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Two Regions of the Adenovirus Early Region 1A Proteins Are Required for Transformation

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Regions of the adenovirus type 5 early region 1A (E1A) proteins that are required for transformation were defined by using a series of deletion mutants. Deletion mutations collectively spanning the entire protein-coding region of E1A were constructed and assayed for their ability to cooperate with an activated *ras* oncogene to induce transformation in primary baby rat kidney cells. Two regions of E1A (amino acids 1 to 85 and 121 to 127) were found to be essential for transformation. Deletion of all or part of the region from amino acids 121 to 127 resulted in a total loss of transforming ability. An adjacent stretch of amino acids (residues 128 to 139), largely consisting of acidic residues, was found to be dispensable for transformation but appeared to influence the efficiency of transformation. Amino acids 1 to 85 made up a second region of the E1A protein that was essential for transformation. Deletion of all or part of this region resulted in a loss of the transforming activity. Even a mutation resulting in a single amino acid change at position 2 of the polypeptide chain was sufficient to eliminate transformation. Deletion of amino acids 86 to 120 or 128 to 289 did not eliminate transformation, although some mutations in these regions had lowered efficiencies of transformation. Foci induced by transformation-competent mutants could be expanded into cell lines that retained their transformed morphology and constitutively expressed the mutant E1A proteins.

An extensive body of research exists to support the involvement of a multistep process in the conversion of normal cells to the tumorigenic phenotype (5, 28). Molecular models supporting this theory were first provided by studies on two DNA tumor viruses, adenovirus and polyomavirus. Transformation of primary cells by adenovirus requires the expression of both the early region 1A (E1A) and 1B (E1B) genes (10, 19, 55). Similarly, expression of both the large T and middle T antigens is necessary for transformation by polyomavirus (38). Although no overall structural homologies are apparent between the transforming genes of these two viruses, functional relationships do appear to exist. These functional relationships are also shared by a number of cellular oncogenes. Thus, E1A can also cooperate with the polyomavirus middle T antigen or activated H-*ras* gene to transform primary cells (44). Furthermore, polyomavirus large T, p53, and members of the *myc* gene family can substitute for E1A in these assays (7, 20, 27, 35, 44, 47, 59). These observations suggest the involvement of multiple functions in the transformation process and indicate that members of these groups of oncogenes express related biological functions.

The adenovirus E1A gene codes for several related proteins to which a number of interesting properties have been attributed. In addition to its ability to complement a second oncogene in transformation, a closely related function allows E1A to immortalize primary cells (19, 44, 62). Introduction of E1A into primary cells provides these cells with an unlimited proliferative capacity when cultured in the presence of serum. Although the relationship between this function and the activity involved in transformation is not clear, the two appear to be closely linked (62). Interestingly, *myc*, p53, and polyomavirus large T antigen also possess immortalization functions (20, 27, 39). It appears quite likely that the immortalization activity is an essential part of the function(s) involved in transformation of cultured primary cells.

Another well-studied function of E1A is *trans*-activation. Expression of E1A stimulates transcription from a variety of viral and cellular promoters, including the adenovirus early-region promoters and the major late promoter (for a review, see reference 2). However, *trans*-activation by E1A is not universal for all promoters. In some instances, E1A causes a decrease in transcription from promoters that are linked to enhancer elements (3, 11, 18, 57). Mutagenic analysis has suggested that transformation and *trans*-activation are carried out by distinct functions of E1A (13, 29, 31, 33, 40). Several other activities have been attributed to E1A, including the induction of cellular DNA synthesis and secretion of a cellular growth factor (4, 21, 36, 51). Presently, it is not clear whether these are additional functions of E1A or whether they are additional manifestations of the activities responsible for *trans*-activation or transformation.

In transformed cells and at early times during viral infection, two mRNAs, referred to by their sedimentation values of 13S and 12S, are produced from the E1A gene (for a review, see reference 2). These two mRNAs, 13S and 12S, arise through differential splicing of a common precursor and code for related proteins of 289 and 243 amino acids, respectively. The two proteins differ internally by 46 amino acids that are unique to the 13S mRNA product. A number of E1A protein species can be resolved on one- and two-dimensional gels and presumably arise as a result of extensive posttranslational modification of the primary translation products (12, 14, 16, 43, 46, 48, 50, 61). Like the products of the *myc*, p53, and polyomavirus large T antigen genes, the E1A proteins are located in the nucleus of the cell (1, 6, 8, 14, 41, 42). Several proteins of cellular origin have been shown to stably interact with the E1A proteins and can be coimmunoprecipitated with E1A by using either monoclonal antibodies or antipeptide serum specific for E1A (15, 60). Although little is known concerning the nature of these

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protein-protein interactions, it is possible that these proteins are involved in one or more functions of E1A.

The T24 H-*ras* oncogene is a powerful transforming agent when introduced into certain established cell lines, such as the NIH 3T3 cell line (53). However, in primary baby rat kidney (BRK) cells, efficient transformation requires the expression of a second oncogene, such as the E1A oncogene. As a preliminary step in investigating the biochemical nature of the role of E1A in transformation, we have determined which E1A protein sequences are required for cooperation with an activated *ras* oncogene in the transformation of primary BRK cells. Using a strategy of deletion mutagenesis, we demonstrate that two small, distinct regions of the E1A protein are essential for cooperation with the T24 H-*ras* oncogene to transform primary BRK cells.

