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Identification of Separate Domains in the Adenovirus E1A Gene for Immortalization Activity and the Activation of Virus Early Genes

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The transformation and early adenovirus gene transactivation functions of the E1A region were analyzed with deletion and point mutations. Deletion of amino acids from position 86 through 120 had little effect on the lytic or transforming functions of the E1A products, while deletion of amino acids from position 121 through 150 significantly impaired both functions. The sensitivity of the transformation function to alterations in the region from amino acid position 121 to 150 was further indicated by the impairment of transforming activity resulting from single amino acid substitutions at positions 124 and 135. Interestingly, conversion of a cysteine residue at position 124 to glycine severely impaired the transformation functions without affecting the early adenovirus gene activating functions. Single amino acid substitutions in a different region of the E1A gene had the converse effect. All the mutants produced polypeptides of sufficient stability to be detected by Western immunoblot analysis. The single amino acid substitutions at positions 124 and 135, although impairing the transformation functions, did not detectably alter the formation of the higher-apparent-molecular-weight forms of the E1A products.

The E1A and E1B genes of adenovirus types 2 and 5 (Ad2 and Ad5) are sufficient for complete transformation of rodent cells (6, 16, 17, 33, 47, 55). In addition, the E1A gene alone is capable of producing a partially transformed phenotype in rat cells (24, 43). E1A products are also involved in the regulation of expression of other adenovirus gene products during productive infection in human cells. In the absence of functional E1A products, expression of other early genes and transcription from the major late promoter are reduced (1, 26, 32, 37, 57).

Early in infection two mRNAs are made from E1A, the 12S and 13S products (2, 7, 28, 39). These products are predicted to encode proteins 243 and 289 amino acids long, respectively, which differ only by the presence of the 46 amino acids at positions 140 through 185 which are unique to the 13S product (Fig. 1).

The 13S product has been shown to be sufficient for the activation of other adenovirus genes required for lytic infection of HeLa cells (5, 9, 16, 25, 35, 36, 41, 52, 60, 63). Several reports have indicated that both the 12S and 13S products have partial transformation activities. Both the 12S and 13S products have been shown to induce immortalization of rodent cells, although some differences have been noted in the phenotypes of the cells immortalized by the separate products of the E1A region (20, 25, 34, 60, 63). Our experiments and those of others indicate that the 12S product does not efficiently activate other adenovirus genes in HeLa cells (20, 34, 36, 52, 63). One interpretation of these results is that at least some aspects of the transformation functions reside in the region common to the 12S and 13S products, while the 13S unique region is required in addition for efficient activation of the early adenovirus genes. The importance of the 13S unique region for efficient activation of adenovirus early genes is further emphasized by the demonstration that single amino acid changes in the 13S unique region severely impair the ability of the E1A region to activate adenovirus early genes (13). Other evidence indicates that the 12S product actually does contain a significant ability to transactivate adenovirus early genes (11, 31, 60). No mutations have been obtained which specifically impair the immortalization function of the E1A products, so the question is still unresolved whether the transformation and early adenovirus gene transactivating functions are separable in the E1A products.

To learn more about the relationship between the transformation and early adenovirus gene transactivation functions of the 12S and 13S E1A cDNA products, we constructed deletion and point mutations. Our results indicate that deletion of the amino acids from positions 86 through 120 has little effect on the lytic or transforming functions of the E1A products, while deletion of the amino acids from positions 121 through 150 significantly impairs both functions. The sensitivity of the transformation function to alterations in the region from amino acid position 121 to 150 is further indicated by the impairment of transforming activity that results from point mutations altering amino acids at positions 124 and 135. Most interestingly, our results indicate that severe impairment of the transformation functions can occur without impairment of the early adenovirus gene activating functions. Conversely, missense mutations which severely impair the ability of the 13S product to activate early adenovirus genes do not appear to impair its transformation function.

MATERIALS AND METHODS

Cells and viruses. Monolayer cultures of cell lines 293 (19) and HeLa were grown in Dulbecco modified Eagle medium supplemented with 10% calf serum, streptomycin (100 μg/ml), and penicillin (100 μg/ml). Ad5dl309 (27) was propagated on HeLa monolayers. New viruses were constructed as described previously (36, 51). Mutant viruses were identified by restriction digest analysis and dot-blot hybridization. Ad5dl312 (27) and the new E1A mutant and cDNA viruses were propagated on 293 cells. Ad5dl309 and Ad5dl312 were kindly provided by T. Shenk. Viral infections and titrations were performed essentially as described pre-

