JOURNAL OF VIROLOGY, Sept. 1990, p. 4421–4427
0022-538X/90/094421-07$02.00/0

Vol. 64, No. 9

# 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

ROLAND W. STEIN,[1] MARY CORRIGAN,[2] PETER YACIUK,[2] JAMES WHELAN,[1] AND ELIZABETH MORAN[2]*

*Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, Tennessee 37322-0615,[1] and Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724[2]*

Received 26 April 1990/Accepted 14 June 1990

**Adenovirus E1A transforming function requires two distinct regions of the protein. Transforming activity is closely linked with the presence of a region designated conserved domain 2 and the ability of this region to bind the product of the cellular retinoblastoma tumor suppressor gene. We have investigated the biological properties of the second transforming region of E1A, which is located near the N terminus. Transformation-defective mutants containing deletions in the N terminus (deletion of residues between amino acids 2 and 36) were deficient in the ability to induce DNA synthesis and repress insulin enhancer-stimulated activity. The function of the N-terminal region correlated closely with binding of the 300-kilodalton E1A-associated protein and not with binding of the retinoblastoma protein. These results indicate that transformation by E1A is mediated by two functionally independent regions of the protein which interact with different specific cellular proteins and suggest that the 300-kilodalton E1A-associated protein plays a major role in E1A-mediated cell growth control mechanisms.**

The adenovirus E1A gene products are the major transcriptional regulators synthesized early during infection. These proteins mediate both the transcriptional activation of virus early promoters and the inhibition of enhancer-stimulated transcription (1, 2, 17, 21, 26, 35, 44, 51, 53). The E1A proteins are also sufficient to establish an extended growth potential in primary cells (18, 39) and are able to cooperate with the products of second oncogenes such as the adenovirus E1B gene or an activated *ras* gene product to induce a more fully transformed phenotype in primary cells (11, 39, 43, 49).

The techniques of in vitro mutagenesis have facilitated the localization of some of these biological functions to specific regions in the E1A protein products (reviewed in reference 33). This analysis has been aided by the availability of sequence information for the E1A genes from a variety of adenovirus serotypes (23, 50), which encode proteins containing three distinct regions of highly conserved amino acid sequence termed conserved domains 1, 2, and 3. The location of these regions in the E1A proteins is illustrated schematically in Fig. 1.

Conserved domain 3 distinguishes the larger of the two major (13S and 12S) early E1A mRNA products. It is closely associated with the function required for activation of other virus transcription units (3, 12, 19, 28, 29, 32, 47) and is dispensable for E1A transforming functions (14, 29, 60) as well as enhancer inhibition functions (2, 51).

Conserved domain 2 is required for E1A transformation functions (20, 25, 27, 33, 34, 41). This domain appears to constitute a discrete structural and genetic unit common to the transforming proteins of several divergent classes of DNA tumor viruses (10, 31, 36). The transforming function of domain 2 and its homologs appears to derive from their capacity to bind the approximately 105-kilodalton (kDa) product of the retinoblastoma (RB) tumor susceptibility gene (4, 7, 31, 55).

Although domain 2 plays an essential role in E1A transforming functions, it is not sufficient for these functions. A second region N-terminally distal from and not contiguous with domain 2 is also required for transformation activity in primary cells (20, 30, 41, 42, 46, 56). Loss of transforming activity resulting from deletion of the N-terminal region can occur without tertiary disruption of domain 2 structure. The evidence supporting this is the demonstration that an N-terminal deletion peptide is able to cooperate in *trans* with a domain 2 deletion peptide (30). This unusual *trans*-cooperating activity supports the argument that each biologically active region is functioning autonomously during transformation. If so, then conserved domain 2 and the N-terminal region may mediate distinctly different biological activities required in transformation.

To investigate whether the N-terminal transforming region of the E1A products may mediate biological events distinct from those associated with conserved domain 2, we constructed and analyzed a series of deletion mutants upstream of domain 2 for their ability to transform primary BRK cells, induce DNA synthesis, repress insulin enhancer-stimulated transcription, and bind to E1A-associated cellular proteins. Our results confirm the identification of a class of N-terminal E1A transformation-defective mutants that retain the ability to bind the 105-kDa RB gene product (8, 57). We show further that these mutants are defective compared with domain 2 mutants in their ability to induce DNA synthesis and inhibit insulin enhancer-stimulated transcription. These activities correlate with binding of a previously noted (16, 59) E1A-associated protein of 300 kDa. These results suggest that the N-terminal region and conserved domain 2 play essential but independent roles during E1A-mediated transformation.

* Corresponding author.

TABLE 1. Focus formation on primary-cultured BRK cells

| Plasmid cotransfected with pT24-H-*ras* | No. of foci from 10 plates in expt no.: | | Mean % induced[a] |
|---|---|---|---|
| | 1 | 2 | |
| pE1A.WT | 61 | 88 | 100 |
| pE1A.81-120 | 22 | | 36.1 |
| pE1A.76-120 | 18 | | 35.9 |
| pE1A.73-120 | 27 | | 39.4 |
| pE1A.51-116 | 39 | | 51.9 |
| pE1A.15-35 | 0 | 0 | <1.5 |
| pE1A.2-36 | 0 | 0 | <1.5 |
| pE1A.CXdl(121-150) | 0 | 0 | <1.5 |
| pE1A.CXdl(121-150) + pE1A.15-35 | | 28 | 22.7 |
| pE1A.CXdl(121-150) + pE1A.2-36 | | 12 | 13.6 |

[a] Data are expressed as percentages of numbers of wild-type foci, as described in Materials and Methods. Each mutant was assayed in at least three independent experiments, including those whose results are shown here.