MATERIALS AND METHODS

Construction of mutants. Mutations were introduced into the protein-coding region of the adenovirus serotype 5 (Ad5) E1A gene in either plasmid p1A or plasmid pC1A. Plasmid p1A contains Ad5 sequences from nucleotides 1 to 1834 (see reference 56 for the numbering of Ad5 nucleotides) cloned into the *Eco*RI and *Pst*I sites of the plasmid pAT153 (30). Plasmid pC1A contains the same adenovirus sequences cloned into the *Eco*RI and *Pst*I sites of the pUC18 vector. Protocols used in these procedures were those described by Maniatis et al. (30) except where otherwise noted. The nucleotide sequence of each mutant was confirmed by sequencing the mutated regions of the plasmid by the method of Sanger et al. (45).

A series of mutants with nucleotide sequences encoding truncated protein products were constructed by inserting the synthetic *Xba*I linker, CTCTAGAG, into either the *Sma*I site at nucleotide 1009 (CT*dl*1009) or positions upstream and ligating the upstream sequences to the *Xba*I site located at nucleotide 1339. Carboxy-terminal deletion (CT*dl*) mutants CT*dl*979, CT*dl*976, CT*dl*961, CT*dl*952, and CT*dl*877 were created by digestion from the *Sma*I site with the exonuclease *BAL* 31, followed by the insertion of an *Xba*I linker, and joining to downstream sequences via the *Xba*I site located at nucleotide 1339 (Fig. 1). Mutants CT*dl*940, CT*dl*934, and CT*dl*931 were constructed in a similar manner, except that after insertion of the *Xba*I linker, oligonucleotide-directed mutagenesis (63) was used to delete a single nucleotide 5′ to the *Xba*I linker. All carboxy-terminal deletion mutants terminate translation within the linker sequences. Mutants *dl*975/1339 and *dl*891/1339 were created in an identical manner to the carboxy-terminal mutants but contain the *Xba*I linker in a reading frame that allows readthrough into the sequences downstream of the *Xba*I site located at nucleotide 1339, resulting in a large in-frame deletion.

Point mutant 563 (*pm*563) was created by using oligonucleotide-directed mutagenesis (63) to change the nucleotide sequence surrounding the E1A translational start site, at nucleotide 560, from AAA ATG AGA to ACC ATG GGA. This change of three nucleotides created an *Nco*I site and preserved the translational start signal. Amino-terminal deletion (NT*dl*) mutants contain deletions in the sequences encoding the amino-terminal region of the E1A protein and were created by *BAL* 31 exonuclease digestions from the *Hin*dIII site of MTEBE1A (62), followed by insertion of the synthetic *Nco*I linker, CCCATGGG, and joining of these sequences to upstream adenovirus sequences by ligating to the *Nco*I site of mutant *pm*563. Mutant NT*dl*814 was created by inserting an *Nco*I linker into the *Nae*I site at nucleotide 814 and ligating to the upstream sequences of *pm*563. The mutant *dl*813/919 was constructed by cutting with *Cla*I (at 919), filling in with Klenow fragment, and ligating to the *Nae*I site at 813, deleting sequences 814 to 918.

The mutants *dl*922/947 and *dl*955/986 were constructed by using oligonucleotide-directed mutagenesis to loop out nucleotides 923 to 946 and 956 to 985, respectively. Mutagenesis was essentially as described by Zoller and Smith (63), except that the efficiency-enhancing method of Kunkel (25) was incorporated into the mutagenesis procedure. In both cases, 33-base oligonucleotides were used to loop out the desired regions from a single-stranded clone of M13mp18 containing the E1A gene.

Primary BRK cell cultures. Cultures of BRK cells were prepared from 6-day-old baby Fisher rats (Taconic Farms, Germantown, N.Y.). After removing the kidneys from the baby rats, the surrounding capsule was removed, and the kidneys were minced. Cells were dispersed by treatment in a collagenase-dispase solution for 30 to 45 min at 37°C with a spinner bar to provide agitation. Any remaining clumps of tissue were allowed to settle, and the suspended cells were plated at a density of 3×10^5 to 5×10^5 cells per 60-mm-diameter tissue culture plate in Dulbecco modified Eagle medium supplemented with 5% fetal bovine serum.

