washed with PBS at 24 h posttransfection, scraped from the dish, pelleted, and resuspended in 100 μl of 0.25 M Tris (pH 7.8). Chloramphenicol acetyltransferase (CAT) activity was measure with 50 μl of cell extracts as previously described (76). Acetylated [^{3}H]chloramphenicol was resolved by thin-layer chromatography and visualized by autoradiography of En^{3}Hance (NEN Dupont)-treated chromatography plates.

RESULTS

An E1a amino-terminal function is required for virus growth. To assay the contribution of amino-terminal sequences of E1a during the infectious cycle, we assayed the growth of a series of viruses (Fig. 1) which contain substitution/deletion mutations within residues 20 to 70 of E1a. The mutant viruses fall into two groups according to the sizes of the deletions. We show below that viruses within each group are phenotypically related. The first group consists of viruses with small deletions of 5 to 7 aa (*sub/dl*1005, *sub/dl*1032, and *sub/dl*1085), and the second consisted of mutants with large deletions, ranging from 25 to 48 aa (*sub/dl*1004, *sub/dl*1006, *sub/dl*1008, and *sub/dl*1015).

We assayed the expression of late viral functions by these mutants during infections of Chang liver cells (16), a human cell line which is permissive for Ad5 growth but which is not virally transformed. Viruses which encode a wild-type 289-aa E1a peptide or a mutant peptide with one of the small amino-terminal deletions efficiently expressed late viral proteins and a viral RNA abundant at late times, VA1-RNA (data not shown). In contrast, viruses with large amino-terminal deletions expressed greatly reduced levels of these late products. Late protein expression was independent of CR2 inasmuch as mutant virus *dl*922 with a deletion in CR2 expressed late proteins at levels equivalent to that of the wild type (data not shown; see below). These results suggested that in Chang liver cells, progression to the late phase is dependent on a function provided by the amino terminus which includes CR1.

Viral DNA replication is a prerequisite for late-phase transcription and protein synthesis. To determine whether viral DNA replication was dependent on the amino-terminal region, levels of replicated viral DNA in mutant virus-infected Chang liver cells were assayed by Hirt extraction and Southern blotting. Wild-type virus *dl*309, which encodes the 243R and the 289R proteins, and *dl*348, which encodes only the 289R protein, both replicated DNA efficiently by 24 h postinfection (Fig. 2A). *dl*347, which expresses only the 243R protein and lacks CR3, did not replicate. Viruses which expressed mutant E1a proteins with small deletions (*sub/dl*1005, *sub/dl*1032, and *sub/dl*1085) replicated comparably to the wild type, *dl*309 (Fig. 2A). In contrast, viruses encoding large deletion mutant E1a proteins, *sub/dl*1004, *sub/dl*1006, *sub/dl*1008, and *sub/dl*1015, were defective for replication. Mutant *dl*922, which has a CR2 deletion, replicated as efficiently as *dl*309. Equivalent levels of viral replication from the different mutants during infections of 293 cells, an E1a-transformed human cell line (28) (not shown), demonstrate that differences in viral replication in Chang liver cells can be attributed to differences in E1a function. From the above findings, we conclude that late protein, late RNA synthesis, and viral DNA replication in Chang liver cells require an E1a amino-terminal function provided by the wild-type and small deletion mutant viruses but lost in the large deletion mutants, in addition to the CR3 function.

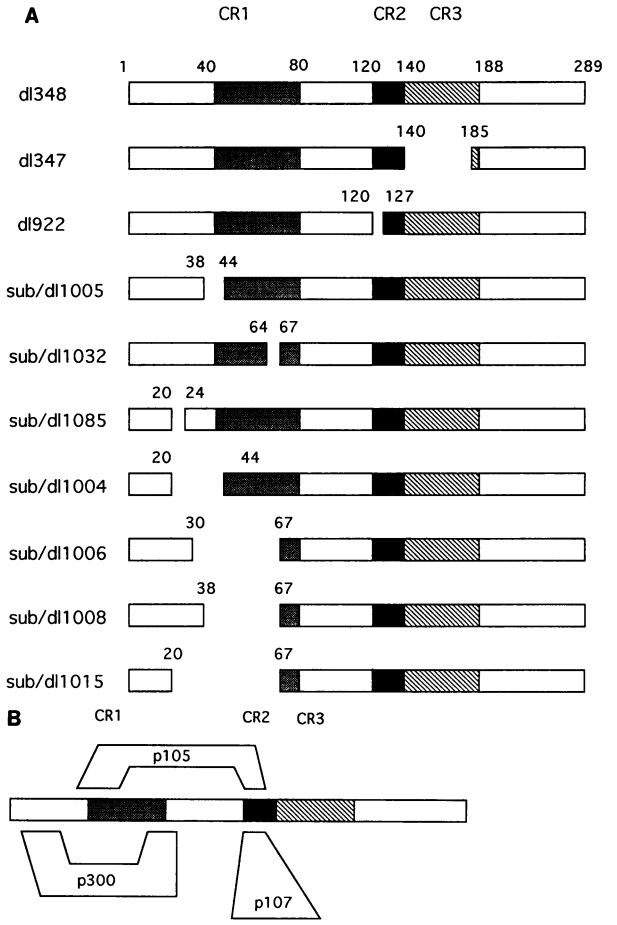

FIG. 1. Structure of adenovirus E1A mutations. (A) Protein coding regions of the E1A gene of the genomic, wild-type *dl*309 strain of Ad5 and of *dl*348 and *dl*347, which are respectively 13S and 12S cDNA recombinants, and of deletion-substitution mutant viruses, which are shown in the 13S cDNA background. Corresponding viruses which encode the same mutations in the 12S cDNA background were also employed. The structures of the in-frame deletion-substitution mutants have been previously reported (83). The E1a amino acids which directly border the deleted region are identified by the amino acid number. (B) Approximate sites of binding of cellular proteins p300, Rb, and p107 relative to CR1 (grey box), CR2 (black box), and CR3 (hatched box) are shown.

Virus replication in diploid fibroblasts. Cell-specific properties may account for the observed phenotypes of the mutant viruses in Chang liver cells. To determine whether the dependence of virus late functions on the amino terminus occurs in other cell types, we assayed viral DNA replication in two nontransformed fibroblast cell lines: AG1523 human diploid fibroblast cells that were isolated from human foreskin and exhibit growth properties of untransformed cells, and the murine BALB/c3T3 cells. Both cell lines were assayed to determine if the defective late function phenotype is restricted to fully permissive cells. In both fibroblast cell lines, the replication kinetics were slow compared with those of Chang liver cells. In the human fibroblast cell line, replication of the