## MATERIALS AND METHODS

**Cells and viruses.** Monolayer cultures of the 293, HeLa, and HIT T-15 2.2.2 cell lines were maintained as described previously (6, 32). All viruses were propagated on 293 monolayers. Ad5dl309 and Ad5dl312 (22) were obtained from T. Shenk. Primary BRK cells were prepared by collagenase-dispase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) treatment of kidneys from 6-day-old Fisher rats.

**Transformation assays.** Cells were transfected 2 to 3 days after plating as described previously (34). All transformation assays were done in cooperation with pT24-H-*ras* originally obtained from Fasano et al. (9). The *trans*-cooperation assay has also been described previously (30). Results in Table 1 are indicated as percentages of wild-type activity. This is derived from the ratio of the average number of foci per plate in the test transfection to the average number of foci per plate in a parallel transfection with a wild-type E1A plasmid. All percentages represent the averaged results from at least three independent experiments. The total number of wild-type-induced foci in each experiment was in the range of 25 to 75. Several stable cell lines were grown out from individual foci induced by each mutant or combination of mutants giving positive results, and the E1A protein products expressed were analyzed by immunoprecipitation. The lack of detectable contaminating wild-type products and the presence of appropriate mutant products were verified by comparison with the products expressed in BRK cells infected with the analogous mutant viruses as described previously (30).

**Construction of mutants.** Mutant E1A.CXdl(121-150) has been described previously (30, 34). The E1A mutants pSVXL.124(153-289) and pSVE1A (wild type) were generously provided by Anna Velcich and have been described elsewhere (52). The remaining mutants were generated by "loop-out" mutagenesis (62) using synthetic oligonucleotides ranging in length from 20 to 40 nucleotides. The oligonucleotides were hybridized to single-stranded template DNA generated from pUC118 vectors (containing a bacteriophage M13 origin of replication) grown in the presence of M13 helper phage, in some cases according to the method of Kunkel (24) to increase the efficiency of mutant isolation. Restriction fragments containing the deletions were transferred by standard recombinant DNA techniques to a wild-type genomic E1A plasmid, pE1A.WT, and the coding region of the entire transferred fragment was sequenced (40) to verify that there were no second-site mutations. The plasmids were named for the amino acid residues deleted: pE1A.81-120, pE1A.76-120, etc. E1A mutant plasmids were rebuilt into complete viruses as described previously (32). The retention of the proper translation frame was verified by nucleotide sequencing of the plasmids and by the demonstration that the expressed proteins maintain a monoclonal antibody epitope (M73) near the C terminus.

**Immunoprecipitations.** Monolayer cell cultures (diameter, 6 cm) were incubated with 0.1 mCi of Tran[$^{35}$S] label (ICN) in methionine-free Dulbecco modified Eagle medium from 17 to 19 h postinfection. Immunoprecipitations were done as described previously (16). Samples from equal numbers of cells were loaded in each lane. The monoclonal antibodies M73, pAB419, and C36 have been described previously (15, 16, 55) and were provided by E. Harlow.

**DNA synthesis.** Viral Hirt assays and [$^{3}$H]thymidine (ICN) incorporation experiments were done as described previously (61). The maximum standard deviation in these experiments was 24%.

**Repression of insulin enhancer-stimulated activity.** Transfections were performed by the calcium phosphate precipitation method described by Wigler et al. (58). Ten micrograms of total DNA containing 5 μg of the −700 CAT rat II insulin enhancer expression plasmid (54) and 1.25 μg of either an E1A expression plasmid or carrier DNA (pUC19) was coprecipitated and added to a 100-mm monolayer of HIT T-15 2.2.2 cells at 20 to 30% confluency. The precipitates were removed 4 h later, and the cells were treated with 20% glycerol and Dulbecco modified Eagle medium for 2 min before addition of fresh medium. Cells were harvested for chloramphenicol acetyltransferase (CAT) assays 40 to 48 h later. CAT enzymatic assays were performed as described by Gorman et al. (13). The first-exon mutants with the exception of pE1A.CXdl(121-150) were assayed as 12S cDNA expression plasmids. The E1A.CXdl(121-150) mutation makes a 13S cDNA but deletes part of the 13S unique region in addition to domain 2. The second-exon mutant, pSVXL.124(153-289), is a genomic E1A expression plasmid which expresses truncated versions of both the 12S and 13S E1A mRNA products. The activities of the first-exon mutants were compared with that of p12S.WT (34). The activities of pE1A.CXdl(121-150) and the second-exon mutant, pSVXL.124(153-289), were compared with that of pSVE1A (45). Expression of the reporter plasmid was reduced by a factor of 9 by p12S.WT and by a factor of 5 by pSVE1A.

## RESULTS

**N-terminal E1A sequences required for transformation activity.** We and others have shown that there are two regions within the first exon of E1A that are required for transformation. One biologically active site corresponds closely with the boundaries of the highly conserved sequences in domain 2 (Fig. 1). In an attempt to localize the active site in the upstream E1A region, we have made a systematic series of deletion mutants in the region upstream of domain 2. The endpoints of the deletions are indicated in Fig. 1. These mutant plasmids were assayed for biological activity in a *ras* cotransformation assay.