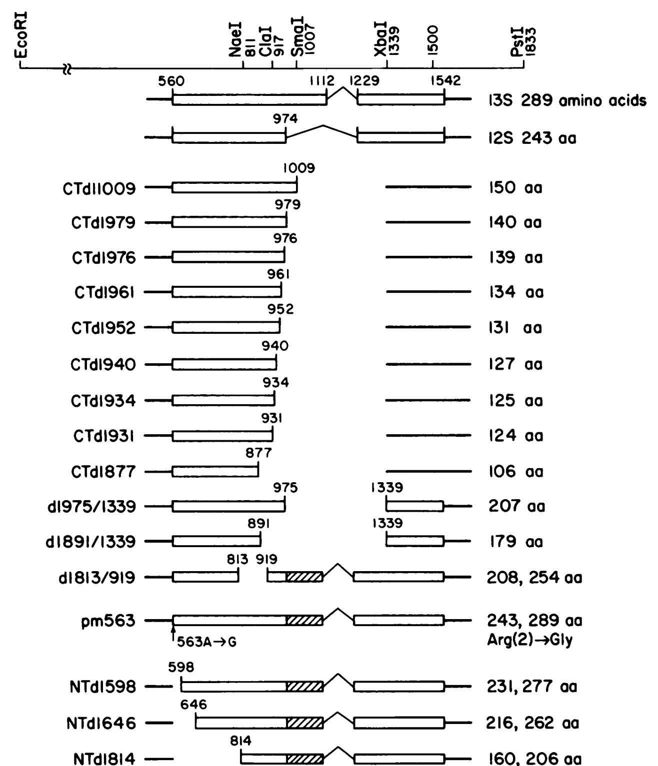

FIG. 1. Adenovirus E1A deletion mutants and their translation products. Schematic representations of the E1A gene, its early mRNAs, and mRNAs produced by deletion mutants of E1A are shown. Symbols: ▭, translated region of mRNA; ——, untranslated portion of mRNA; ▨, 13S unique region in constructs in which the alternative splicing pattern of the 13S and 12S mRNAs is left intact. Numbers represent the terminal adenovirus nucleotide where deletions have been made, except in *pm*563 where it represents the position of a point mutation. To the right of each mutant is the length (in amino acids [aa]) of the predicted mutant E1A protein product. The polypeptide lengths of the CT*dl* mutants do not include the linker-encoded leucine located at the carboxy terminus.

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Transfection of BRK cells. Transfections were carried out by the calcium phosphate precipitation method of Wigler et al. (58). At 24 h after the initial plating of the BRK cells, the medium on the BRK cells was changed, and approximately 4 h later, the cells were transfected with a calcium phosphate precipitate containing 2 μg of each plasmid being tested plus 8 to 10 μg of sheared salmon sperm DNA as the carrier in a 0.5-ml volume. The precipitate was left overnight before removal and replacement with fresh medium. Medium was changed every 3 days, and the assay was scored at 3 to 4 weeks after the transfection. Visualization of transformed foci was enhanced before scoring the assay by fixing the cells with methanol and staining with Giemsa stain.

Transformed cell lines were established by removing the cells from individual foci with cloning cylinders and a trypsin-EDTA solution. The cells isolated from these foci were then grown in Dulbecco modified Eagle medium plus 5% fetal bovine serum.

Immunoblot analysis of E1A proteins. Approximately 5×10^7 cells were lysed in RIPA buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris [pH 8.0]), and the lysates were cleared of cellular debris by low-speed centrifugation. The E1A proteins were immunoprecipitated with a cocktail of monoclonal antibodies that recognize different epitopes of the E1A proteins (14). Immunoprecipitated proteins were dissolved in sample buffer and run on a 15% sodium dodecyl sulfate-polyacrylamide gel (26). Proteins were transferred to nitrocellulose by electrophoretic transfer as previously described (54). E1A and mutant E1A proteins were detected by probing with ^{125}I-labeled anti-E1A monoclonal antibodies.

RESULTS

Construction of mutants. A series of deletion mutants spanning the entire protein-coding region of E1A were constructed to define the regions of the protein that are essential for transformation of primary BRK cells when cotransfected with the T24 H-*ras* oncogene. Previous studies have shown that plasmids containing either the E1A 13S or 12S cDNA are capable of cooperating with the T24 H-*ras* oncogene to transform primary BRK cells (62). Furthermore, the E1A mutant *hr*A, which expresses a truncated protein missing most of the 13S unique region and all the exon 2-encoded sequences, is also capable of inducing transformed foci in this assay (62). Together these results suggest that the E1A functions required to cooperate with the *ras* oncogene reside in the region of the protein encoded by exon 1 of the 12S mRNA, i.e., in the amino-terminal 139 residues. To establish a carboxy-terminal boundary for the essential transforming region of E1A, a nested set of deletions was created by inserting the synthetic *Xba*I linker, CTCTAGAG, into positions from nucleotide 1009 to 877. Carboxy-terminal deletion mutants (CT*dl*) are named for the 3′ nucleotide of the E1A sequences joined to the synthetic *Xba*I linker (Fig. 1). In each case, the *Xba*I linker was inserted into the coding region in the appropriate reading frame to code for a leucine residue and then terminate translation. Mutants *dl*975/1339 and *dl*891/1339 were created in a similar manner, except that they contained the *Xba*I linker in a reading frame that preserved readthrough of the coding sequences downstream of the *Xba*I site (i.e., the carboxy-terminal 67 amino acids), resulting in large in-frame deletions.

To assess the functional requirement for the amino-terminal sequences of E1A, a similar strategy of mutagenesis was used. By using oligonucleotide-directed mutagenesis, the sequence surrounding the natural translational start signal for E1A, located at nucleotide 560, was changed from AAA ATG AGA to ACC ATG GGA (mutant *pm*563). This change of three nucleotides resulted in the creation of an *Nco*I site, while retaining the correct sequences thought to be important in initiating translation (23). The mutation at position 563 also resulted in the substitution of the amino acid glycine in place of the arginine normally found in position 2 of the polypeptide chain. Amino-terminal deletions were created by introducing *Nco*I linkers coding for translational initiation sites into positions within the coding region of the E1A gene (see Materials and Methods). The amino-terminal deletion (NT*dl*) mutants are named for the 5′ nucleotide of E1A joined to the *Nco*I linker (Fig. 1). For each mutant in this series, the synthetic linker encodes the initiating methionine followed by a glycine before joining the E1A sequences particular to each individual mutant.