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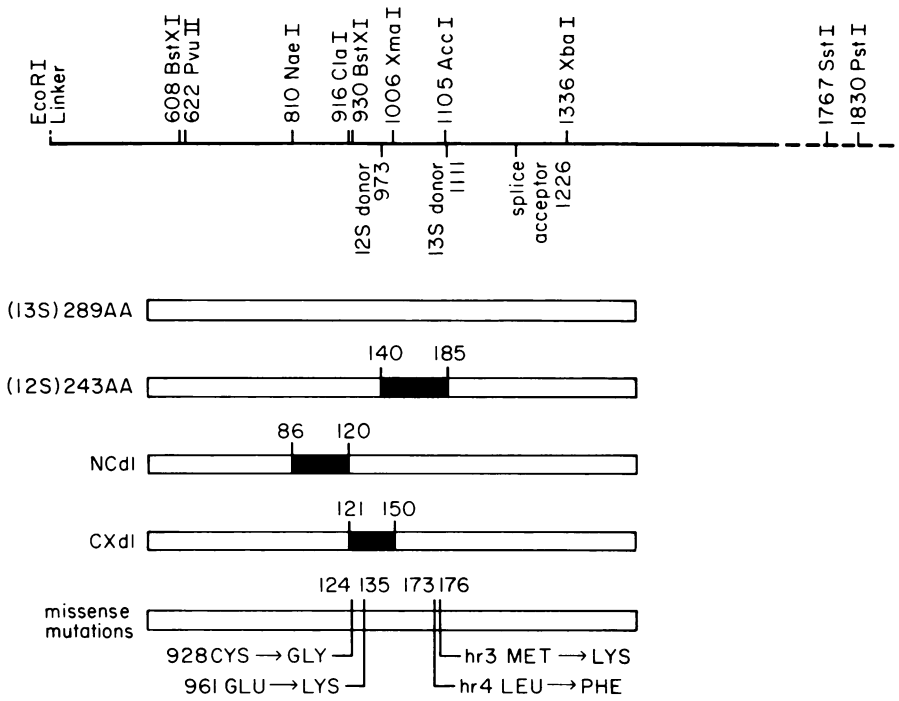

FIG. 1. Schematic representation of mutant products of the E1A region. Nucleotide positions of the 12S and 13S splice junctions and of selected restriction sites are indicated in the top line. The positions of E1A mutants discussed in the text are indicated by amino acid (AA) position numbers. All amino acid position numbers refer to the position as it would occur in the 289-amino acid product. The E1A-CXdl mutation deletes the 12S splice donor. Several of the mutants were constructed in one or more E1A cDNA products as described in the text. Positions of the hr3 and hr4 mutations were obtained from reference 13. The locations of the splice sites for the E1A 13S and 12S mRNAs are from reference 39.

viously (59) except that $MgCl_2$ was omitted for plaque assays on 293 cells.

Construction of mutations. (i) Wild-type cDNA plasmids. To remove most of the E1B sequences, the *Eco*RI-to-*Pst*I region (the locations of selected restriction sites in the E1A region are indicated in Fig. 1) of pLA1 (53) and the *Eco*RI-to-*Sst*I region of pLE13S and pLE12S (36) were subcloned into pUC18 to make plasmids pE1A-WT, p13S-WT, and p12S-WT, respectively.

(ii) In-frame deletion mutations. pMTE1A (63) was digested with *Cla*I and *Xma*I (all restriction enzymes used were obtained from New England BioLabs, Inc., Beverly, Mass.). The single-stranded ends were filled in and joined by using *Escherichia coli* DNA polymerase large fragment and T4 DNA ligase (New England BioLabs). After transfection into $CaCl_2$-competent *E. coli* cells, purified DNA from single-colony isolates showing an appropriate mutant restriction digest pattern was transferred to M13 and sequenced by the dideoxy method (44) to confirm that the deletion was in frame. The resultant plasmid was designated pMTE1A-CXdl. The *Bst*XI fragment of pMTE1A-CXdl was substituted for the wild-type *Bst*XI fragment of pLA1 to make plasmid pLECXdl, and the adenovirus *Eco*RI-to-*Pst*I fragment of pLECXdl was subcloned into pUC118 to make plasmid pE1A-CXdl. The *Nae*I-to-*Cla*I fragment of pE1A-WT was excised in the manner just described to form the plasmid pE1A-NCdl. The sequence of the new junction in this mutation was also confirmed by dideoxy sequencing in M13.

(iii) Oligonucleotide-directed synthesis of single point mutations. The *Hin*dIII-to-*Pst*I or *Hin*dIII-to-*Bam*HI region (both fragments extend from base pair 498 to base pair 1830) of pMT13S and pMTEB12S (63), respectively, were subcloned into M13mp8 and M13mp9 (61) to make substrates for mutagenesis. The mutagenesis procedure has been described previously (64). For the 961 mutation, a 17-mer complementary to the coding strand of E1A from nucleotides 950 to 966 with a single noncomplementary base at position 961 was synthesized (on an Applied Biosystems 380-A DNA synthesizer). It was then annealed along with the M13 universal primer to 12S and 13S M13 templates, extended, ligated, and transfected into *E. coli* JM107 (61). Individual plaques were cultured, and the M13 bacteriophage DNA was screened by dot-blot hybridization to ^{32}P-end-labeled mutant oligonucleotide. Positive isolates were plaque purified again and rescreened. Single-stranded DNA from the positive isolates was sequenced to confirm the mutation. Restriction digest fragments of replicative form DNA containing the mutant sequence were subcloned into wild-type E1A plasmids. The *Pvu*II-to-*Xba*I fragment of M13-12S-961 was subcloned into pMTE1A to make pMT12S-961, from which the *Cla*I-to-*Xba*I fragment was subcloned into pLA1 to make pLE12S-961, from which the *Eco*RI-to-*Pst*I fragment was subcloned into pUC118 to make p12S-961. The *Nae*I-to-*Acc*I fragment of M13-13S-961 was subcloned into an E1A plasmid from which the *Cla*I-to-*Xba*I fragment was subcloned into pE1A-WT to make pE1A-961.