The results (Table 1) show that a deletion extending from residue 51 to residue 116 retains substantial transforming activity. Smaller deletions extending from within conserved domain 1 to the upstream border of domain 2 also retain

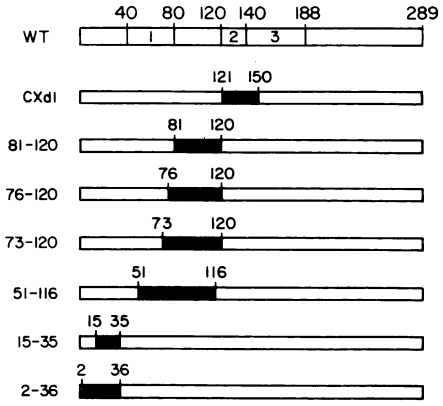


FIG. 1. Summary of the structure of E1A mutants upstream of domain 2. The E1A proteins (□) contain three domains of highly conserved amino acid sequence alternating with less-conserved regions. Conserved domains 1 and 2 occur in both of the major early E1A splice products, while domain 3 is unique to the 13S product. The numbers above the bars indicate the endpoints of various E1A deletion mutants. The deleted regions are indicated (■). All deletions retain the original frame of translation. Amino acid position numbers refer to the positions as they would occur in the 13S product. WT, Wild type.

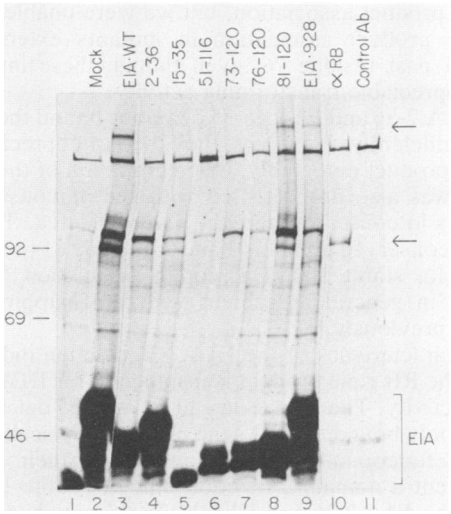


FIG. 2. Immunoprecipitations of E1A mutant peptides from virus-infected HeLa cells. Extracts of infected HeLa cells were immunoprecipitated as described previously (31) with E1A fusion protein-specific mouse monoclonal antibody (16) series M73 (lanes 1 to 9). Extracts from E1A wild-type (Ad5.dl309, the parental virus for all the mutant constructs)-infected cells were also immunoprecipitated with a control simian virus 40 T-antigen-specific mouse monoclonal antibody, pAB419 (lane 11), which does not recognize any products specifically in host cells, or a human RB product-specific mouse monoclonal antibody, C36 (lane 10). Immunoprecipitates were run on sodium dodecyl sulfate-7.5% polyacrylamide gels. The labels above lanes 3 to 9 indicate the E1A amino acid residues deleted. The endpoints of the deletions are indicated schematically in Fig. 1. The position of the specific RB gene product band at approximately 105 kDa is indicated by the lower arrow on the right. The higher arrow indicates the position of the unidentified 300-kDa cellular protein which also associates with the E1A products. Numbers on the left indicate the positions of molecular weight markers in kilodaltons. The area in which the heterogeneous products of the E1A gene run is indicated by a bracket on the right. WT, Wild type; Ab, antibody.

activity. In contrast, as shown before, a deletion removing domain 2, pE1A.CXdl(121-150), shows almost no activity. In addition to helping define sequences nonessential for transformation, this analysis helps demonstrate the autonomy of domain 2 structure and function. The critical residues for domain 2 function begin at position 121. Each deletion in the series in which residues 51 to 116, 73 to 120, 76 to 120, 81 to 120, and 86 to 120 are deleted introduces a different context of protein sequence close or immediately adjacent to domain 2 yet does not seriously impair domain 2 function.

We also made deletions near the extreme N terminus. In contrast to the deletion of residues 51 to 116, deletions extending from residues 2 to 36 or 15 to 35 showed no activity at all (Table 1), although they do encode stable proteins (Fig. 2 and additional data not shown). Both of these mutations remove only sequences outside of conserved domain 1, suggesting that the strictly essential upstream sequences lie toward the extreme N terminus of the E1A products rather than across the whole region of conserved domain 1.

In order to exclude the possibility that loss of function in the N-terminal mutants was merely a consequence of tertiary effects resulting in impairment of domain 2 function, we determined the activity of the mutant constructs in the *trans*-cooperation assay. Deletion of conserved domain 2 in E1A.CXdl(121-150) renders the E1A products profoundly defective for transformation activity. The requirement for domain 2 function can, however, be supplied in *trans*. This cooperating activity is not a result of a recombination event, since proper translation of the peptide products is required for activity (30). The E1A.2-36 and E1A.15-35 mutants were analyzed in this assay to determine whether their loss of transforming function was a consequence of an impairment in domain 2 function. As illustrated in the results in Table 1, the E1A.2-36 and E1A.15-35 mutants were able to supply the *trans*-cooperating domain 2 function efficiently to the E1A.CXdl(121-150) mutant. This result indicates that the E1A.2-36 and E1A.15-35 mutants are not transformation defective as a consequence of a disruption in region 2 tertiary structure and supports the proposal that there are at least two separate active sites required for E1A transforming function.