The largest amino-terminal mutant had a deletion of the protein-coding sequences to nucleotide 813. To assess the function of the region found between the mutations of the amino-terminal mutants and the carboxy-terminal mutants, an internal deletion mutation was created. Mutant *dl*813/919 had nucleotides 814 to 918 deleted, and along with the mutants described above, completed a set of deletion mutants that collectively spanned the entire protein-coding region of E1A.

To examine the ability of these mutants to direct the synthesis of mutant E1A proteins, recombinant adenoviruses containing each of the E1A mutants were constructed. Proteins of the expected relative mobility were expressed and could be immunoprecipitated from infected HeLa or BRK cells (T. Grodzicker, M. Quinlan, and P. Whyte, unpublished observations).

Establishment of a carboxy-terminal boundary for E1A sequences essential for cooperation with the T24 H-*ras* oncogene. To establish a carboxy-terminal boundary for the E1A sequences involved in the transforming functions, the carboxy-terminal mutants were assayed for their ability to cooperate with the T24 H-*ras* oncogene in a focus-forming assay (Table 1). Many of the transformed foci observed in these experiments consisted of cells which did not firmly adhere to the tissue culture dish and during media changes could easily be spread to other parts of the plate. As a result, the foci observed on a particular dish may include some secondary foci, in addition to the primary foci. For this reason, the data have been expressed both as the total number of foci observed and as the number of plates in the assay with one or more foci.

As previously reported, the T24 H-*ras* plasmid was unable to produce foci in this assay when transfected alone; however, when cotransfected with the wild-type E1A gene, numerous foci were observed (29, 33, 44, 62). Mutants CT*dl*1009, CT*dl*979, and CT*dl*976 also cooperated with the *ras* oncogene to form numerous foci (Table 1), although the frequency was consistently lower than that observed with wild-type E1A. Mutant CT*dl*976 has nucleotide sequences encoding a protein that would be identical to the polypeptide encoded by exon 1 of the 12S mRNA, plus the linker-encoded leucine at the terminus. This indicated that the regions of E1A that are essential for transformation are located in the amino-terminal 139 amino acids, although other regions of the protein may also be required to attain wild-type efficiency. Deletion of a further five amino acids resulted in a notable decrease in the frequency of foci observed; however, foci were consistently observed (mutant

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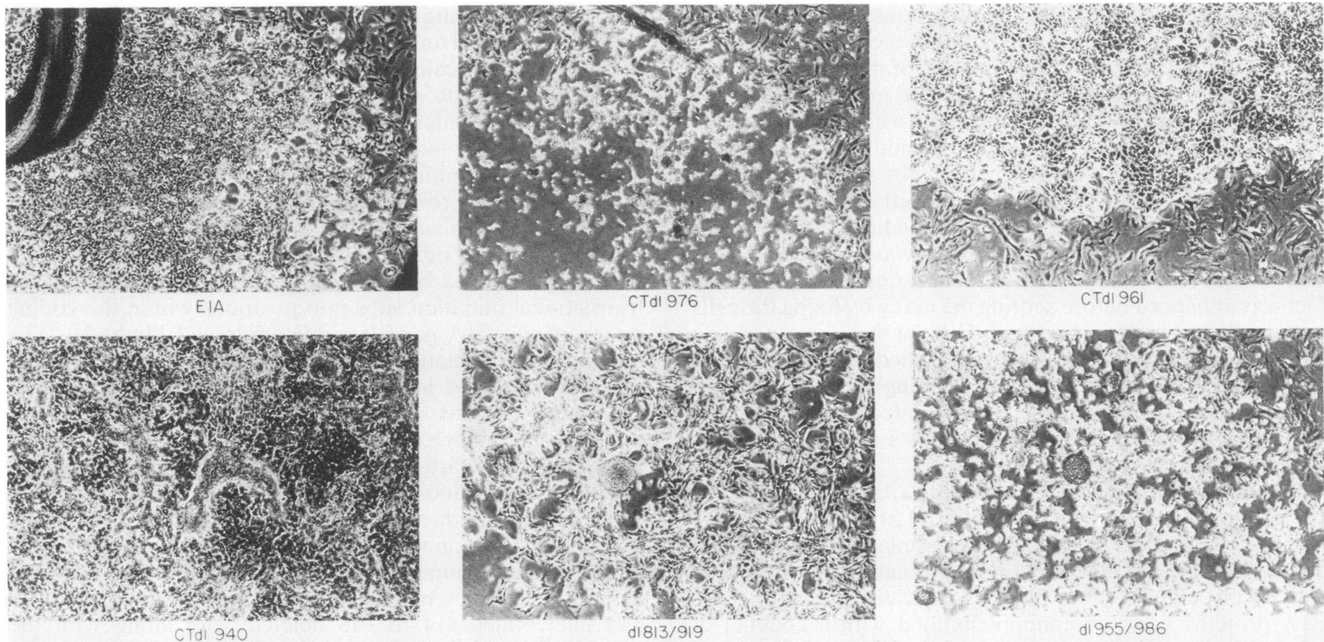

FIG. 2. Transformed foci induced by E1A or mutants of E1A with T24 H-*ras*. BRK cells were cotransfected with plasmids expressing E1A or mutants of E1A and *ras*. Photographs of transformed foci induced by E1A and several key mutants are shown after several weeks in culture. Below each photograph is the E1A construct that was cotransfected with the *ras* oncogene. Photographs are from different experiments and were taken at different times after transfection. Magnification, 40×.