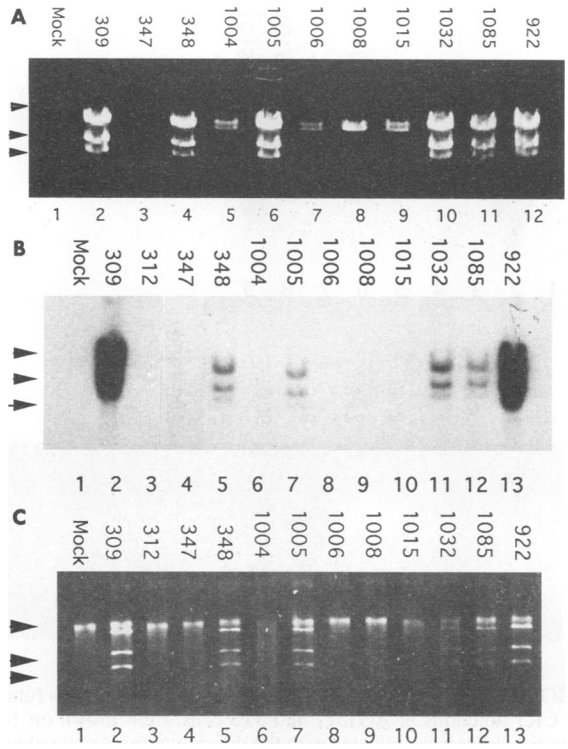

FIG. 2. Analysis of mutant virus replication in different cell lines. (A) Chang liver cells were infected with different mutant viruses and lysed at 24 h postinfection with neutral lysis buffer. Viral DNA was isolated from the supernatant of high-salt extraction as described in Materials and Methods. Viral DNA was restricted with *Sal*I enzyme, and the fragments were resolved by agarose gel electrophoresis. The distinctive fragments of viral DNA indicated by arrows on the left are 19,400, 9,400, and 7,200 bp in length. (B) Cells were infected with different viruses at 20 PFU per cell. Cells were lysed at 40 h postinfection, and the viral DNA was extracted. Viral DNA was restricted with *Sal*I and resolved by agarose gel electrophoresis. Viral fragments were transferred to a nitrocellulose membrane and probed with *dl*309 DNA ^{32}P labeled by the random priming method and visualized by autoradiography. DNA in AG1523 cells was analyzed at 40 h postinfection. (C) Ethidium bromide agarose gel electrophoresis of *Sal*I-restricted Hirt extract viral DNA from BALB/c3T3 cells at 48 h postinfection with the noted viruses at 20 PFU per cell.

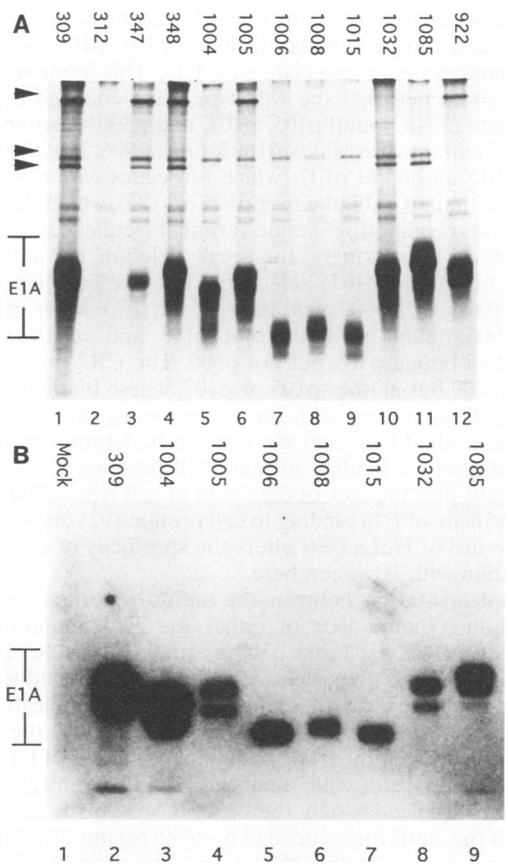

FIG. 3. Analysis of mutant E1A protein binding to cellular factors in HeLa cells and E1a expression in Chang liver cells. (A) HeLa cells were infected at 10 PFU per cell with the noted viral strains and labeled with [^{35}S]methionine for 4 h during early infection. Nuclear extracts were prepared as described in Materials and Methods, and E1a complexes were immunoprecipitated with M73 monoclonal antibody and fractionated by SDS-PAGE. The positions of migration of p300, p107, and p105 are noted (arrowheads), as are the positions of the E1a products themselves. (B) Nuclear extracts (10 μg) isolated from virus-infected Chang liver cells were resolved on SDS–8% PAGE and transferred to a nitrocellulose membrane. The membrane was then probed with M73 antibody to detect E1a proteins and then probed with ^{125}I-labeled protein A to detect the M73 antibody. The filters were washed and visualized by autoradiography.

wild-type strain *dl*309 was barely detectable at 24 h postinfection (not shown) but became apparent at 40 h postinfection (Fig. 2B). Despite a delay in the onset of replication, the phenotypes of the mutant viruses paralleled those seen in Chang liver cell infections. Replication is dependent on a CR3 function, since the 12S cDNA virus, *dl*347, was inactive at 40 h postinfection and replicated only after 70 h, and then at a level lower than the 13S cDNA virus, *dl*348 (data not shown). Significantly, the viruses with small deletions replicated to the same level as *dl*348, whereas the large deletion mutant viruses were defective in both the AG1523 cells and in BALB/c3T3 cells (Fig. 2C). The fact that the replication phenotypes found in Chang liver cells were reproduced in AG1523 and BALB/c3T3 cells indicates that the amino-terminal 70 aa of E1a provide a function necessary for virus growth in several types of nontransformed cells.

The defect in growth in BALB/c3T3 and AG1523 cells was not due to a lack of E1a protein expression, since immunoprecipitation from infected HeLa cells (Fig. 3A) and Western immunoblotting of infected Chang liver cell extracts (Fig. 3B) demonstrated that E1a proteins are expressed by each of the mutant viral strains. Immunofluorescence detection studies with infected cells demonstrated that all mutant E1a proteins were localized to the nucleus, including infections by mutants which fail to replicate (data not shown). Thus, the defect did not arise from improper E1a localization. The kinetics of replication in BALB/c3T3 and AG1523 cells were analyzed to determine whether the reduction simply reflected an initial lag in the onset of replication. Measurement of the kinetics of [^{3}H]thymidine uptake by infected cells and dot blot analysis of viral DNA at different times after infection showed that cells infected with wild-type virus *dl*309 and small deletion mutant viruses begin to incorporate [^{3}H]thymidine and commence replication at 24 h postinfection and that this increase is not seen with viruses bearing the large E1a deletions or with *dl*312 even at 72 h postinfection (data not shown).

Cellular proteins associated with the amino-terminal deletion mutant E1a proteins. The immunoprecipitation of E1a

from infected HeLa cells in Fig. 3A also revealed the previously characterized cellular proteins pRb, p107, and p300, which noncovalently associate with E1a. This immune precipitation confirmed that the wild-type E1as encoded by *dl*309, *dl*347, and *dl*348 bound p105, p107, and p300. Also, the large deletion mutant viruses *sub/dl*1004, *sub/dl*1006, *sub/dl*1008, and *sub/dl*1015 all bound p107 (which associates with CR2 exclusively) but failed to bind either p105 or p300, which depend on CR1 and neighboring amino-terminal sequences for their association. Interestingly, the small deletion mutant viruses (which all bound p107) selectively lost the association with either p105 or p300 such that of the two latter proteins, *sub/dl*1005 bound p300 but not p105 and *sub/dl*1032 and *sub/dl*1085 bound p105 but not p300. The CR2 mutant *dl*922 bound p300 but neither p105 or p107. These binding patterns are in full agreement with the structures of the mutant E1a proteins studied here and the previously determined sites on E1a required for binding to these cell proteins (24, 96). This agreement plus the lack of detection of cell type influences on the specificity of E1a binding to cell protein (92) make unlikely that the use of HeLa cells alters the specificity of cell protein association with E1a seen here.