**The upstream region involved in transforming function correlates with binding of the 300-kDa E1A-associated protein.** The E1A proteins have the ability to bind a number of cellular proteins (16, 59), one of which has a molecular mass of 105 kDa and has been identified as the product of the RB tumor suppressor gene (7, 55). The mutants described in the section above were rebuilt back into virus constructs to facilitate assay of the E1A proteins and their association with cellular products. The ability of the mutant E1A products to bind to the RB gene product, p105-RB, and another prominent, but as yet unidentified, product with a molecular mass of 300 kDa was assayed with infected HeLa cells (Fig. 2). There is also a prominent p107-associated protein which binds to a site in domain 2 close to or overlapping with the p105-RB-binding site (8, 57). The binding of p107 was not severely affected in the N-terminal mutants described here or in E1A.928 (Fig. 2) and will not be considered further.

Sequences in domain 2 are required for stable association with the RB product but not with the 300-kDa product (8, 57). This pattern can be seen in Fig. 2 in the domain 2 missense mutant, E1A.928. Results with the mutants described here indicate that the region between conserved domains 1 and 2 (residues 81 to 120) is not required for stable

RB gene product association, but we were unable to detect RB gene product association in mutants extending into domain 1 past residue 76, even though these mutants retained appreciable transforming activity.

The E1A.2-36 and E1A.15-35 deletions bound the RB gene product efficiently. However, they did not coprecipitate the 300-kDa product detectably. Coprecipitation of the 300-kDa product was also not detected with the mutants in which sequences in conserved domain 1 were deleted. The region between conserved domains 1 and 2 (residue 81 to 120) is not required for stable 300-kDa protein association. These results are in general agreement with the mapping studies reported previously (8, 57).

The characteristics of the E1A.2-36 deletion indicate that binding the RB gene product is insufficient for E1A transformation activity. The E1A.2-36 and E1A.15-35 deletion products not only bound the RB gene product physically but also bound it functionally, as determined by their ability to complement a domain 2 deletion mutant (Table 1). Nevertheless, the E1A.2-36 and E1A.15-35 mutants had no transforming activity by themselves (Table 1). The results shown in Fig. 2 suggest that both the 300-kDa and p105-RB E1A-associated proteins may be important for E1A-mediated transformation. If so, they appear to function through interactions with two independently acting regions within the E1A proteins, the N-terminal region and conserved domain 2. To address the question of autonomy more directly, we compared mutants resulting from deletions in these regions for their ability to induce DNA synthesis and repress insulin enhancer-stimulated transcriptional activity.

**N-terminal deletion mutants are impaired in the ability to induce DNA synthesis and viral replication.** We assayed the ability of the N-terminal mutant E1A.2-36 to induce DNA synthesis and viral replication in BRK cells. We compared its activity with those of two domain 2 mutants, E1A.928 and E1A.CXdl(121-150). The 928 mutation is a point mutation in residue 124 that affects only domain 2 activity. The E1A.CXdl(121-150) mutation deletes all of domain 2 and part of domain 3. The transformation defect in the E1A.928 point mutant is as severe as that in the complete deletion mutant (34). Previously, we have shown that these domain 2 mutants retain an appreciable ability to induce DNA synthesis in BRK cells despite their severe transforming defect (30, 61).

Viral DNA was selectively extracted from [$^3$H]thymidine-labeled infected cells by a modified Hirt procedure. The levels of [$^3$H]thymidine incorporation into viral DNA from Ad5dl309 (the E1A.WT virus parent for all the E1A mutants used in this study), E1A.928 (a domain 2 mutant), and the N-terminal mutant E1A.2-36 were compared. The results shown in Fig. 3A indicate that the E1A.2-36 deletion impairs the ability of the E1A products to induce virus DNA replication by a factor of about 10.

The ability to replicate viral DNA in infected quiescent cells requires two E1A functions: the capacity to induce the cellular DNA synthesis functions and the ability to transactivate other viral proteins. The latter activity is a function of conserved domain 3. To determine that the impairment in the E1A.2-36 deletion mutant is actually a defect in the ability to induce cellular DNA synthesis functions, we did a complementation experiment with E1A.2-36 and a domain 3-defective mutant, E1A.hr3. The E1A.hr3 mutant is severely defective in the ability to transactivate viral products (12) and therefore in the ability to replicate viral DNA (Fig. 3B). This mutant, however, retains the ability to induce cellular DNA synthesis sufficient to support rapid and extensive proliferation of BRK cells (61). Coinfection of the E1A.2-36 and E1A.hr3 viruses resulted in near-wild-type levels of viral DNA synthesis (Fig. 3B), indicating that these two sets of products indeed contribute separate functions.