CT*dl*961 [Table 1]). No significant reduction of focus-forming efficiency was observed when the next seven amino acids (mutant CT*dl*940) were deleted, indicating that the amino-terminal 127 amino acids are capable of cooperating with the T24 H-*ras* oncogene to transform BRK cells, albeit with lowered efficiency. Deletion of an additional two amino acids in mutant CT*dl*934 resulted in the elimination of transformation. Activity was not restored when further carboxy-terminal sequences were deleted (mutants CT*dl*931 and CT*dl*877), indicating that the lack of transforming function was not a unique property of mutant CT*dl*934.

Although all the mutants of the CT*dl* series expressed lower levels of E1A protein compared with wild-type constructions when assayed by infection (data not shown) or in transformed cell lines (see Fig. 3a), no difference was observed between transforming and nontransforming members of this group. Therefore, protein levels cannot explain the observed differences in transforming abilities of these mutants, although the levels may contribute to their lower transformation efficiency compared with that of the wild type.

Photographs of typical foci induced by E1A and representatives of the transforming mutants are shown in Fig. 2. Cells transformed by E1A displayed a variety of morphologies, ranging from small rounded cells with a refractile appearance to cells with an epitheliallike appearance. Foci induced by

TABLE 1. Cooperation between adenovirus E1A carboxy-terminal deletion mutants and H-*ras* to transform primary BRK cells

Plasmid cotransfected[a]	Expt 1		Expt 2		Expt 3		Expt 4	
	No. of plates with foci[b]	Total no. of foci[c]	No. of plates with foci	Total no. of foci	No. of plates with foci	Total no. of foci	No. of plates with foci	Total no. of foci
None (carrier DNA only)	0	0	0	0	0	0	0	0
E1A (wild type)	10	45	10	74	10	82	10	78
CT*dl*1009	5	6	6	10	5	9	9	42
CT*dl*979	10	33	8	27	7	35	8	28
CT*dl*976	6	11	5	11	6	8	9	42
CT*dl*961	3	3	2	2	2	7	9	26
CT*dl*952	(2)[d]	(2)	0	0	0	0	ND[e]	ND
CT*dl*940	3	8	3	5	2	3	5	18
CT*dl*934	0	0	0	0	0	0	0	0
CT*dl*931	0	0	0	0	0	0	0	0
CT*dl*877	0	0	0	0	0	0	0	0
*dl*975/1339	7	36	9	32	8	16	ND	ND
*dl*891/1339	0	0	0	0	0	0	ND	ND

[a] Plasmid cotransfected into BRK cells with a plasmid containing the T24 H-*ras* oncogene.
[b] Number of plates in the assay with one or more foci. In each experiment, 10 plates were transfected with each mutant.
[c] Total number of foci per 10 plates in each experiment.
[d] Numbers in parentheses refer to mutants that induced abortive transformants only. See text for details.
[e] ND, Not done.

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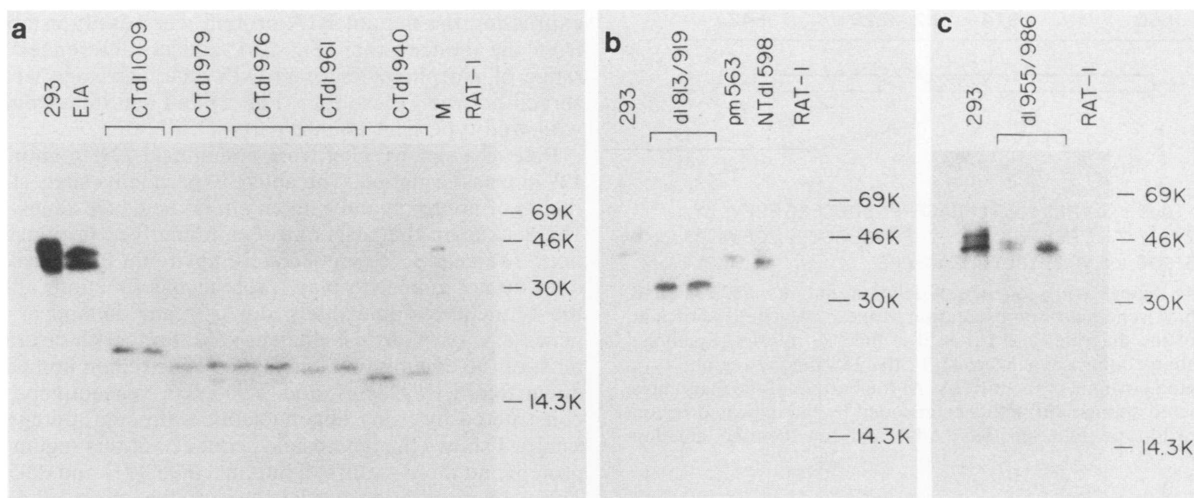

FIG. 3. Immunoblot analysis of mutant E1A proteins produced from transformed cell lines. Transformed cell lines were established by cloning foci from cotransfected BRK cells. Approximately 2.5×10^7 cells were lysed in RIPA buffer, immunoprecipitated with anti-E1A monoclonal antibodies, and run on a 15% polyacrylamide gel. After transfer to nitrocellulose, the blot was probed with ^{125}I-labeled monoclonal antibody M58 (a and c) or M1 (b). At the top of each lane is the mutant used to transform the cell line. Two independently arising transformed cell lines were analyzed for each mutant. E1A immunoprecipitated from approximately 3×10^6 to 5×10^6 293 cells (9, 49) was run as a positive control in each of the three immunoblots; RAT-1 cells were used as a negative control. The bands in lane M in panel a are standard size markers, 69,000-, 46,000-, 30,000-, and 14,300-molecular-weight proteins (69K, 46K, 30K, and 14.3K, respectively).