The 928 mutation was made in a similar manner. A 19-mer made complementary to the noncoding strand of E1A from nucleotides 919 to 937 with a single noncomplementary base at position 928 was used to direct the synthesis of M13-13S-928. An *Nae*I-to-*Acc*I replicative form DNA fragment containing the 928 mutation was subcloned into an E1A plasmid from which the *Cla*I-to-*Pst*I fragment was subcloned

into pE1A-WT to make pE1A-928. The *Bst*XI fragment of pE1A-928 was substituted for the wild-type *Bst*XI fragment of p12S-WT to make p12S-928. The *Acc*I-to-*Pst*I fragment of pMT13S was substituted for the *Acc*I-to-*Pst*I fragment of pE1A-928 to make p13S-928.

In all cases the mutation in the final plasmid was confirmed by sequencing in M13 or pUC118. The entire region of the final plasmid originally derived from the mutated M13 substrate rather than from other wild-type plasmids was sequenced to ensure the absence of secondary mutations.

(iv) p13S-hr3 and p13S-hr4. The *Eco*RI-to-*Pst*I regions of pHR3 and pHR4 (13) were subcloned into pUC118. The *Acc*I-to-*Pst*I fragment of pMT13S was substituted into each of these plasmids to make p13S-hr3 and p13S-hr4.

Transient expression assay of chloramphenicol acetyltransferase (*cat*) activity. Transfections were carried out by a modified calcium phosphate coprecipitation technique (18, 58) as described previously (36). Cells were harvested 36 to 48 h later and assayed for *cat* activity (15, modified in reference 57), with the further modification that half of the lysate resulting from a 6-cm plate was incubated with 0.1 μCi of [^{14}C]chloramphenicol (approximately 40 mCi/mmol; New England Nuclear Corp., Boston, Mass.), 48 μl of distilled water, and 1 μl of 40 mM acetyl-coenzyme A (Sigma Chemical Co., St. Louis, Mo.). The extent of conversion of chloramphenicol to its acetylated forms was quantitated by cutting the respective spots from the thin-layer chromatography plates for assay by liquid scintillation spectroscopy.

Transformation of primary baby rat kidney cells. Primary baby rat kidney (BRK) cells were prepared as described previously (36) by collagenase-dispase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) treatment of kidneys from 6-day-old Fisher rats. A total of 3×10^5 to 5×10^5 cells per 60-mm dish were plated 2 days before infection. Cells were fixed with methanol-acetone (1:1 vol/vol) and stained with 10% Giemsa 3 to 4 weeks postinfection.

Transfection of primary baby rat kidney cells. Cells were prepared as described above for the transformation assay. For transfection, cells were given fresh medium 2 days after plating and transfected approximately 4 h later. T24 H-*ras* plasmid (10, 14) DNA (4 μg), 8 μg of the E1A plasmid to be tested, and 80 μg of high-molecular-weight salmon sperm DNA (Sigma) were precipitated with calcium phosphate and distributed evenly among eight 60-mm BRK plates. The plates were incubated at 37°C overnight, and then the cells were fed to remove excess precipitate. Cells were fed twice weekly, and foci were counted 3 to 4 weeks posttransfection.

Analysis of virus late proteins. HeLa cells infected at a multiplicity of 1 PFU per cell were labeled for 1 h before harvesting with 20 μCi of [^{35}S]methionine (New England Nuclear Corp.) in Dulbecco modified Eagle medium minus methionine per 6-cm plate. Cells were lysed in boiling 20% glycerol–1.5 M β-mercaptoethanol–6% Sodium dodecyl sulfate–0.125 M Tris hydrochloride (pH 6.8) and drawn through an 18-gauge needle. Samples corresponding to approximately 5×10^4 cells were resolved by electrophoresis through 12.5% SDS-polyacrylamide gels and processed for fluorography.

Western analysis of viral proteins. Protein extracts were prepared 16 h postinfection from cultures of primary BRK cells infected as described below at a multiplicity of 25 PFU per cell. Infected cells were lysed in RIPA buffer (1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris [pH 7.4], 150 mM NaCl). RIPA lysate supernatants corresponding to approximately 5×10^5 cells were separated in 10% SDS-polyacrylamide gels, electrophoretically transferred to nitrocellulose (54), and incubated with anti-*trpE* E1A fusion protein antibodies (49). The immune complexes were detected with ^{125}I-labeled protein A (New England Nuclear Corp.).

Immunoprecipitation and two-dimensional gel electrophoresis. BRK cells infected at a multiplicity of 25 PFU per cell were labeled from 12 to 14 h post infection with 300 μCi of [^{35}S]methionine in Dulbecco modified Eagle medium minus methionine per 6-cm plate. Infected cells were lysed in RIPA buffer and precleared with IgG-Sorb (The Enzyme Center, Boston, Mass.); cleared lysates were absorbed with anti-E1A-*trpE* fusion protein mouse monoclonal antibodies, series M73 (21), and precipitated on protein A-Sepharose (Pharmacia Uppsala, Sweden). Immune complexes were solubilized in isoelectric focusing buffer (0.3% SDS, 9.95 M urea, 4% Nonidet P-40, 2% ampholytes, 100 mM dithiothreitol). The electrophoretic procedures were performed as described previously (12, 38). Gels contained LKB pH 3.5 to 10 ampholytes in the first dimension and 10% acrylamide in the second dimension.