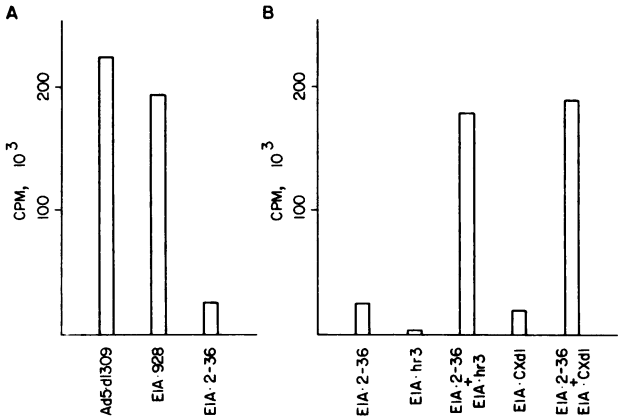


FIG. 3. Analysis of virus DNA production in infected quiescent cells. Primary BRK cells were infected at a multiplicity of infection of 10 PFU per cell. Coinfection experiments were done at a total multiplicity of 10, i.e., 5 PFU of each virus per cell. [$^3$H]thymidine was added approximately 1 h postinfection, when the unabsorbed virus was removed. At 30 h postinfection, virus DNA was extracted and samples were counted. The counts per minute were normalized to those from cells infected with a near-total E1A deletion virus, Ad5dl312.

If the activity impaired in the E1A.2-36 mutant were indeed independent of both domain 3 and domain 2 activities, we would expect that E1A.2-36 could also be complemented for viral replication by the E1A.CXdl(121-150) mutation, which inactivates both domains 2 and 3. Figure 3B shows that such complementation can indeed occur. These results suggest that induction of DNA synthesis correlates more closely with the N-terminal transforming function than with the domain 2 transforming function.

**N-terminal E1A mutants are significantly impaired in the ability to repress insulin enhancer-stimulated activity.** We have demonstrated previously that both the 13S and 12S E1A mRNA protein products repress insulin enhancer-stimulated transcription in pancreatic β cells (44, 45). To identify more precisely the region(s) in E1A that is required for repression, we transfected the deletion mutants with −700 CAT, a plasmid that contains the coding region of the bacterial CAT gene linked to the rat insulin II gene enhancer-promoter. The insulin expression plasmid was introduced into an insulin-producing β cell line, HIT T-15 2.2.2, by calcium phosphate coprecipitation with or without the E1A expression plasmids. Protein extracts were prepared 48 h after transfection and assayed for CAT enzyme levels.

The mutants with deletions at residues 73 to 120, 76 to 120, 81 to 120, 86 to 120, 121 to 150 [E1A.CXdl(121-150)], and 153 to 289 repressed insulin enhancer-stimulated transcription as efficiently as their respective wild-type parental plasmids did (Fig. 4) (see Materials and Methods for description of plasmids). However, the E1A.2-36, E1A.15-35, and E1A.51-116 N-terminal region mutants repressed −700 CAT expression at less than 10% of the wild-type level. These results suggest that insulin enhancer repression, like the ability to induce DNA synthesis, correlates more closely with the N-terminal transforming function than with the domain 2 transforming function.

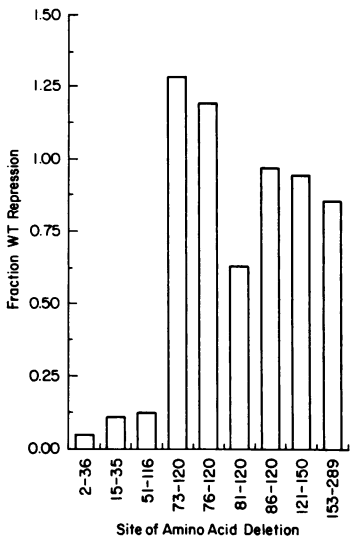


FIG. 4. Ability of N-terminal E1A mutants to repress insulin enhancer-stimulated transcription. HIT T-15 2.2.2 cells were transfected as described in Materials and Methods by using insulin enhancer plasmid DNA, −700 CAT, E1A mutant plasmid DNA, and carrier pUC19 DNA. Reporter gene activity was assayed by measuring CAT enzyme levels. The results are expressed as fractions of E1A.WT repression. The experiments were repeated on five different occasions; data shown are from one representative experiment.

## DISCUSSION

Two physically independent regions of E1A must both be functional in order for its transforming activity to be manifest (30). One of these regions constitutes the relatively well-defined sequences designated conserved domain 2. Domain 2 sequences are shared by several DNA tumor virus transforming proteins, and the transforming function of this region is closely correlated with its RB gene product-associating activity (4, 5, 7, 31, 55).

A second region, noncontiguous and N-terminally distal to domain 2, is likewise absolutely required for E1A transforming function (20, 30, 41, 42, 46, 56). Here we have analyzed the biological activities associated with the required N-terminal sequences. Our results suggest that the ability to induce DNA synthesis in quiescent cells and to repress insulin enhancer-stimulated transcription colocalize rather closely and correlate with the N-terminal transforming function. They are independent of the RB gene product-associating activity of the E1A products and correlate much more closely with binding of the unidentified 300-kDa cellular product.

The summary of this evidence is as follows. First, the required N-terminal activity can be abolished absolutely with little apparent physical or functional disruption of RB gene product binding. Second, the ability to induce DNA synthesis is severely impaired in a mutant (E1A.2-36) which has lost the N-terminal transforming activity, although this activity is relatively unaffected in mutants which have lost domain 2 function and RB gene product-associating activity. In fact, a complete domain 2 deletion mutant can complement the E1A.2-36 N-terminal mutant for viral replication in quiescent cells. Third, mutants with defects in the N-terminal transforming function are severely impaired in their ability to repress insulin enhancer-stimulated transcription; in contrast, domain 2 mutants are nearly wild type for repression in this assay.