each of the transforming mutants differed little from those produced by wild-type E1A, except that foci with the refractile morphology induced by carboxy-terminal mutants often appeared to have a lower cell density than wild-type E1A-induced foci. Upon continued incubation, these foci grew progressively larger but did not exhibit the dense growth pattern of the transformed cells seen in the wild-type E1A-induced foci. This did not appear to represent any reduced proliferative potential, since these foci could be expanded into established cell lines that maintained their transformed morphology. Only mutant CT*dl*952 failed to produce foci that could be easily expanded into cell lines. The relatively few foci produced by this mutant (Table 1) failed to survive the initial passages in attempts to establish cell lines. This is somewhat surprising, since the mutant CT*dl*940, which produces a shorter polypeptide than CT*dl*952, repeatedly produced foci which could be easily expanded into cell lines. One possible explanation for this result is that the carboxy terminus of CT*dl*952 consists of several hydrophobic amino acids which may result in aberrant folding or destabilize the mutant protein product.

An immunoblot analysis of the mutant E1A proteins from transformed cell lines showed that each of the cell lines was actively synthesizing the appropriate mutant protein (Fig. 3). The aberrant mobility of E1A on sodium dodecyl sulfate-polyacrylamide gels makes it difficult to predict expected sizes for each of the mutant proteins; however, the mutant E1A proteins detected in these lines increased in relative mobility with the extent of the deletion in E1A, as would be expected.

A previous study has implicated the carboxy-terminal sequences of the E1A protein as being important for efficient nuclear localization (24). To examine the role of this region in transformation by E1A, two mutants were constructed in which sequences from exon 1 were joined to the sequences encoding the carboxy-terminal 67 amino acids (mutants *dl*891/1339 and *dl*975/1339 [Fig. 1]). Consistent with previous studies, immunofluorescence studies with recombinant viruses expressing these mutant E1A proteins showed that proteins containing the carboxy-terminal 67 amino acids were efficiently translocated to the nucleus. Mutant proteins lacking these sequences were found distributed throughout the cytoplasm and nucleus (data not shown). The mutant *dl*975/1339 is identical to mutant CT*dl*976, except that its nucleotide sequence encodes the 67 carboxy-terminal amino acids of E1A, rather than terminating after the first 139 amino acids. This mutant induced only slightly higher numbers of foci than mutant CT*dl*976 (Table 1), suggesting that the addition of these carboxy-terminal sequences plays only a minor role in enhancing the transforming potential of the essential upstream sequences. The mutant *dl*891/1339, which contains the amino-terminal 111 amino acids linked to the carboxy-terminal 67 amino acids, failed to induce foci. This result supports the above observation of an essential role for the sequences extending to amino acid 127.

Deletion analysis of amino-terminal sequences of E1A and their role in transformation. To examine the role of amino-terminal sequences in transformation, a set of mutants was constructed in which various portions of the amino-terminal sequences of the E1A proteins were deleted, and a translational start site was inserted (Fig. 1). Plasmids containing these mutants were cotransfected with the T24 H-*ras* oncogene into BRK cells and scored for their ability to induce foci (Table 2). All of these mutants were found to be defective for transformation, including the point mutant *pm*563. This mutant contains only a single point mutation in the protein-coding region, resulting in the substitution of a glycine residue for the arginine residue normally found at position 2. A recombinant adenovirus expressing the *pm*563 protein produced slightly more E1A than wild-type adenovirus, indicating that the failure of this mutant to transform was not due to a translational artifact (data not shown). Only rarely were foci observed in cells cotransfected with this mutant, as was also the case with mutant NT*dl*598. Deletion of the sequences encoding amino acids 2 to 28 (NT*dl*646) or amino acids 2 to 85 (NT*dl*814) eliminated the ability of these mutants to score in this assay. Furthermore, foci induced by the mutants *pm*563 and NT*dl*598 appeared to be abortive.

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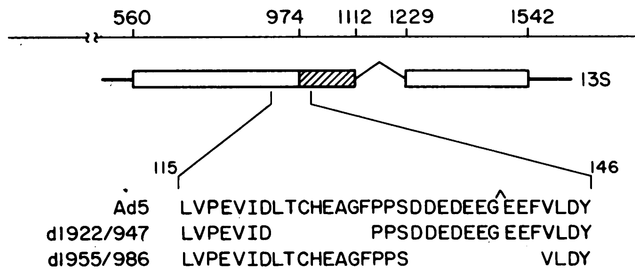

FIG. 4. Amino acid sequence of deletion mutants *dl*922/947 and *dl*955/986. A schematic representation of the E1A gene is shown at the top of the diagram, and below it is the 13S mRNA. Symbols: ▭, protein-coding sequences; ▨, the 13S unique region; —, untranslated portions of the mRNA. At the bottom of the diagram is the predicted amino acid sequence encoded by the indicated region for the wild-type E1A protein (Ad5) and for the two deletion mutants.