Complementation between the amino terminus and CR3. The finding that a lack of either the E1a amino-terminal binding sites for p105 and p300 or the lack of CR3 leads to inactivity suggests that the amino terminus, including CR1, cooperates with CR3 to induce viral DNA replication and subsequent late functions. Interestingly, in Fig. 2 the viruses which encode both the 243R and the 289R forms of E1a, *dl*309 and *dl*922, replicate with significantly greater efficiency than *dl*348, which encodes only the 289R form. This is despite the fact that the 289R form encoded by *dl*348 retains all amino acid sequences present in the 243R form. This suggests that functions of the amino terminus (which may be provided by both the 289R and the 243R forms) and CR3 functions (provided by the 289R form exclusively) can work most efficiently if they are on separate molecules and that some form of *trans* cooperation between CR1 and CR3 takes place.

To compare the capacities of the CR1 and CR3 conserved regions to function in *trans* and in *cis*, complementation studies were performed. Cells were coinfected with two virus types: (i) *dl*347, which encodes the 243R protein and provides the amino-terminal but not the CR3 function, and (ii) viruses with a large deletion mutation of the 289R protein, which provide CR3 but not amino-terminal function. Synergistic effects by the two viruses on viral replication, measured in AG1523 fibroblasts, are seen in Fig. 4A. While no replication is seen for *sub/dl*1015-13 or *dl*347 alone, when the two viruses are coinfected (lane 8), replication is readily observed. It is likely that amino terminus CR1 and CR2 functions are limiting under the conditions of this assay, since coinfection of *dl*347 with *dl*348 (lane 6), a combination which would provide increased intact amino terminus and CR2 function relative to *dl*348 alone, synergized relative to the *dl*348 single infection (lane 5). Also, coinfection with *sub/dl*1015-13 and *dl*348 (lane 9), a combination which would provide increased CR3 function, gave increased replication relative to *dl*348 alone, implying CR3 is also a limiting factor. A similar complementation effect was noted when VA1 RNA synthesis was measured by S1 analysis with BALB/c3T3 cells (Fig. 4B). These experiments indicate that in both AG1523 and BALB/c3T3 cells, the amino-terminal CR1 functions and the CR3 functions can complement in *trans*. This reinforces the notion that the amino-terminal function is distinct from the CR3 function and synergizes with it.

Early viral protein synthesis. Because Ad5 replication is dependent on E2 region products (15, 86), insufficient E2

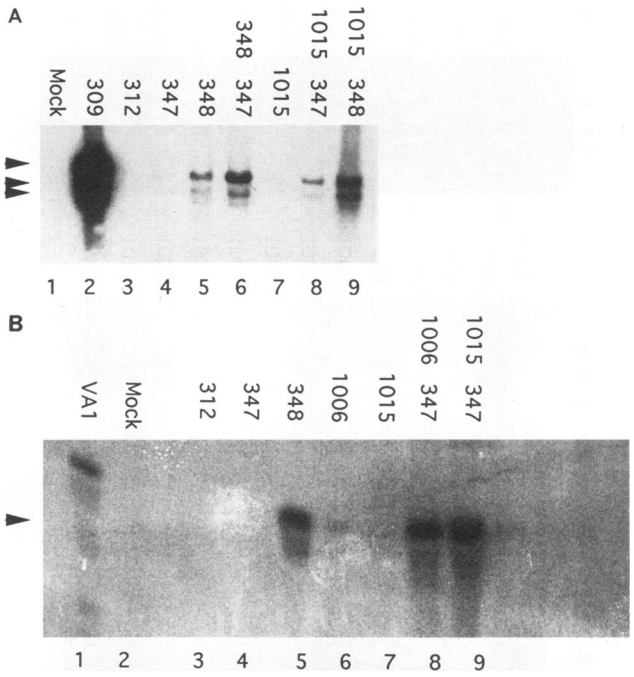

FIG. 4. Analysis of cooperation between amino terminus functions and CR3 functions in AG1523 and 3T3 cells. Cells grown on 10-cm-diameter dishes were infected with the different viruses or combination of viruses as indicated. *dl*312 is a virus with deletion of the E1a gene. *dl*347 is a virus encoding only the wild-type 12S E1A product, whereas *dl*348 encodes only the wild-type 13S E1a protein. *sub/dl*1015-13 is a mutant virus encoding E1a protein with functional CR2 and CR3. (A) Complementation for viral replication in AG1523 fibroblasts. Viral DNA was extracted, restricted with *Sal*I, resolved on an agarose gel, transferred to nitrocellulose, and probed for viral sequences. (B) VA1 RNA synthesis in rodent 3T3 cells, quantitated by S1 analysis.

expression could block viral DNA synthesis. To assay for such a block, the levels of the E2 region-encoded 72-kDa DNA-binding protein (DBP) in wild-type and mutant virus-infected cells were determined. Immunoprecipitation of extracts from [^{35}S]methionine-labeled, infected Chang liver cells (Fig. 5A) and Western analyses of DBP in infected BALB/c3T3 cells (Fig. 5B) both show that viruses defective for replication are also defective for synthesis of high levels of DBP. This indicates that functions provided by the amino terminus are required for the efficient synthesis of E2 region-encoded protein. Most significantly, in both analyses, the small deletion mutant viruses *sub/dl*1005, *sub/dl*1032, and *sub/dl*1085 synthesized DBP at levels comparable to those of the wild-type virus, *dl*309, and comparable to those of the 289R-specific virus, *dl*348. The CR2 mutant *dl*922 expressed DBP at this high level as well, while the large deletion mutant viruses *sub/dl*1004, *sub/dl*1006. *sub/dl*1008, and *sub/dl*1015 all expressed greatly reduced levels of DBP.

Analysis of early gene transcription. The reduced levels of DBP expression by large deletion mutant viruses suggested a defect in early-region E2 transcription or mRNA production. Northern (RNA) analysis of virus-infected BALB/c3T3 cells showed that wild-type and small deletion viruses express E2 mRNA at similar levels, while large deletion mutant virus E2a mRNA levels were decreased (data not shown). To determine whether early gene transcription was blocked, we performed nuclear run-on assays. Run-on RNA synthesized in nuclei from infected cells was hybridized to filters containing cDNA probes

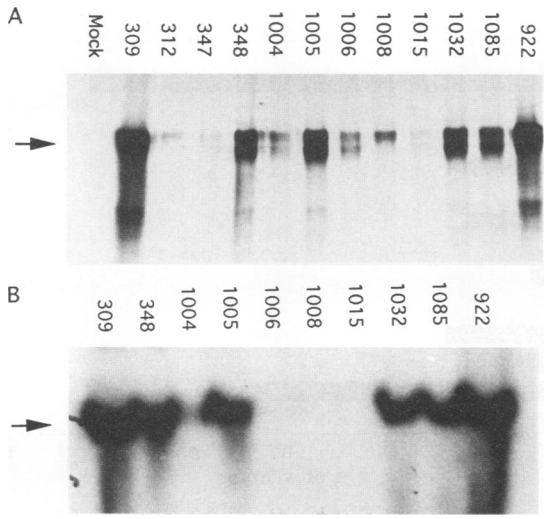

FIG. 5. Synthesis of the E2 72K early replication protein in mutant adenovirus-infected cells. (A) Immunoprecipitation of E2-72K DBP from Chang liver cells. Monolayer Chang liver cells were infected with the different viruses at a multiplicity of 10 PFU per cell. At early times, 8 h postinfection, cells were labeled with [^{35}S]methionine for 2 h. Cells were lysed in E1a lysis buffer (250 mM NaCl, 50 mM HEPES [pH 7.0], and 0.1% Nonidet P-40) on ice, and extracts were immunoprecipitated with H219, a monoclonal antibody against E2-DBP. Immunoprecipitated DBP protein was resolved by SDS-PAGE and fluorographed, and DBP was visualized by autoradiography. (B) Western immunoblotting for E2-72K DBP in BALB/c3T3 cells. Monolayer cells were infected with mutant adenoviruses at 20 PFU per cell, and at 24 h postinfection, cells were lysed in 2× Laemmli buffer and the proteins were resolved by SDS-PAGE. The proteins were transferred to nitrocellulose, and the filter was probed for E2-72K DBP with H219 and ^{125}I-protein A as described in Materials and Methods.