Since the E1A proteins associate with several cellular products, it is tempting to speculate that the second E1A-mediated transforming activity involves association with one of these products. A strong candidate for a required N-terminal association is the 300-kDa cellular product whose association with the E1A products requires sequences from residue 2 to approximately residue 70 (8, 57).

In addition to its biological consequences, the E1A.2-36 deletion severely impairs the ability of the E1A products to bind the 300-kDa product. However, the suggestion that association with the 300-kDa product may be a basic part of the biochemical mechanism by which E1A mediates these functions is not entirely consistent with the properties of mutants such as E1A.51-116, which lacks sequences within conserved domain 1. E1A.51-116 does not appear to bind the 300-kDa or RB products but is at least partially positive in the biological functions assayed here.

One possibility is that the conserved sequences of domain 1 play an important role in stabilizing the binding of the E1A-associated proteins but do not encode their actual required binding sites. The apparent lack of RB and 300-kDa product binding with domain 1 mutants could be only a consequence of the stringency of the coimmunoprecipitation assay compared with the sensitivity of the biological assays. This suggestion is supported directly by the results and analysis of Egan et al. (8), who found that domain 1 sequences are not strictly required for RB gene product association. This suggestion is also consistent with the intermediate phenotype of the E1A.51-116 mutant. In contrast, the severely defective phenotype of the E1A.2-36 mutant suggests that this deletion affects the active site more directly.

The colocalization of the biological activities required for induction of DNA synthesis and repression of the insulin enhancer suggests that these activities may be related. It seems likely that E1A transforms cells by directly or indirectly modulating the expression of cellular genes involved in growth control. It is possible that association of the 300-kDa product and the E1A proteins negatively regulates transcription of a set of cellular genes important in cell growth control. Possible cellular targets include the JE, c-*myc*, and stromelysin genes, whose transcription is strongly repressed by E1A during adenovirus-mediated transformation. This repression activity is impaired in E1A mutants near the N terminus but does not require sequences in domain 2 (48).

A strong consensus of data now indicates that an E1A enhancer repression function localizes to the extreme N terminus. However, the N terminus may not be the only region of E1A required for repression of enhancer-stimulated transcription. While several studies have found that domain 2 and second-exon sequences are dispensable, or nearly so, for repression function (20, 38, 46, 48), other studies have indicated a more important role of domain 2 and second-exon sequences in repression of simian virus 40, polyomavirus, and immunoglobulin enhancer activity (27, 52). We have obtained the domain 2 and second-exon mutants that were defective for repression in those studies from those investigators (27, 52) and found that they are wild type for repression in our system (data not shown). It may be that the region of E1A required for repression is enhancer dependent. Possibly, during E1A-mediated transformation, the negative regulation of different cellular genes requires different sequence domains of E1A.

In broad outline, available data indicate that the extreme N terminus of the E1A message encodes an essential trans-

forming function. The essential region may overlap with but does not appear to be centered directly in conserved domain 1, as several investigators (20, 41, 56) have also found that deletions of conserved domain 1 sequences from about residue 60 to 80 are less deleterious than deletions closer to the extreme N terminus, even ones such as E1A.2-36 which do not actually include the highly conserved sequences. The majority, at least, of the sequences in conserved domain 1 may play an important supporting but not strictly essential role in transforming function. The distinction between the actual N terminus and the conserved region that lies near the N terminus is important in understanding the structural significance of the E1A protein. The actual N terminus is a structural unit distinct from conserved domain 1. Its protein properties are entirely different in terms of net charge and predicted secondary structure (for example, conserved region 1 is highly proline enriched and contains several predicted β turns, while the N terminus has no predicted turns). In addition, these regions are physically separable by a completely nonessential region extending at least from residue 25 to 35 (8, 20).

An important corollary to the distinction between the N terminus and conserved region 1 as the active site is the ability to study independently the effects of binding of different cellular proteins. Conserved region 1 plays a role in binding both the RB and 300-kDa proteins, while the N terminus is not required for RB product binding. The majority of mutations examined in previous studies lie within conserved region 1. We now know that they affect both binding functions and that this complicates the ability to make correlations between biological activities and specific binding functions. The usefulness of this separation of functions is seen in the ability of the E1A.2-36 deletion to block completely the repressive effects of transforming growth factor β1 on *myc* expression in human keratinocytes (37), suggesting that the ability of E1A and related proteins to block transforming growth factor β1 function correlates with the RB product-binding region of E1A rather than with transforming function generally.

The conclusion emerging is that there is a striking correlation between specific biological activities of E1A and the binding of the 300-kDa product. The specific biological activities include induction of DNA synthesis and the repression of certain enhancers; both activities are essentially independent of domain 2 function and RB gene product binding. We are continuing work to determine the biochemical nature of the required N-terminal activity and to identify the precise amino acid sequences encoding this function. It will also be of interest to understand the structural and functional relationships between E1A active sites and those of the transforming proteins of other classes of DNA tumor viruses.