RESULTS

Deletion mutations. Our previous experiments (36, 63) and those of others (20, 34) have indicated that although the 12S cDNA product (243R) of the E1A gene has little of the adenovirus early gene-activating function found in the 13S cDNA E1A product (289R), it does have a very active immortalization function. To learn more about the relationship between the transformation and early adenovirus gene transactivation functions of the 12S and 13S products, we constructed deletion and point mutations. In particular, we were interested to know what effect, if any, impairment of the transformation functions would have on the early adenovirus gene transactivation activity of the E1A products.

We have shown (63) that a plasmid carrying the E1A region of the mutant virus hr-A (46), which expresses only the N-terminal 140 amino acids of the 289R product, 139 of which are common to the 243R product, is able to cooperate with the *ras* oncogene to transform primary BRK cells in culture. This result suggested that the active site for at least this aspect of the transformation functions is located in the N-terminal exon common to the 12S and 13S products. We constructed in-frame deletion mutants in this region to define the active site further.

An E1A restriction fragment extending from the *Nae*I site located at base pair 810 to the *Cla*I site located at base pair 916 was removed to create the plasmid pE1A-NCdl. Removal of these nucleotide sequences is predicted to result in the deletion of 35 amino acids, from amino acid positions 86 through 120 in the E1A region (Fig. 1).

Another in-frame deletion mutant plasmid, pE1A-CXdl, was constructed by removing a restriction fragment extending from the *Cla*I site at base pair 916 to the *Xma*I site at base pair 1006. Removal of this fragment is predicted to result in the deletion of 29 amino acids from positions 121 through 150 in the E1A region. (A new codon for alanine is created by the new nucleotide junction; an alanine previously occurred at position 127.) The nucleotide deletion also includes the donor splice site for the 12S cDNA product, so the mutant would be expected to produce only the 13S product.

These mutant plasmids, pE1A-NCdl and pE1A-CXdl, were each assayed for the ability to cooperate with the *ras* oncogene to transform primary BRK cells in culture. Complementation of the *ras* oncogene is a very sensitive assay

TABLE 1. Focus formation on primary cultured BRK cells: cotransfection of E1A mutant plasmids with the *ras* oncogene

Plasmids	No. of foci in expt:						
	1	2	3	4	5	6	7
H-*ras* + pE1A-WT	53	104	44	61	33	75	49
H-*ras* + p12S-WT	89	104			26	86	
H-*ras* + p13S-WT	16	30			19	14	18
H-*ras* + pE1A-NCdl	28	34	18		15		
H-*ras* + pE1A-CXdl					0	0	0
H-*ras* + pE1A-961					5	15	12
H-*ras* + pE1A-928			1	1	0		
H-*ras* + p13S-928					0	0	0
H-*ras* + p13S-hr3					30	48	
H-*ras* + p13S-hr4					21	48	
H-*ras* + pUC18	0	0	0	0	0	0	0

for E1A transformation functions, and since expression of the *ras* gene does not appear to be dependent on the E1A products, use of this assay enables the transformation functions of the E1A products to be assayed in a system which does not depend on the ability of the E1A products to activate other early adenovirus genes such as E1B. The characteristics of cultured primary BRK cells transformed by the individual E1A cDNA products plus the *ras* oncogene have been described previously (63).

The results shown in Table 1 indicate that pE1A-NCdl is still very active in its ability to complement the *ras* gene, although its activity is reduced about 2.5-fold from that of the wild-type E1A plasmid. pE1A-CXdl, however, is at least 15-fold impaired in its ability to cooperate with the *ras* gene to induce transformation of primary BRK cells, even when its complementation ability is compared with that of the 13S cDNA product, which typically induces about a third as many foci as the wild-type E1A region.

The ability of the E1A-NCdl and E1A-CXdl mutant plasmids to activate expression from the early region 3 (E3) adenovirus promoter was tested in HeLa cells in a transient expression assay (57) in which expression of the bacterial chloramphenicol acetyltransferase (*cat*) gene is dependent on activation of the E3 promoter. pE1A-NCdl activated E3-*cat* as well as the wild-type E1A plasmid does, while pE1A-CXdl had little ability to stimulate expression of E3-*cat* (Fig. 2).

These results suggest that the transformation function is particularly sensitive to alterations in the region of amino acids from positions 121 to 139. Several studies (20, 34, 36, 52, 63) have indicated that the 13S unique region (amino acid positions 140 through 185) is essential for the early adenovirus gene activating function. The impairment of E3-*cat*-stimulating activity in pE1A-CXdl suggests that amino acids between positions 121 and 139 are also important for this activity. Amino acids from position 86 through 120 appear to be dispensable, or nearly so, for both activities.