for early regions E1a, E2, E3, and E4; a probe for the late RNA, VA1; and a pBR322 control DNA. Results with AG1523 cells (Fig. 6) and BALB/c3T3 cells (data not shown) revealed transcription from early regions E2, E3, and E4 by replication-competent viruses at 24 h after infection, a time prior to the onset of DNA replication. The level of E1a transcription was below the threshold of detection. No VA1 RNA transcripts

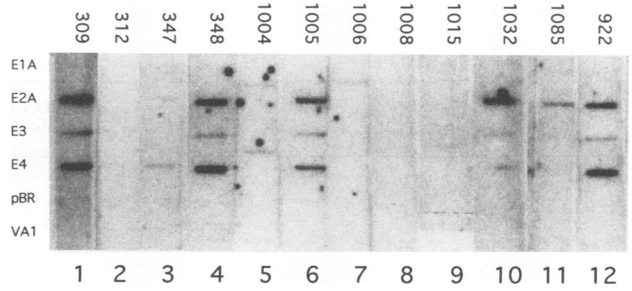

FIG. 6. Nuclear transcription run-off analysis of early viral genes. AG1523 human diploid fibroblasts were infected with the mutant viruses at 50 PFU per cell. The nuclei were isolated at 24 h postinfection, and nascent transcripts were elongated in the presence of [^{32}P]UTP as described in Materials and Methods. Each filter was hybridized with equal counts from the run-off reaction. The filters were washed and autoradiographed. The filters contained cDNA for the viral genes indicated on the left. VA1 was used as a control indicator of postreplicative transcription.

were detected in wild-type or other infected cells, confirming that the cells were in the early phase of infection. Interestingly, with the large deletion mutant viruses, nascent transcripts from early regions 2, 3, and 4 were undetectable. In contrast, E2 transcripts were readily detected for *sub/dl*1005, *sub/dl*1032, and *sub/dl*1085 as well as the CR2 mutant *dl*922. E3 and E4 were also activated in the small deletion and *dl*922 mutant infections, with the possible exception of *sub/dl*1085.

To determine whether the coordinate reduction of early gene transcription by the large deletion mutant viruses is a property specific to factors affecting the viral template and viral chromatin, we assayed transactivation of a viral promoter residing in cell chromosomal DNA. We stably integrated into BALB/c3T3 cells the vector, pE2-CAT. In the resulting cell line, the surrogate CAT gene is expressed from the E2a promoter during infection by viruses expressing wild-type E1a. As shown in Fig. 7, control E2-CAT cells do not express basal CAT enzyme activity. However, when E2-CAT cells were infected with *dl*309, CAT activity significantly increased, indicating that the reporter gene is responsive to virally expressed E1a. The responsiveness of the chromosomally integrated E2a promoter to the different mutant E1a proteins was assayed by mutant virus infection of the E2-CAT cells. CAT assays of infected E2-CAT cell extracts shown in Fig. 7A and quantitated in Fig. 7B demonstrate that expression of the mutant 289-aa E1a proteins which failed to activate early promoters during infection also failed to activate the chromosomally integrated E2a promoter in the E2-CAT cells. Viruses expressing the small deletion mutant E1a 243R proteins were active at levels comparable to those of *dl*348, which expresses the wild-type 289R protein, and *dl*922, the CR2 mutant virus.

DISCUSSION

We have analyzed the E1a amino-terminal region corresponding to residues 20 to 70, including CR1, for its role in activating Ad5 early gene expression and the viral life cycle. The locations of the conserved regions CR1, CR2, and CR3 within E1a and the sites of interaction with cell proteins are summarized in Fig. 1B. The locations of deletions in mutant viruses are given in Fig. 1A. Our results indicate that multiple E1a regions which bind different cell proteins function in the induction of early promoter activity. Notably, these regions can substitute for one another functionally and synergize with CR3 to transactivate.

We have previously shown that our large deletion mutant viruses which neither activate early transcription nor replicate DNA in nontransformed cells were phenotypically wild type in HeLa cells (83). HeLa cells express the human papillomavirus type 18 sequences (78) encoding the E7 protein, which, like E1a, binds p105 and p107 (23). Thus, in HeLa cells, the human papillomavirus type 18 E7 protein may provide functions lacking in the E1a large deletion mutants. E7 and E1a can dissociate E2F from its complexes with the p105Rb and p107 proteins (18, 42, 98), complexes in which E2F is repressed (see below). In agreement, both E7 and E1a can transactivate the Ad5 E2 promoter (70, 71). Furthermore, expression of E7 in Chang liver cells confers permissive growth on the large deletion mutant viruses (97a).

Previous studies have indicated a role for the E1a amino terminus in transactivation (11). The derepression of E2F following E1a binding to pRb provides a mechanism for CR1- and CR2-dependent transactivation of E2F-regulated promoters (73). In addition to regulating the E2a promoter, E2F regulates a number of cellular genes associated with G_1/S progression, including the c-Myc and DHFR genes (9, 36, 88).

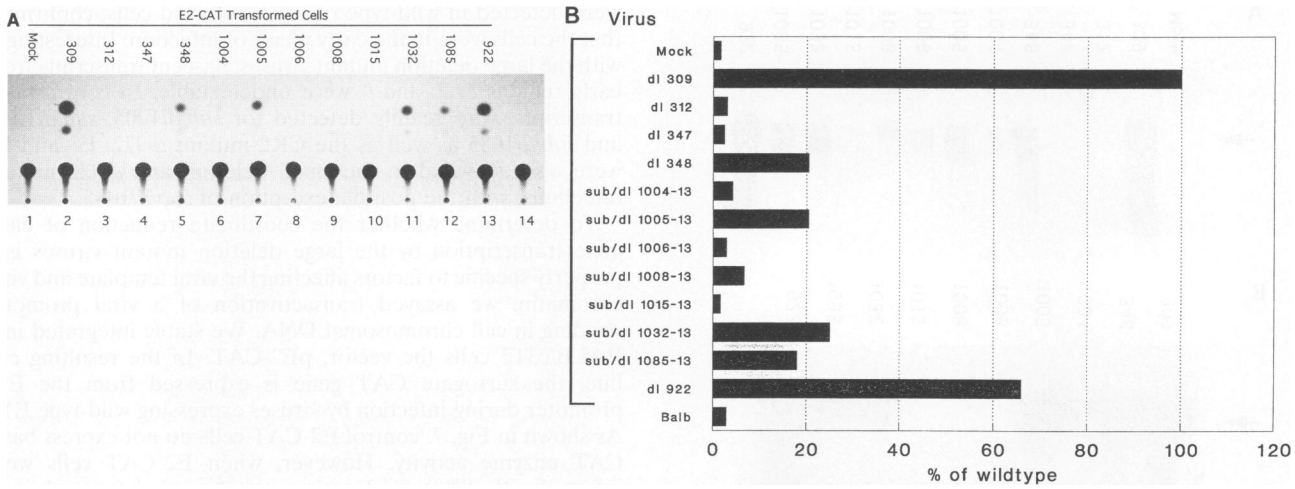

FIG. 7. E1a stimulation of a stably integrated chloramphenicol gene regulated by the E2 promoter is dependent on CR1. Monolayer BALB/c3T3 cells were stably transformed by pE2CAT and pRSVNeo. Geneticin (GIBCO)-resistant cells were clonally expanded and analyzed for CAT enzyme production when infected by *dl*309. One response cell line was isolated of a total of six analyzed. The cell line was subsequently expanded for studies of transactivation by the different mutant adenoviruses. The E2-CAT cells were infected, and 50% of cell lysate from a 10-cm-diameter dish was prepared as described in Materials and Methods for CAT enzyme analysis. (A) Acetylated [^{3}H]chloramphenicol was resolved on thin-layer chromatography and treated with aerosol En^{3}Hance (Dupont, NEN) for autoradiography. The BALB/c3T3 lane 14 represents cell extracts prepared from the parental cell. (B) Relative conversion of [^{3}H]chloramphenicol was determined with respect to that of wild-type *dl*309. The converted and unconverted [^{3}H]chloramphenicol spots were excised and counted. The percent of total [^{3}H]chloramphenicol converted was determined. The percentage of chloramphenicol converted for a mutant virus was then divided by the percent converted for *dl*309 to give the relative value shown on the bar graph.