## ACKNOWLEDGMENTS

This work was supported by Public Health Service grants CA-46436 and CA-13106 (to E.M.) and GM-30257 (to R.W.S.) from the National Institutes of Health and grant 47095 from the Juvenile Diabetes Foundation (to R.W.S.). E.M. is the recipient of a Junior Faculty Research Award (JFRA-206) from the American Cancer Society. R.W.S. is the recipient of a Career Development Award from the Juvenile Diabetes Foundation. J.W. was supported by a postdoctoral fellowship from the Juvenile Diabetes Foundation. P.Y. was supported by Public Health Service training grant CA-09311 from the National Institutes of Health.

We thank Regina Whittaker, Nancy Dawkins, Theresa Lavenue, and Howard Masouka for excellent technical assistance and Eileen White and Ed Harlow for helpful discussions and critical reading of the manuscript.

## LITERATURE CITED

1. **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.
2. **Borrelli, E., R. Hen, and P. Chambon.** 1984. Adenovirus-2 E1a gene products repress enhancer-induced stimulation of transcription. Nature (London) **312**:608–612.
3. **Carlock, L. R., and N. C. Jones.** 1981. Transformation-defective mutant of adenovirus type 5 containing a single altered E1A mRNA species. J. Virol. **40**:657–664.
4. **DeCaprio, J. A., J. W. Ludlow, J. Figge, J.-Y. Shew, C.-M. Huang, W.-H. Lee, E. Marsilio, E. Paucha, and D. M. Livingston.** 1988. SV40 large T antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. Cell **54**:275–283.
5. **Dyson, N., P. Howley, K. Munger, and E. Harlow.** 1989. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science **243**:934–937.
6. **Edlund, T., M. D. Walker, P. J. Barr, and W. J. Rutter.** 1985. Cell-specific expression of the rat insulin gene: evidence for the role of two distinct 5′-flanking sequences. Science **230**:912–916.
7. **Egan, C., S. T. Bayley, and P. E. Branton.** 1989. Binding of the RB1 protein to E1A products is required for adenovirus transformation. Oncogene **4**:383–388.
8. **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.
9. **Fasano, O., E. Taparowsky, J. Fiddes, M. Wigler, and M. Goldfarb.** 1983. Sequence and structure of the coding region of the human H-*ras*-1 gene from T24 bladder carcinoma cells. J. Mol. Appl. Genet. **2**:173–180.
10. **Figge, J., T. Webster, T. F. Smith, and E. Paucha.** 1988. Prediction of similar transforming regions in simian virus 40 large T, adenovirus E1A, and *myc* oncoproteins. J. Virol. **62**:1814–1818.
11. **Gallimore, P. H., P. A. Sharp, and J. Sambrook.** 1974. Viral DNA in transformed cells. III. A study of the sequences of adenovirus 2 DNA in nine lines of transformed rat cells using specific fragments of the viral genome. J. Mol. Biol. **89**:49–72.
12. **Glenn, G. M., and R. P. Ricciardi.** 1985. Adenovirus 5 early region 1A host range mutants *hr3*, *hr4*, and *hr5* contain point mutations which generate single amino acid substitutions. J. Virol. **56**:66–74.
13. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
14. **Haley, K. P., G. 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.
15. **Harlow, E., B. R. Franza, Jr., and C. Schley.** 1985. Monoclonal antibodies specific for adenovirus early region 1A proteins: extensive heterogeneity in early region 1A products. J. Virol. **55**:533–546.
16. **Harlow, E., P. Whyte, B. R. Franza, Jr., and C. Schley.** 1986. Association of adenovirus early-region 1A proteins with cellular polypeptides. Mol. Cell. Biol. **6**:1579–1589.
17. **Hen, R., E. Borrelli, and P. Chambon.** 1985. Repression of the immunoglobulin heavy chain enhancer by the adenovirus-2 E1a products. Science **230**:1391–1394.
18. **Houweling, A., P. J. van den 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.
19. **Jelsma, T. N., J. A. Howe, C. M. Evelegh, N. F. Cunniff, M. H. Skiadopoulos, M. R. Floroff, J. E. Denman, and S. T. Bayley.** 1988. Use of deletion and point mutants spanning the coding region of the adenovirus 5 E1A gene to define a domain that is essential for transcriptional activation. Virology **163**:494–502.
20. **Jelsma, T. N., J. A. Howe, J. S. Mymryk, C. M. Evelegh,**