Point mutations. To obtain point mutations which might be defective in transformation activity, we used oligonucleotide-directed site-specific mutagenesis to change certain amino acids in the region from positions 121 to 139. This region of amino acids is highly conserved among the adenovirus serotypes (56) and includes a string of acidic amino acids from positions 133 to 138. The c-*myc* oncogene contains a similar stretch of acidic amino acids (40), and it has been shown that the *myc* oncogene, like E1A, will complement the *ras* oncogene in transforming primary cells (30, 43). Point mutations were introduced at base pair 928 (T to G) to change the amino acid at position 124 from cysteine to glycine and at base pair 961 (G to A) to change the acidic amino acid at position 135 from glutamic acid to the basic amino acid lysine (Fig. 1).

The resulting plasmids, pE1A-928 and pE1A-961, were tested for their ability to cooperate with the *ras* oncogene to transform primary BRK cells in culture. The results of these experiments are included in Table 1. Plasmid pE1A-928 is about 50-fold less active than the wild type in the ability to complement the *ras* gene; pE1A-961 is also somewhat impaired in this ability, but only about fivefold. The ability of these plasmids to activate adenovirus early genes was also tested in the E3-*cat* transient expression assay. Both point mutation plasmids had the ability to activate E3-*cat* expression (Fig. 2) to approximately the same level as wild-type E1A. Thus, from the evidence of the 928 mutation in particular, it appears that severe impairment of the transformation function of E1A products can occur without accompanying impairment of the early gene transactivation function.

Analysis of transformation function in 12S cDNA mutant virus constructions. To study the transformation defect of the 928 and 961 point mutations more quantitatively, we rebuilt the mutant plasmids into viruses. The transformation function of the 12S cDNA virus is particularly amenable to study because this virus is not lytic and induces proliferation of primary BRK cells in culture at very high efficiency as we have described previously (36). The 928 and 961 mutations were therefore introduced into 12S cDNA viruses. Figure 3 shows the ability of these viruses to induce proliferation of primary BRK cells infected at various representative multiplicities. Both mutants were defective in this activity at all multiplicities tested. At a mutiplicity of infection (MOI) of 2 PFU per cell, the number of proliferating foci induced by the 12S wild-type virus was 45-fold higher than the number induced by the 12S-961 virus. The 12S-961 virus appeared to be a little less defective relative to wild-type 12S virus when both were infected at an MOI of 10 PFU per cell. The 12S-928 virus was more defective in this function than the 12S-961 virus. Infection with the 12S-928 virus or Ad5dl312, like mock infection, had little effect on the quiescent layer of primary cells, but failed to induce the more deeply staining colonies of rapidly proliferating cells seen after infection

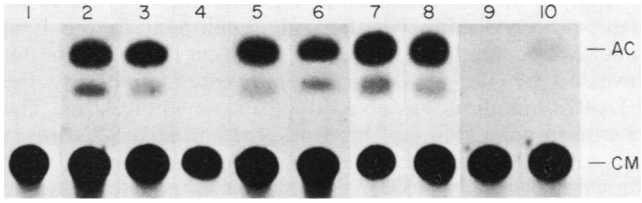

FIG. 2. Transactivation by E1A of an adenovirus E3-promoted chloramphenicol acetyltransferase (*cat*) gene. HeLa cells were cotransfected with pKCAT23 (57) and pUC18 (lane 1), pE1A-WT (lane 2), pE1A-NCdl (lane 3), pE1A-CXdl (lane 4), pE1A-928 (lane 5), pE1A-961 (lane 6), p13S-WT (lane 7), p13S-928 (lane 8), p13S-hr3 (lane 9), or p13S-hr4 (lane 10). Cell lysates were incubated with [^{14}C]chloramphenicol and acetyl-CoA and analyzed by thin-layer chromatography to separate chloramphenicol (CM) from its acetylated forms (AC). In an average of at least three experiments, including the ones illustrated, the measurement of *cat* activity was 8.3-fold and 9.7-fold higher after cotransfection with pE1A-WT or p13S-WT, respectively, than after cotransfection with pUC18. The ratios of mutant to wild-type activity were: pE1A-NCdl, 1.35; pE1A-CXdl, 0.01; pE1A-928, 1.24; pE1A-961, 0.89; p13S-928, 0.77; p13S-hr3, 0.01; p13S-hr4, 0.10.