We have previously shown that Ad5's ability to induce cell DNA synthesis is lost by the large deletion mutant viruses (83) which also do not replicate viral DNA (Table 1). This suggests that E1a induction of viral DNA synthesis parallels E1a induction of cell DNA synthesis. If common E1a-dependent mechanisms control viral promoters for replicative functions and cellular genes for cell DNA replication functions, E1a could coordinately induce viral and cell replication to provide synergy between viral and cellular growth programs.

Other mechanisms besides E1a-dependent relief of Rb repression of E2F must, however, operate. We find that the E2a promoter is active in viruses with mutant E1as that fail to associate with p105, indicating a more complex regulatory relationship between Rb binding and early gene expression. Virus *dl*922, which has a deletion in CR2 and virus *sub/dl*1005, with a small deletion in CR1, both express E1a proteins that fail to bind p105, yet they efficiently express E2a transcripts and DBP protein and also replicate viral DNA. Virus mutant *dl*922 also fails to bind p107, which like Rb forms a complex with E2F (13, 79, 82). E1a mutant *dl*922 is also expected to fail to bind p130, which is related to p107 and p105 and associates with CR2 (79). E1a mutant *dl*922 does associate with p300, a cellular protein which binds DNA (1, 74) and undergoes cell cycle-dependent phosphorylation (100). However, E1a mutants *sub/dl*1032 and *sub/dl*1085, neither of which associate with p300 but nonetheless can activate E2a transcription and late viral functions, show that binding of p300 is also dispensable for E2a activity.

The fact that individual cell protein binding to E1a is dispensable for early promoter activation is brought into focus

TABLE 1. Binding ability[a]

Virus	E1a mutation	Binding			E2a activation	NIH 3T3 cell DNA synthesis	Viral DNA synthesis
		p300	p107	p105			
*dl*309	Wild type	+	+	+	+	ND	+
*dl*312	Δ 1-289[b]	−	−	−	−	−	−
*dl*347	Δ 140-185[c]	+	+	+	−	+	−
*dl*348	289R only[c]	+	+	+	+	ND	+
*sub/dl*1005	Δ 38-44	+	+	−	+	+	+
*sub/dl*1032	Δ 64-67	−	+	+	+	+	+
*sub/dl*1085	Δ 20-24	−	+	+	+	+	+
*sub/dl*1004	Δ 20-44	−	+	−	−	−	−
*sub/dl*1006	Δ 30-67	−	+	−	−	−	−
*sub/dl*1008	Δ 38-67	−	+	−	−	−	−
*sub/dl*1015	Δ 20-67	−	+	−	−	−	−
*dl*922	Δ 120-127	+	−	−	+	ND	+

[a] The structure of substitution/deletion mutants and assay of NIH 3T3 cell DNA synthesis are described elsewhere (83). The first and last wild-type residues deleted from substitution/deletion mutants are indicated, and these are replaced in the mutant viral proteins by 2- or 3-aa insertions encoded by linkers. ND, not determined.

[b] *dl*312 has a deletion of the E1a gene.

[c] *dl*347 expresses only the 12S 243R protein, and *dl*348 expresses the 13S 289R protein.

by the phenotypes summarized in Table 1. The small deletion mutants are all capable of activating the early promoters, although one small deletion mutant subclass fails to bind Rb, while a second subclass fails to bind p300. Also, both classes of mutants bind p107. When this is considered together with the results from mutant *dl*922, it is apparent that pRb, p107, and p300 binding are individually dispensable for early promoter activation. This suggests that any functions provided by binding of pRb, p107, or p300 are dispensable, are overlapping, or are provided by another pathway, such as E1a binding of a second cell protein. Other aspects of E1a function show a similar relationship between E1a CR1 genotype and phenotype, in which small but not large deletion mutants are functional. Repression of the differentiated phenotype of PC12 cells (10, 35), the ability to induce viral and cellular DNA synthesis (41, 83), and the activation of proliferating cell nuclear antigen PCNA (47) also share this pattern.

E2F is the most extensively characterized transcription factor regulated by the Rb protein (4, 43, 80), but its functions alone cannot account fully for the general dependence of the activity of the early promoters besides E2a on the E1a amino terminus reported here and by Kraus et al. (52). The other Ad5 early transcription units, including E1b, E3, and E4, are dependent on the amino-terminal functions, although they lack E2F binding sites in their respective promoters. Each early promoter utilizes a combination of transcription factors to activate transcription (46). It is therefore possible that the other early promoters are dependent on transcription factors different from E2F which are also regulated by pRb through an E1a amino-terminal function. A broader role for pRb in transcription is indicated by evidence that additional DNA-binding proteins interact with pRb. Examples of these include Elf-1 (91), PU.1 (31), Sp1 (49, 89), MyoD (30), and ATF-2 (50). Alternatively, it is possible that CR1 or flanking residues in the amino terminus act directly or indirectly on a component of the general transcription machinery which is necessary for viral early promoter activity.

Our results suggest functional redundancy in cell protein interaction with the amino terminus. The small deletion E1a mutants bind different combinations of cell proteins; nevertheless, each mutant virus efficiently activates its early promoters. This apparent redundancy between E1a-pRb and E1a-p300 interaction could arise by several mechanisms. It is possible that the cellular proteins which bind to E1a (e.g., p300K, p105Rb, and p107) have several distinct transcription factors as targets, and a subset of the transcription factors may be sufficient for early gene expression. The fact that p300 can interact with the TATA factor provides direct evidence that the function of this E1a-associated protein affects transcriptional regulation (1). Alternatively, combinations of the cell proteins, for example, p300 and pRb, may cooperate to repress gene expression. Sequestration of any one protein by a particular E1a region would disrupt the functional cooperation, leading to derepression of gene expression.

Our data show that different domains of the E1a amino terminus are capable of functioning synergistically with CR3. CR3 contains a transactivation domain and interacts with other components of the transcriptional machinery, including the ATF-2 transcription factor (59, 60) and the general transcription factor TBP (39, 54). ATF-2 employs a basic region-zipper DNA-binding domain to associate with Ad5 early promoters (46, 104) and interacts with TBP to facilitate the formation of a preinitiation complex (38). These findings raise the possibility that CR3 acts as a bridge between ATF-2 and TBP (54). If CR3 provided a bridging function or other function providing access to TBP for transcription factors which are controlled by p105, p107, and p300 and which are derepressed by the E1a amino terminus, the observed synergy between the different E1a amino-terminal domains with CR3 would be explained. Whatever mechanism of CR1-CR3 synergy operates, it must account for the coordinate control of all early promoters by E1a. Coordinate regulation suggests that the different early promoters have common regulatory features responsive to E1a despite the differences in transcription factor proteins which bind to their regulatory regions. To determine general mechanisms of early promoter controls, it will be necessary to establish how E1a regulates early promoters other than E2a, for which the targets of action of CR1 are not yet known.