Upon continued incubation, the cells in these foci ceased proliferating and detached from the dish. Attempts to establish transformed cell lines from these foci were unsuccessful. One focus each from the *pm*563 and NT*dl*598 transformants gave rise to established lines; however, at early passage, the cells in both of these lines underwent a crisis when many of the cells detached from the tissue culture dish and were lost. The remaining cells reverted from the rounded, transformed morphology to a flat, nontransformed morphology. These cells appeared to be immortalized and continued to grow well after numerous passages but were contact inhibited and could be growth arrested in low serum. Immunoblot analysis of lysates from these cells indicated that the mutant E1A proteins were being synthesized in these cells (Fig. 3b). Thus, mutants *pm*563 and NT*dl*598 appeared to be incapable of inducing stable transformants, although, on rare occasions, unstable transformed foci were observed. It therefore appears that the sequences at the amino terminus of E1A are essential for cooperation with an activated *ras* oncogene.

To assess the function of the sequences between limits of the carboxy-terminal and amino-terminal mutants, the mutant *dl*813/919 was created. This mutant predicts a protein product with an internal deletion of amino acids 86 to 120. An apparently identical mutant has been recently described by Moran et al. (33). Consistent with the results obtained by these authors, our experiments indicated that this mutation resulted in only a modest decrease in the number of foci induced (Table 2), suggesting that these sequences play little or no role in the transforming activity of E1A. Cell lines expressing the mutant E1A protein were easily established from the induced foci (Fig. 3b), and no differences in the range of morphologies or growth characteristics were observed between these foci (Fig. 2) and cell lines compared with wild-type E1A counterparts.

Role of a small region from amino acid 121 to amino acid 127 in transformation. The above experiments suggest that a region of approximately seven amino acids (residues 121 to 127) makes up the basis of a region involved in transformation. The sequences immediately upstream from these residues do not appear to play a role in this function, although the sequences immediately downstream do appear to be necessary for wild-type efficiency. Mutants with deletions of most of the essential region or the acidic region immediately downstream, *dl*922/947 and *dl*955/986, respectively, were constructed by using oligonucleotide-directed mutagenesis. Figure 4 shows the amino acid sequence of this region of the protein and the structure of mutants *dl*922/947 and *dl*955/986. These mutants were used to assess the effect of deleting these regions when all surrounding sequences were present. Results of cotransfection assays with these mutants are presented in Table 3. Deletion of residues 122 to 129 (mutant *dl*922/947) resulted in virtual elimination of the appearance of foci. The one focus apparently induced by this mutant was observed after staining, and therefore, could not be further characterized. Deletion of the stretch of acidic amino acids (residues 133 to 142 [Fig. 4]) resulted in a reduction of the number of foci observed, yet foci were consistently observed. This result is consistent with the results obtained with the carboxy-terminal deletion mutants. No differences in morphology between these foci and those induced by wild-type E1A were observed (Fig. 2), and transformed cell lines expressing the mutant protein (Fig. 3c) were easily established.

DISCUSSION

Deletion mutagenesis was used to determine which regions of E1A are necessary for cooperation with a second oncogene to bring about morphological transformation of primary cells. Previous studies involving mutagenesis of the E1A gene have implicated different regions of the protein as being important for different activities (for a review, see reference 32). This has led to speculation that E1A is composed of multiple functional domains. The results presented in this study support this model by demonstrating that much of the E1A protein-coding region is dispensable for transformation and that only two regions of the E1A polypeptide, amino acids 1 to 85 and amino acids 121 to 127, are

TABLE 2. Cooperation between adenovirus E1A amino-terminal deletion mutants and T24 H-*ras* to transform primary BRK cells

Plasmid cotransfected[a]	Expt 1		Expt 2		Expt 3		Expt 4	
	No. of plates with foci[b]	Total no. of foci[c]	No. of plates with foci	Total no. of foci	No. of plates with foci	Total no. of foci	No. of plates with foci	Total no. of foci
None (carrier DNA only)	0	0	0	0	0	0	0	0
E1A (wild type)	9	167	10	45	10	74	10	82
*pm*563	(5)[d]	(6)	0	0	0	0	(2)	(2)
NT*dl*598	(2)	(2)	0	0	0	0	0	0
NT*dl*646	0	0	0	0	0	0	0	0
NT*dl*814	0	0	0	0	0	0	0	0
*dl*813/919	ND[e]	ND	9	35	10	65	10	62

[a] Plasmid cotransfected into BRK cells with a plasmid containing the T24 H-*ras* oncogene.
[b] Number of plates in the assay with one or more foci. In each experiment, 10 plates were transfected with each mutant.
[c] Total number of foci per 10 plates in each experiment.
[d] Numbers in parentheses refer to mutants that induced abortive transformants only. See text for details.
[e] ND, Not done.

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TABLE 3. Cooperation between adenovirus E1A internal deletion mutants and T24 H-*ras* to transform primary BRK cells

Plasmid cotransfected[a]	Expt 1		Expt 2		Expt 3	
	No. of plates with foci[b]	Total no. of foci[c]	No. of plates with foci	Total no. of foci	No. of plates with foci	Total no. of foci
None (carrier DNA only)	0	0	0	0	0	0
E1A (wild type)	10	133	10	136	10	137
*dl*922/956	0	0	1	1	0	0
*dl*955/986	3	3	6	10	3	10

[a] Plasmid cotransfected into BRK cells with a plasmid containing the T24 H-*ras* oncogene.
[b] Number of plates in the assay with one or more foci. In each experiment, 10 plates were transfected with each mutant.
[c] Total number of foci per 10 plates in each experiment.

required for cooperation with the T24 H-*ras* oncogene to transform BRK cells.