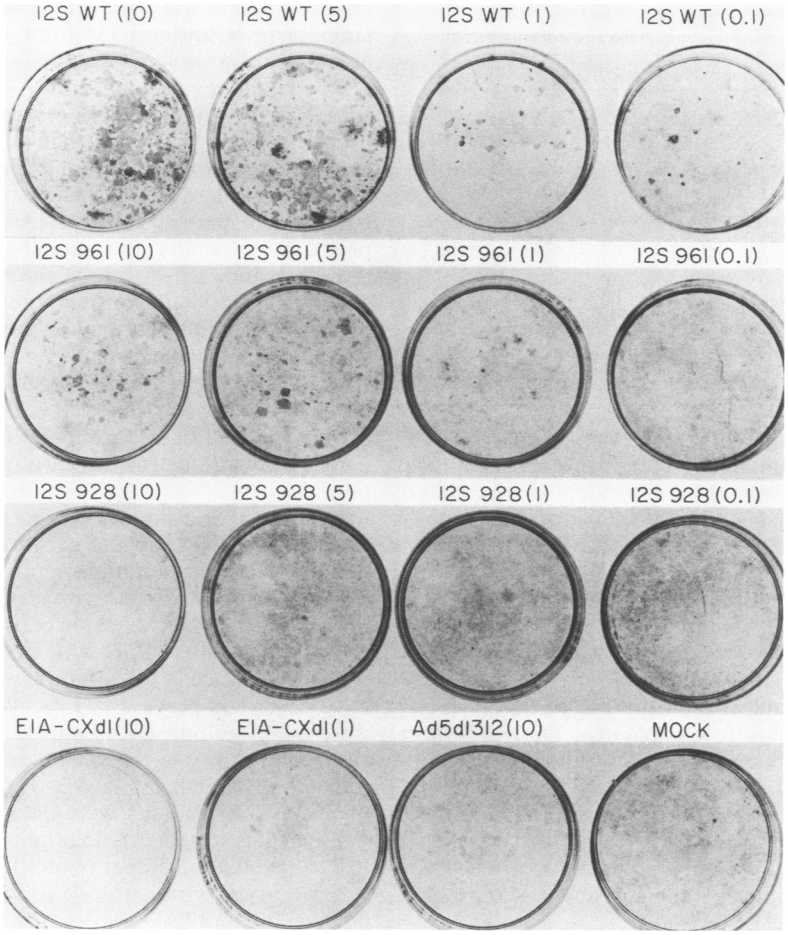

FIG. 3. Immortalization of primary BRK cells by virus infection. Primary BRK cells from 6-day-old rats were infected with wild-type 12S cDNA virus, 12S-961 virus, 12S-928 virus, E1A-CXdl virus, or Ad5dl312 virus at the MOI indicated in the figure or were mock infected. Cells were incubated at 37°C in Dulbecco modified Eagle medium plus 5% fetal calf serum. The medium was changed twice weekly, and plates were stained with 10% Giemsa 3 weeks postinfection.

with the wild-type 12S virus. The difference in the number of foci induced by the 12S wild type and the 12S-928 viruses was much greater than 100-fold, even at an MOI of 50.

Analysis of lytic functions in E1A mutant virus constructions. To determine whether an impairment in the lytic functions accompanies the transformation defect but is not revealed by the E3-*cat* transient expression assay, the E1A-928 mutation was also rebuilt into a whole virus. The plaque-forming ability of the wild-type and E1A-928 viruses was compared in HeLa and 293 cells, and the results are shown in Table 2. The E1A-928 mutant virus, like wild-type Ad5dl309 virus, formed plaques as well on HeLa cells as on 293 cells. The plaque-forming ability of the 12S cDNA mutant viruses is also shown in Table 2. Both the 12S-961 and 12S 928 viruses, like the wild-type 12S cDNA virus, were defective for plaque formation in HeLa cells relative to 293 cells.

TABLE 2. Plaque-forming ability of mutant viruses

Virus	Infectivity (PFU/ml)	
	293	HeLa
Ad5dl309	1.7×10^9	6.8×10^8
E1A-NCdl	1.2×10^9	3.2×10^8
E1A-CXdl	3.2×10^9	3.6×10^6
E1A-928	3.4×10^8	1.1×10^8
12S-WT	1.4×10^8	3.7×10^4
12S-928	3.7×10^8	1.8×10^5
12S-961	1.0×10^8	1.8×10^5
Ad5dl312	4.9×10^8	1.7×10^4

The growth properties of the E1A-928 virus were also studied by analysis of the appearance of virus late proteins during infection of HeLa cells. The results (Fig. 4) indicate that the relative levels of expression of late proteins at 24, 36, and 48 h postinfection are the same during E1A-928 virus infection as during wild-type virus infection. Neither the plaque-forming ability nor the course of infection indicated by the level or time of appearance of virus late proteins appeared to be affected by the existence of the 928 point mutation, even though this mutation severely affects the transformation properties of the E1A gene.

The plasmids pE1A-NCdl and pE1A-CXdl were also rebuilt into whole virus to determine whether the deleted amino acid region, from positions 86 through 120, is completely dispensable for the lytic functions of the virus in HeLa cells and to determine the effect of these deletions on the stability of the E1A proteins (see below). In Table 2 the plaque-forming ability of the wild-type, E1A-NCdl, and E1A-CXdl viruses is compared in HeLa and 293 cells. The