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REFERENCES

1. **Abraham, S. E., S. Lobo, P. Yaciuk, H. G. Wang, and E. Moran.** 1993. p300 and p300-associated proteins are components of TATA-binding protein (TBP) complexes. Oncogene **8**:1639–1647.
2. **Ausubel, F., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1988. Current protocols in molecular biology. Green Publishing Associates and Wiley-Interscience, New York.
3. **Bagchi, S., P. Raychaudhuri, and J. Nevins.** 1990. Adenovirus E1A proteins can dissociate heteromeric complexes involving the E2F transcription factor: a novel mechanism for E1A transactivation. Cell **62**:659–669.
4. **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.
5. **Bandara, L. R., J. P. Adamczewski, T. Hunt, and N. L. Thangue.** 1991. Cyclin A and the retinoblastoma gene product complex with a common transcription factor. Nature (London) **352**:249–251.
6. **Berk, A. J.** 1986. Adenovirus promoters and E1A transactivation. Annu. Rev. Genet. **20**:45–79.
7. **Berk, A. J., F. Lee, T. Harrison, J. Williams, and P. A. Sharp.** 1979. Pre-early adenovirus 5 gene product regulates synthesis of early viral messenger RNAs. Cell **17**:935–944.
8. **Berk, A. J., and P. A. Sharp.** 1978. Structure of the adenovirus 2 early mRNAs. Cell **14**:695–711.
9. **Blake, M. C., and J. C. Azizkhan.** 1989. Transcription factor E2F is required for efficient expression of the hamster dihydrofolate reductase gene in vitro and in vivo. Mol. Cell. Biol. **9**:4994–5002.
10. **Boulukos, K. E., and E. B. Ziff.** 1993. Adenovirus 5 E1a proteins disrupt the neuronal phenotype and growth factor responsiveness of PC12 cells by a conserved region 1-dependent mechanism. Oncogene **8**:237–248.
11. **Braithwaite, A. W., C. C. Nelson, and A. J. D. Bellet.** 1991. E1a revisited: the case for multiple cooperative transactivation domains. New Biol. **3**:18–26.
12. **Buchkovich, K., N. Dyson, P. Whyte, and E. Harlow.** 1990. Cellular proteins that are targets for transformation by DNA tumor viruses. CIBA Found. Symp. **150**:262–278.
13. **Cao, L., B. Faha, M. Dembski, L. H. Tsai, E. Harlow, and N. Dyson.** 1992. Independent binding of the retinoblastoma protein and p107 to the transcription factor E2F. Nature (London) **355**:176–179.
14. **Carlock, L. R., and N. C. Jones.** 1981. Transformation-defective mutant of adenovirus type 5 containing a single altered E1A mRNA species. J. Virol. **42**:106–113.