The region between amino acids 121 and 139 was found to be required for efficient transformation by analysis of mutants with amino acids progressively removed from the carboxy terminus of the protein and by analysis of mutants carrying small deletions within this region. Amino acids 121 to 127 appeared to be essential for transformation. Deletion of this region or parts of it resulted in the loss of transforming ability. Mutants missing residues 128 to 139 were capable of inducing stable transformants; however, these amino acids appeared to influence the levels of foci observed. Cells transformed by mutants lacking this region were easily expanded into cell lines that grew as well as their wild-type counterparts. One possibility is that this region is more important in the initiation of transformation than maintenance of transformation. The results obtained by using deletion mutations in the region between 121 and 139 are consistent with two recent reports of transformation-defective point mutants that yield amino acid substitutions at positions 124, 127, 132, or 135 (29, 33). Together, studies involving deletion and point mutations implicate this region as being critical to the transforming properties of E1A.

Several interesting homologies have been described between this region of E1A and regions of other relevant proteins. First, the region encompassing amino acids 121 to 139 is strongly conserved among the E1A proteins of serotypes from the different subgroups of adenovirus (22). Second, although no overall homology exists between papovaviral T antigens and E1A, T antigens from several papovaviruses contain a region homologous to amino acids 116 to 137 of E1A (51). Third, the region of amino acids 133 to 141 is mostly made up of acidic residues. A similar stretch of acidic amino acids in the c-*myc* protein forms part of the basis for a limited homology between E1A and the c-*myc* protein (37). Our results demonstrate that this region is not essential for E1A to cooperate with the *ras* oncogene, although it does appear to influence the number of foci induced. Two recent reports indicate that the homologous region of the c-*myc* protein is not necessary for transformation of either chicken or rat embryo fibroblasts but is required for transformation of bone marrow cells (17, 52). It is possible that different regions of E1A are required for transformation of different cell types.

A second region of E1A necessary for transformation is located between amino acids 1 and 85. Deletion of all or part of this region abolished the formation of stable transformants. A single amino acid substitution at position 2 that changes the arginine residue to glycine (mutant *pm*563) was sufficient to eliminate transformation, and mutants that had deletions further into the coding region did not restore activity. The carboxy terminus of this region at amino acid 85 is based on results with the mutant *dl*813/919, which removes amino acids 86 to 120 and has little effect on transformation. Although our data indicate that the carboxy-terminal boundary of this region does not extend past amino acid 85, the mutants used in these experiments do not establish a carboxy-terminal boundary for this region. It is possible that this boundary is actually somewhere further toward the amino terminus.

The results obtained with mutants *pm*563 and NT*dl*598 appear to conflict with results previously reported by Osborne et al. (34). These authors found that a mutant E1A protein with an internal translational initiation site (at methionine 15) was capable of transforming at near-wild-type levels. The failure of *pm*563 and NT*dl*598 to transform was not due to inability of these plasmids to express the mutant proteins, since recombinant adenoviruses had slightly higher levels of the mutant E1A proteins than did the wild type (T. Grodzicker and P. Whyte, unpublished observations). While no explanation for this apparent discrepancy is readily available, it should be noted that the results of Osborne et al. involved transformation of rat embryo cells by a recombinant adenovirus, whereas our results were obtained by cotransfecting the E1A mutants with *ras* into BRK cells. The different results may reflect different functions of E1A needed to cooperate with the T24 H-*ras* oncogene compared with functions needed to transform in an adenovirus background. Another possibility is that the different cell types used have different susceptibilities to transformation.

Whereas only two regions of E1A appeared to be essential for the induction of stable transformants, none of our mutants transformed as well as wild-type E1A, suggesting that other regions of the protein make contributions to the transformation frequency. For example, *dl*975/1339, which includes the carboxy-terminal 67 amino acids, was able to induce higher levels of foci than mutant CT*dl*976, which encodes a similar protein but lacks the carboxy-terminal 67 amino acids. The strong nuclear localization imparted by these sequences may be responsible for the somewhat higher levels of transformation observed with *dl*975/1339 compared with CT*dl*976. Although this and other regions may influence the frequency of transformation, only mutations in the two regions designated as being essential were capable of eliminating transformation.

The demonstration that two regions of E1A are important for transformation suggests several possible models. First, in the folded structure of the protein, the two regions may make up parts of the same functional domain and contribute to a single biochemical function. In this model, multiple events involved in transformation would not be separable by mutagenesis. Alternatively, each of these regions may consist of separate domains capable of independently carrying out their respective functions, and the combination of their

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activities results in transformation. This model implies that multiple functions are involved in transformation and predicts that separation of these functions by mutagenesis is possible. Interestingly, deletion of the sequences between the two regions involved in transformation (mutant *dl*813/919) did not affect the transforming activity, demonstrating the lack of requirement for the intervening sequences. One possible interpretation of this result is that the transformation activity is not dependent on a critical physical interaction between the two regions involved in transformation. Experiments addressing this issue are currently under way.

Definition of the transforming regions of the E1A proteins should be useful in distinguishing between the models described above and should also be useful in initiating biochemical studies on the nature of the transforming activity of E1A.

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ADDENDUM

Mutations within the amino-terminal region of E1A that are transformation defective have also been recently described by Schneider et al. (EMBO J. **6**:2053–2060, 1987) and Lillie et al. (Cell **50**:1091–1100, 1987).

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