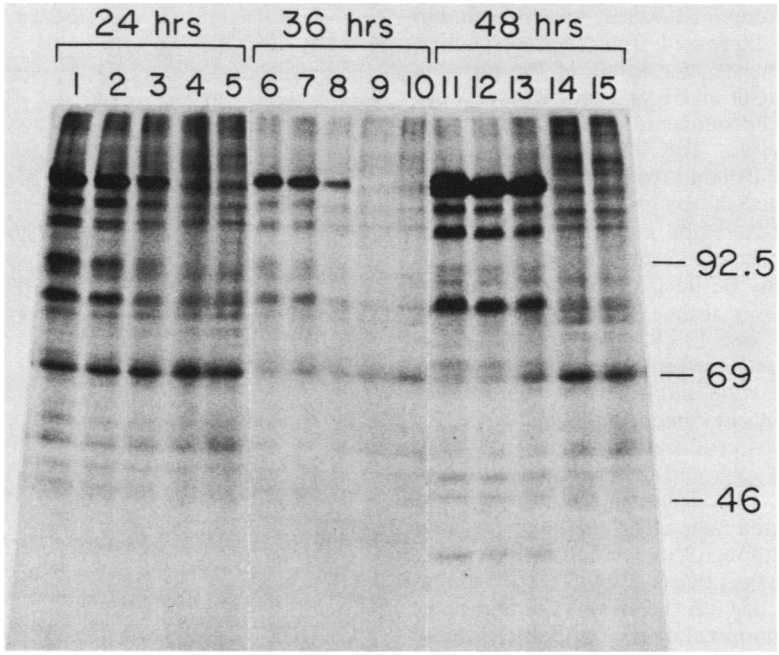

FIG. 4. Analysis of virus late proteins. HeLa cells infected at a multiplicity of 1 PFU per cell with Ad5dl309 virus (lanes 1, 6, and 11), E1A-928 virus (lanes 2, 7, and 12), E1A-NCdl virus (lanes 3, 8, and 13), E1A-CXdl virus (lanes 4, 9, and 14), or Ad5dl312 virus (lanes 5, 10, and 15) were labeled with [^{35}S]methionine in methionine-free Dulbecco modified Eagle medium for 1 h before being harvested at the times indicated in the figure. Samples were lysed and resolved by electrophoresis through 15% SDS-polyacrylamide gels. Numbers on the right indicate kilodaltons.

E1A-CXdl virus was defective for plaque formation in HeLa cells relative to 293 cells. The E1A-NCdl mutant virus, like wild-type Ad5dl309 virus, formed plaques with approximately the same efficiency on HeLa cells as on 293 cells, indicating that the amino acid region from positions 86 through 120 is not essential for lytic growth of the virus. The production of late proteins was also measured during infection of HeLa cells with the E1A-NCdl and E1A-CXdl mutant viruses (Fig. 4). The time of appearance of virus late proteins did not appear to be greatly affected by the presence of the NC deletion, again indicating that amino acids 86 through 120 are nearly dispensable for virus lytic functions in HeLa cells. No virus-specific late proteins were detected during infection with the E1A-CXdl virus.

Infection with either E1A-928 or E1A-NCdl virus, as with wild-type Ad5dl309 or 13S virus (36) was cytotoxic to primary cultured BRK cells. The E1A-CXdl virus, although less cytotoxic than Ad5dl309 or 13S virus, failed to induce immortalized foci on BRK cells (Fig. 3).

13S cDNA point mutations. Since the E1A-928 plasmid is far less efficient than the 13S wild-type cDNA plasmid in complementing the transforming functions of the *ras* oncogene (Table 1), the results with the E1A-928 mutation imply that transformation functions are lost in both the 13S and 12S cDNA products, while the transactivation function of the 13S cDNA product is unaffected. This implication was tested directly with the construction of a 13S-928 cDNA plasmid. This plasmid was able to activate E3-*cat* expression (Fig. 2), but failed to complement the *ras* oncogene to induce transformation of primary BRK cells in culture (Table 1).

Analysis of the E1A cDNA products has indicated that the loss of the 13S unique region, converting the 13S product to the 12S product, results in the impairment of transactivating activity but not in the impairment of certain transformation activities (20, 34, 36, 63). The 13S product has some of the same transformation activities seen in the 12S product (20, 25, 34, 63), but it is not known whether the transformation activity seen in the 13S product is dependent on its transactivation function. To discover whether loss of transactivation activity in the 13S product can occur without loss of transformation activities, we converted two host-range mutants containing point mutations in the 13S unique region to 13S cDNA plasmids so that transformation and transactivation functions could be measured in the absence of the 12S E1A product. The E1A products of Ad5-hr3 and Ad5-hr4 (13, 22) have previously been shown to be defective for the activation of virus lytic functions (22) and for the induction of E3-*cat* expression (13). These mutant viruses, like the 12S virus, have partial transformation activities (16). The loss of full expression of adenovirus transformation activity probably results, at least in part, from diminished expression of the E1B region owing to impairment of the E1A early virus gene transactivation function. The relative contributions of the wild-type 12S product and the mutant 13S products to the remaining transformation activity cannot be distinguished with the genomic E1A-hr3 and E1A-hr4 constructs. Therefore, we substituted a 13S cDNA junction into plasmids (13) containing the E1A region of Ad5-hr3 and Ad5-hr4 to make plasmids p13S-hr3 and p13S-hr4. Both of these plasmids were impaired for the activation of E3-*cat* (Fig. 2) but were at least as active as the wild-type 13S product in the ability to complement the *ras* oncogene to induce stable transformation of primary BRK cells (Table 1).

Analysis of mutant E1A proteins. To determine whether the reduced transforming activity of the 961 and 928 mutations results from reduced cellular levels of the mutant poteins during BRK cell infection, we assayed the levels of wild-type and mutant proteins by Western analysis of infected primary BRK cells. The results are shown in Fig. 5. Both the 928 and 961 mutant proteins expressed from the 12S

cDNA viruses and the full-length E1A virus were detected in amounts similar to those expressed from their respective wild-type counterparts. The cellular levels of the proteins produced from the E1A-NCdl and E1A-CXdl viruses were also assayed and found to be similar to the levels produced by wild-type Ad5dl309 virus. The 928 and 961 mutant proteins migrated indistinguishably from the corresponding wild-type proteins through SDS-polyacrylamide gels. The protein products of the E1A region migrated much slower than expected from their predicted molecular weights (8, 9, 45), and this difference may be due in part to posttranslational modifications. However, if the transformation activity observed in the 12S-961 and 12S-928 mutant viruses is associated with any difference in protein modification, this effect is not detectable as an alteration in the rate of migration of the proteins in one-dimensional gels.