15. **Challberg, M. D., and T. J. Kelly.** 1989. Animal virus DNA replication. Annu. Rev. Biochem. **58**:671–717.
16. **Chang, R. S.** 1954. Continuous cell cultivation of epithelial-like cell from normal human tissue. Proc. Soc. Exp. Virol. Med. **87**:440–443.
17. **Chellappan, S., S. Hiebert, M. Mudryj, J. Horowitz, and J. Nevins.** 1991. The E2F transcription factor is a cellular target for the RB protein. Cell **65**:1053–1061.
18. **Chellappan, S., K. B. Kraus, B. Kroger, K. Munger, P. M. Howley, W. C. Phelps, and J. R. Nevins.** 1992. Adenovirus E1A, simian virus 40 tumor antigen, and the human papillomavirus E7 protein share the capacity to disrupt the interaction between transcription factor E2F and the retinoblastoma gene product. Proc. Natl. Acad. Sci. USA **89**:4549–4553.
19. **Chittenden, T., D. Livingston, and W. 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.
20. **Chow, L. T., T. R. Broker, and J. B. Lewis.** 1979. Complex splicing patterns of RNAs from the early region of adenovirus-2. J. Mol. Biol. **134**:265–303.
21. **Cobrink, D., P. Whyte, D. S. Peeper, T. Jacks, and R. A. Weinberg.** 1993. Cell cycle-specific association of E2F with the p130 E1A-binding protein. Genes Dev. **7**:2392–2404.
22. **Devoto, S. H., M. Mudryj, J. Pines, T. Hunter, and J. R. Nevins.** 1992. A cyclin A-specific protein kinase complex possesses sequence-specific DNA binding activity: p33cdk2 is a component of the E2F-cyclin A complex. Cell **68**:167–176.
23. **Dyson, N., P. Guida, K. Munger, and E. Harlow.** 1992. Homologous sequences in adenovirus E1a and human papillomavirus E7 proteins mediate interaction with the same set of cellular proteins. J. Virol. **66**:6893–6902.
24. **Egan, C., T. N. Jelsma, J. A. Howe, S. T. Bayley, B. Ferguson, and P. E. Branton.** 1988. Mapping of cellular protein-binding sites on the products of early-region 1A of human adenovirus type 5. Mol. Cell. Biol. **8**:3955–3959.
25. **Ewen, M. E., Y. Xing, J. B. Lawrence, and D. M. Livingston.** 1991. Molecular cloning, chromosomal mapping, and expression of the cDNA for p107, a retinoblastoma gene product-related protein. Cell **66**:1155–1164.
26. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
27. **Giordano, A., C. McCall, P. Whyte, and B. R. Franza.** 1991. Human cyclin A and the retinoblastoma protein interact with similar but distinguishable sequences in the adenovirus E1A gene product. Oncogene **6**:481–486.
28. **Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus 5. J. Gen. Virology **36**:59–72.
29. **Green, M. R., R. Treisman, and T. Maniatis.** 1983. Transcriptional activation of cloned human beta-globin genes by viral immediate-early gene products. Cell **35**:137–148.
30. **Gu, W., J. W. Schneider, G. Condorelli, S. Kaushal, V. Mahdavi, and B. Nadal-Ginard.** 1993. Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. Cell **72**:309–324.
31. **Hagemeier, C., A. J. Bannister, A. Cook, and T. Kouzarides.** 1993. The activation domain of transcription factor PU.1 binds the retinoblastoma (RB) protein and the transcription factor TFIID in vitro: RB shows sequence similarity to TFIID and TFIIB. Proc. Natl. Acad. Sci. USA **90**:1580–1584.
32. **Haley, K. P., J. Overhauser, L. E. Babiss, H. S. Ginsberg, and N. C. Jones.** 1984. Transformation properties of type 5 adenovirus mutants that differentially express the E1A gene products. Proc. Natl. Acad. Sci. USA **81**:5734–5738.
33. **Hannon, G. J., D. Demetrick, and D. Beach.** 1993. Isolation of the Rb-related p130 through its interaction with CDK2 and cyclins. Genes Dev. **7**:2378–2391.
34. **Harlow, E., P. Whyte, B. J. Franza, and C. Schley.** 1986. Association of adenovirus early-region 1A proteins with cellular polypeptides. Mol. Cell. Biol. **6**:1579–1589.
35. **Heasley, L. E., S. Benedict, J. Gleavy, and G. L. Johnson.** 1991. Requirement of the adenovirus E1A transformation domain 1 for inhibition of PC12 cell neuronal differentiation. Cell Regul. **2**:479–489.
36. **Hiebert, S. W., M. Blake, J. Azizhan, and J. R. Nevins.** 1991. Role of E2F transcription factor in E1A-mediated transactivation of cellular genes. J. Virol. **65**:3547–3552.
37. **Hiebert, S. W., S. P. Chellappan, J. M. Horwitz, and J. R. Nevins.** 1992. The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. Genes Dev. **6**:177–185.
38. **Horikoshi, N., T. Hai, Y. S. Lin, M. R. Green, and R. G. Roeder.** 1988. Transcription factor ATF interacts with the TATA factor to facilitate establishment of a preinitiation complex. Cell **54**:1033–1042.
39. **Horikoshi, N., K. Maguire, A. Kralli, E. Maldonado, D. Reinberg, and R. Weinmann.** 1991. Direct interaction between adenovirus E1A protein and the TATA box binding transcription factor IID. Proc. Natl. Acad. Sci. USA **88**:5124–5128.
40. **Houweling, A., P. J. van der Elsen, and A. J. van der Eb.** 1980. Partial transformation of primary rat cells by the leftmost 4.5% fragment of adenovirus 5 DNA. Virology **105**:537–550.
41. **Howe, J. A., and S. T. Bayley.** 1992. Effects of Ad5 E1A mutant viruses on the cell cycle in relation to the binding of cellular proteins including the retinoblastoma protein and cyclin A. Virology **186**:15–24.
42. **Huang, P. S., D. R. Patrick, G. Edwards, P. J. Goodhart, H. E. Huber, L. Miles, V. M. Garsky, A. Oliff, and D. C. Heimbrook.** 1993. Protein domains governing interactions between E2F, the retinoblastoma gene product, and human papillomavirus type 16 E7 protein. Mol. Cell. Biol. **13**:953–960.
43. **Huber, H. E., G. Edwards, P. J. Goodhart, D. R. Patrick, P. S. Huang, M. Ivey-Hoyle, S. F. Barnett, A. Oliff, and D. C. Heimbrook.** 1993. Transcription factor E2F binds DNA as a heterodimer. Proc. Natl. Acad. Sci. USA **90**:3525–3529.
44. **Jelsma, T. N., J. A. Howe, J. S. Mymryk, C. M. Evelegh, N. F. Cunniff, and S. T. Bayley.** 1989. Sequences in E1A proteins of human adenovirus 5 required for cell transformation, repression of a transcriptional enhancer, and induction of proliferating cell nuclear antigen. Virology **171**:120–130.
45. **Jones, N., and T. Shenk.** 1979. An adenovirus type 5 early gene function regulates expression of other early viral genes. Proc. Natl. Acad. Sci. USA **76**:3665–3669.
46. **Jones, N. C., P. W. Rigby, and E. B. Ziff.** 1988. Trans-acting protein factors and the regulation of eukaryotic transcription: lessons from studies on DNA tumor viruses. Genes Dev. **2**:267–281.
47. **Kannabiran, C., G. F. Morris, C. Labrie, and M. B. Mathews.** 1993. The adenovirus E1a 12S product displays functional redundancy in activation the human proliferating cell nuclear antigen promoter. J. Virol. **67**:507–515.
48. **Kao, H. T., and J. R. Nevins.** 1983. Transcriptional activation and subsequent control of the human heat shock gene during adenovirus infection. Mol. Cell. Biol. **3**:2058–2065.
49. **Kim, S. J., U. S. Onwuta, Y. I. Lee, R. Li, M. R. Botchan, and P. D. Robbins.** 1992. The retinoblastoma gene product regulates Sp1-mediated transcription. Mol. Cell. Biol. **12**:2455–2463.
50. **Kim, S. J., S. Wagner, F. Liu, M. A. O'Reilly, P. D. Robbins, and M. R. Green.** 1992. Retinoblastoma gene product activates expression of the human TGF-beta 2 gene through transcription factor ATF-2. Nature (London) **358**:331–334.
51. **Kovesdi, I., R. Reichel, and J. R. Nevins.** 1986. Identification of a cellular transcription factor involved in E1A trans-activation. Cell **45**:219–228.
52. **Kraus, V. B., E. Moran, and J. R. Nevins.** 1992. Promoter-specific *trans*-activation by the adenovirus E1A 12S product involves separate E1A domains. Mol. Cell. Biol. **12**:4391–4399.
53. **Krippl, B., B. Ferguson, N. Jones, M. Rosenberg, and H. Westphal.** 1985. Mapping of functional domains in adenovirus E1A proteins. Proc. Natl. Acad. Sci. USA **82**:7480–7484.
54. **Lee, W. S., C. C. Kao, G. O. Bryant, X. Liu, and A. J. Berk.** 1991. Adenovirus E1A activation domain binds the basic repeat in the TATA box transcription factor. Cell **67**:365–376.
55. **Lees, E., B. Faha, V. Dulic, S. I. Reed, and E. Harlow.** 1992. Cyclin E/cdk2 and cyclin A/cdk2 kinases associate with p107 and