N. F. A. 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 170:120–130.
21. 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.
22. Jones, N., and T. Shenk. 1979. Isolation of adenovirus type 5 host range deletion mutants defective for transformation in rat embryo cells. Cell 17:683–689.
23. Kimelman, D., J. S. Miller, D. Porter, and B. E. Roberts. 1985. E1a regions of the human adenovirus and the highly oncogenic simian adenovirus 7 are closely related. J. Virol. 53:399–409.
24. Kunkel, T. A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA 82: 488–492.
25. Kuppaswamy, M. N., and G. Chinnadurai. 1987. Relationship between the transforming and transcriptional regulatory functions of adenovirus 2 E1A oncogene. Virology 159:31–38.
26. Lewis, J. B., and M. B. Mathews. 1980. Control of adenovirus early gene expression: a class of immediate early products. Cell 21:303–313.
27. 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.
28. Lillie, J. W., P. W. Lowenstein, M. R. Green, and M. Green. 1987. Functional domains of adenovirus type 5 E1a proteins. Cell 50:1091–1100.
29. Montell, C., G. Courtois, C. Eng, and A. Berk. 1984. Complete transformation by adenovirus 2 requires both E1a proteins. Cell 36:951–961.
30. 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.
31. Moran, E. 1988. A region of SV40 large T antigen can substitute for a transforming domain of the adenovirus E1A products. Nature (London) 334:168–170.
32. Moran, E., T. Grodzicker, R. J. Roberts, M. B. Mathews, and B. Zerler. 1986. Lytic and transforming functions of individual products of the adenovirus E1A gene. J. Virol. 57:765–775.
33. Moran, E., and M. B. Mathews. 1987. Multiple functional domains in the adenovirus E1A gene. Cell 48:177–178.
34. Moran, E., B. Zerler, T. M. Harrison, and M. B. Mathews. 1986. Identification of separate domains in the adenovirus E1A gene for immortalization activity and the activation of virus early genes. Mol. Cell. Biol. 6:3470–3480.
35. Nevins, J. R. 1981. Mechanism of activation of early viral transcription by the adenovirus E1A gene product. Cell 26: 213–220.
36. Phelps, W. C., C. L. Yee, K. Munger, and P. M. Howley. 1988. The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1A. Cell 53:539–547.
37. Pietenpol, J. A., R. W. Stein, E. Moran, P. Yaciuk, R. Schlegel, R. M. Lyons, M. R. Pittelkow, K. Munger, P. M. Howley, and H. L. Moses. 1990. TGFβ1 inhibition of *c-myc* transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains. Cell 61:777–785.
38. Rochette-Egly, C., C. Fromental, and P. Chambon. 1990. General repression of enhanson activity by the adenovirus-2 E1A proteins. Genes Dev. 4:137–150.
39. 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.
40. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463–5467.
41. 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.
42. 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.
43. Solnick, D., and M. A. Anderson. 1982. Transformation-deficient adenovirus mutant defective in expression of region 1A but not region 1B. J. Virol. 42:106–113.
44. Stein, R. W., and J. Whelan. 1989. Insulin gene enhancer activity is inhibited by adenovirus 5 E1a gene products. Mol. Cell. Biol. 9:4531–4534.
45. Stein, R. W., and E. B. Ziff. 1987. Repression of insulin gene expression by adenovirus type 5 E1a proteins. Mol. Cell. Biol. 7:1164–1170.
46. Subramanian, T., M. Kuppaswamy, R. J. Nasser, and G. Chinnadurai. 1988. An N-terminal region of adenovirus-E1A essential for cell-transformation and induction of an epithelial-cell growth factor. Oncogene 2:105–112.
47. Svensson, C., and G. Akusjarvi. 1984. Adenovirus 2 early region 1A stimulates expression of both viral and cellular genes. EMBO J. 3:789–794.
48. van Dam, H., R. Offringa, A. M. M. Smits, J. L. Bos, N. C. Jones, and A. J. van der Eb. 1989. The repression of the growth factor-inducible genes, JE, c-*myc* and stromelysin by adenovirus E1A is mediated by conserved region 1. Oncogene 4: 1207–1212.
49. van der Eb, A. J., C. Mulder, F. L. Graham, and A. Houweling. 1977. Transformation with specific fragments of adenovirus DNAs. I. Isolation of specific fragments with transforming activity of adenovirus 2 and 5 DNA. Gene 2:115–132.
50. van Ormondt, H., J. Maat, and R. Dijkema. 1980. Comparison of nucleotide sequences of the early E1a regions for subgroups A, B, and C of human adenoviruses. Gene 12:63–76.
51. Velcich, A., and E. Ziff. 1985. Adenovirus E1a proteins repress transcription from the SV40 early promoter. Cell 40:705–716.
52. Velcich, A., and E. Ziff. 1988. Adenovirus E1a *ras* cooperation activity is separate from its positive and negative transcription regulatory functions. Mol. Cell. Biol. 8:2177–2183.
53. Weeks, D. L., and N. C. Jones. 1983. E1A control of gene expression is mediated by sequences 5′ to the transcriptional starts of the early viral genes. Mol. Cell. Biol. 3:1222–1234.
54. Whelan, J., D. Poon, P. A. Weil, and R. Stein. 1989. Pancreatic β-cell-type-specific expression of the rat insulin II gene is controlled by positive and negative cellular transcriptional elements. Mol. Cell. Biol. 9:3253–3259.
55. Whyte, P., K. Buchkovich, J. M. Horowitz, S. H. Friend, M. Raybuck, R. A. Weinberg, and E. Harlow. 1988. Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. Nature (London) 334:124–129.
56. Whyte, P., H. E. Ruley, and E. Harlow. 1988. Two regions of the adenovirus E1A gene are required for transformation. J. Virol. 62:257–265.
57. Whyte, P., N. M. Williamson, and E. Harlow. 1989. Cellular targets for transformation by the adenovirus E1A proteins. Cell 56:67–75.
58. Wigler, M., A. Pellicer, S. Silverstein, and R. Axel. 1985. Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. Cell 14:729–731.
59. Yee, S., and P. Branton. 1985. Detection of cellular proteins associated with human adenovirus type 5 early region 1A polypeptides. Virology 147:142–153.
60. 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.
61. Zerler, B. R., R. J. Roberts, M. B. Mathews, and E. Moran. 1987. Separate functional domains of the adenovirus E1A gene are involved in regulation of host cell cycle products. Mol. Cell. Biol. 7:821–829.
62. Zoller, M. J., and M. Smith. 1984. Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template. DNA 3:479–488.