E2F in a temporally distinct manner. Genes Dev. **6**:1874–1885.
56. **Leff, T., R. Elkaim, C. R. Goding, P. Jalinot, P. Sassone-Corsi, M. Perricaudet, C. Kedinger, and P. Chambon.** 1984. Individual products of the adenovirus 12S and 13S E1a mRNAs stimulate viral EIIa and EIII expression at the transcriptional level. Proc. Natl. Acad. Sci. USA **81**:4381–4385.
57. **Li, Y., C. Graham, A. M. Duncan, and P. Whyte.** 1993. The adenovirus E1A-associated 130-kD protein is encoded by a member of the retinoblastoma gene family and physically interacts with cyclin A and cyclin E. Genes Dev. **7**:2366–2377.
58. **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.
59. **Lillie, J. W., and M. R. Green.** 1989. Transcription activation by the adenovirus E1A protein. Nature (London) **338**:39–44.
60. **Liu, F., and M. R. Green.** 1990. A specific member of the ATF transcription factor family can mediate transcription activation by the adenovirus E1a protein. Cell **61**:1217–1224.
61. **Mayol, X., X. Grana, A. Baldi, N. Sang, Q. Hu, and A. Giordano.** 1993. Cloning of a new member of the retinoblastoma gene family (pRb2) which binds to the E1A transforming domain. Oncogene **8**:2561–2566.
62. **Montell, C., G. Courtois, C. Eng, and A. Berk.** 1984. Complete transformation by adenovirus 2 requires both E1A proteins. Cell **36**:951–961.
63. **Montell, C., E. F. Fisher, M. H. Caruthers, and A. J. Berk.** 1982. Resolving the functions of overlapping viral gene by site-specific mutagenesis at a mRNA splice site. Nature (London) **295**:380–384.
64. **Moran, B., and B. Zerler.** 1988. Interactions between cell growth-regulating domains in the products of the adenovirus E1A oncogene. Mol. Cell. Biol. **8**:1756–1764.
65. **Moran, E., and M. B. Mathews.** 1987. Multiple functional domains in the adenovirus E1A gene. Cell **48**:177–178.
66. **Nevins, J. R.** 1981. Mechanisms of activation of early viral transcription by the adenovirus E1a gene product. Cell **26**:213–220.
67. **Nevins, J. R.** 1982. Induction of the synthesis of a 70,000 dalton mammalian heat shock protein by the adenovirus E1A gene product. Cell **29**:913–919.
68. **Pagano, M., G. Draetta, and P. Jansen-Durr.** 1992. Association of cdk2 kinase with the transcription factor E2F during S phase. Science **255**:1144–1147.
69. **Perricaudet, M., G. Akusjarvi, A. Virtanen, and U. Pettersson.** 1979. Structure of two spliced mRNAs from the transforming region of human subgroup C adenoviruses. Nature (London) **281**:694–696.
70. **Phelps, W. C., S. Bagchi, J. A. Barnes, P. Raychaudhuri, V. Kraus, K. Munger, 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.
71. **Phelps, W. C., C. L. Lee, K. Munger, and P. M. Howley.** 1988. The human papilloma virus type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1A. Cell **53**:539–547.
72. **Pines, J., and T. Hunter.** 1990. Human cyclin A is adenovirus E1A-associated protein p60 and behaves differently from cyclin B. Nature (London) **346**:760–763.
73. **Raychaudhuri, P., S. Bagchi, S. H. Devoto, V. B. Kraus, E. Moran, and J. R. Nevins.** 1991. Domains of the adenovirus E1A protein required for oncogenic activity are also required for dissociation of E2F transcription factor complexes. Genes Dev. **5**:1200–1211.
74. **Rikitake, Y., and E. Moran.** 1992. DNA-binding properties of the E1A-associated 300-kilodalton protein. Mol. Cell. Biol. **12**:2826–2836.
75. **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.
76. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1988. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
77. **Schneider, J. F., F. Fisher, C. R. Goding, and N. C. Jones.** 1987. Mutational analysis of the adenovirus E1a gene: the role of transcriptional regulation in transformation. EMBO J. **6**:2053–2060.
78. **Schneider-Gadicke, A., and E. Schwarz.** 1986. Different human cervical carcinoma cell lines show similar transcription patterns of human papillomavirus type 18 early genes. EMBO J. **5**:2285–2292.
79. **Schwarz, J. K., S. H. Devoto, E. J. Smith, S. P. Chellappan, L. Jakoi, and J. R. Nevins.** 1993. Interaction of the p107 and Rb proteins with E2F during the cell proliferation response. EMBO J. **12**:1013–1020.
80. **Shan, B., X. Zhu, P. L. Chen, T. Durfee, Y. Yang, D. Sharp, and W. H. Lee.** 1992. Molecular cloning of cellular genes encoding retinoblastoma-associated proteins: identification of a gene with properties of the transcription factor E2E. Mol. Cell. Biol. **12**:5620–5631.
81. **Shenk, T., and J. Flint.** 1990. Transcriptional and transforming activities of the adenovirus E1A proteins. Adv. Cancer Res. **57**:47–85.
82. **Shirodkar, S., M. Ewen, J. A. DeCaprio, J. Morgan, D. M. Livingston, and T. Chittenden.** 1992. The transcription factor E2F interacts with the retinoblastoma product and a p107-cyclin A complex in a cell cycle-regulated manner. Cell **68**:157–166.
83. **Smith, D. H., and E. B. Ziff.** 1988. The amino-terminal region of the adenovirus serotype 5 E1a protein performs two separate functions when expressed in primary baby rat kidney cells. Mol. Cell. Biol. **8**:3882–3890.
84. **Stein, R., and E. B. Ziff.** 1984. HeLa cell β-tubulin gene transcription is stimulated by adenovirus 5 in parallel with viral early genes by an E1a-dependent mechanism. Mol. Cell. Biol. **4**:2792–2801.
85. **Stein, R. W., M. Corrigan, P. Yaciuk, J. Whelan, and E. Moran.** 1990. Analysis of E1A-mediated growth regulation functions: binding of the 300-kilodalton cellular product correlates with E1A enhancer repression function and DNA synthesis-inducing activity. J. Virol. **64**:4421–4427.
86. **Stillman, B.** 1989. Initiation of eukaryotic DNA replication in vitro. Annu. Rev. Cell Biol. **5**:197–245.
87. **Svensson, C., and G. Akusjarvi.** 1984. Adenovirus 2 early region 1A stimulates expression of both viral and cellular genes. EMBO J. **3**:789–794.
88. **Thalmeier, K., H. Synovzik, R. Mertz, E. L. Winnacker, and M. Lipp.** 1989. Nuclear factor E2F mediates basic transcription and trans-activation by E1a of the human MYC promoter. Genes Dev. **3**:527–536.
89. **Udvadia, A. J., K. T. Rogers, P. D. Higgins, Y. Murata, K. H. Martin, P. A. Humphrey, and J. M. Horowitz.** 1993. Sp-1 bind promoter elements regulated by the RB protein and Sp-1-mediated transcription is stimulated by RB coexpression. Proc. Natl. Acad. Sci. USA **90**:3265–3269.
90. **van der Elsen, P. J., A. Houweling, and A. J. van der Eb.** 1983. Morphological transformation of human adenoviruses is determined to a large extent by gene products of region E1a. Virology **131**:242–246.
91. **Wang, C. Y., B. Petryniak, C. B. Thompson, W. G. Kaelin, and J. M. Leiden.** 1993. Regulation of the Ets-related transcription factor Elf-1 by binding to the retinoblastoma protein. Science **260**:1330–1335.
92. **Wang, H. G., Y. Rikitake, M. C. Carter, P. Yaciuk, S. E. Abraham, B. Zerler, and E. Moran.** 1993. Identification of specific adenovirus E1A-N terminal residues critical to the binding of cellular proteins and to the control of cell growth. J. Virol. **67**:476–488.
93. **Weeks, D. L., and N. C. Jones.** 1985. Adenovirus E3-early promoter: sequences required for activation by E1a. Nucleic Acids Res. **13**:5389–5402.
94. **Weintraub, S. J., C. A. Prater, and D. C. Dean.** 1992. Retinoblastoma protein switches the E2F site from a positive to a negative element. Nature (London) **358**:259–261.
95. **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.

96. **Whyte, P., N. M. Williamson, and E. Harlow.** 1989. Cellular targets for transformation by the adenovirus E1A proteins. Cell **56:**67–75.
97. **Winberg, G., and T. Shenk.** 1984. Dissection of overlapping functions within adenovirus type 5 E1A gene. EMBO J. **3:**1907–1912.
97a. **Wong, H., and E. Ziff.** Unpublished data.
98. **Wu, E. W., K. E. Clemens, D. V. Hack, and K. Munger.** 1993. The human papillomavirus E7 oncoprotein and the cellular transcription factor E2F bind to separate sites on the retinoblastoma tumor suppressor protein. J. Virol. **67:**2402–2407.
99. **Yaciuk, P., M. C. Carter, J. M. Pipas, and E. Moran.** 1991. Simian virus 40 large-T antigen expresses a biological activity complementary to the p300-associated transforming function of the adenovirus E1A gene products. Mol. Cell. Biol. **11:**2116–2124.
100. **Yaciuk, P., and E. Moran.** 1991. Analysis with specific polyclonal antiserum indicates that the E1A-associated 300-kilodalton product is a stable nuclear phosphoprotein that undergoes cell cycle phase-specific modification. Mol. Cell. Biol. **11:**5389–5397.
101. **Yee, S. P., and P. E. Branton.** 1985. Detection of cellular proteins associated with human adenovirus type 5 early region 1A polypeptides. Virology **147:**142–153.
102. **Zerler, B., B. Moran, K. Maruyama, J. Moomaw, T. Grodzicker, and H. E. Ruley.** 1986. Adenovirus E1A coding sequences that enable *ras* and *pmt* oncogenes to transform cultured primary cells. Mol. Cell. Biol. **6:**887–899.
103. **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.
104. **Ziff, E.** 1990. Transcription factors: a new family gathers at the cAMP response site. Trends Genet. **6:**69–72.