As expected, the proteins produced from the E1A-NCdl and the E1A-CXdl viruses migrated faster than the protein products of the wild-type virus. The major E1A product of the E1A-CXdl virus migrated faster than the larger product of the E1A-NCdl virus, although the deletion in E1A-CXdl (29 amino acids) is smaller than that in E1A-NCdl (35 amino acids). The E1A products of both the E1A-NCdl and E1A-CXdl viruses were immunoprecipitable with monoclonal antibodies (21) which recognize a site coded by exon 2 of the E1A gene (Fig. 6), verifying that these mutations are in frame. The same immunoprecipitation pattern (not shown) was obtained with antiserum (E1A-C1 serum; 62) raised against a synthetic peptide corresponding to the predicted carboxy terminus of the E1A products.

Proteins precipitated with monoclonal antibodies directed against the E1A proteins present a complex pattern on two-dimensional gels (21). The E1A proteins produced from the E1A-928 and the 12S-928 mutant viruses during infection of primary BRK cells were analyzed by two-dimensional gel electrophoresis of monoclonal antibody-precipitated samples. The significance of the large number of polypeptide products separated on the two-dimensional gels is not known, but the only difference apparent between the pattern produced by the mutant proteins and that produced by the corresponding wild-type proteins was the intensity of the lowest-molecular-weight product detected in the genomic E1A constructs (Fig. 7). The E1A products of the hr3 and hr4 viruses have also been analyzed by one-dimensional and two-dimensional gel electrophoresis (41, 42) and were not detectably different from wild type.

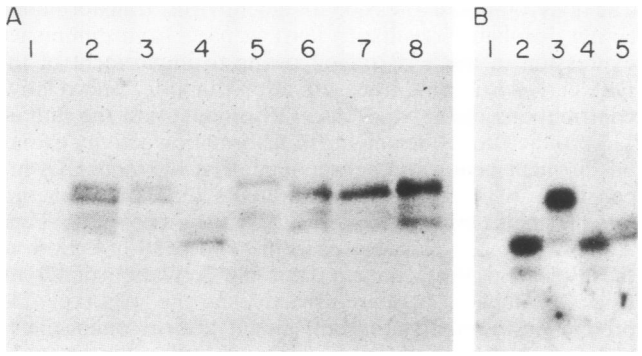

FIG. 5. Western analysis of mutant E1A proteins in virus-infected BRK cells. Protein extracts from BRK cells infected at an MOI of 25 PFU per cell were prepared 16 h postinfection, separated in 10% SDS-polyacrylamide gels, transferred to nitrocellulose, and incubated with anti-*trpE*-E1A fusion protein antibodies (49). (A) Lanes: 1, mock; 2, Ad5dl309; 3, E1A-928; 4, E1A-NCdl; 5, 13S-WT; 6, 12S-WT; 7, 12S-928; 8, 12S-961. (B) Lanes: 1, mock; 2 and 4, E1A-CXdl; 3, Ad5dl309; 5, E1A-NCdl.

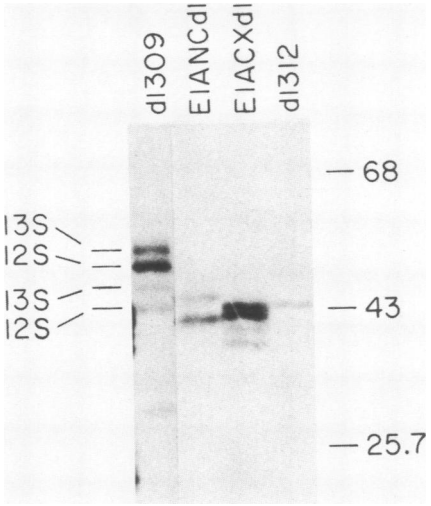

FIG. 6. Immunoprecipitation of E1A deletion mutant products in virus-infected BRK cells. Protein extracts from BRK cells infected at an MOI of 25 PFU per cell with wild-type or mutant viruses as indicated in the figure were labeled with [^{35}S]methionine in methionine-free medium for 2 h before harvesting, absorbed with anti-*trpE*-E1A fusion protein mouse monoclonal antibodies, series 73 (21), precipitated on protein A-sepharose, and separated in 10% SDS-polyacrylamide gels. Numbers on the right indicate kilodaltons.

DISCUSSION

The products of the E1A region have both transformation activity in nonpermissive or semipermissive rodent cells and the ability to transcriptionally activate the products of the adenovirus genome during productive infection of permissive human cells. To better understand the relationship between these activities, we attempted to analyze the functional regions of the E1A products by constructing deletion and single point mutations in the E1A region. We also further analyzed certain missense mutations by rebuilding them into E1A cDNA products.

Our results indicate that a region of 35 amino acids (approximately 12% of the 289R product or 14% of the 243R product) from positions 86 through 120 can be deleted without severely impairing either the transactivation function or the *ras* oncogene complementation function. A similar, but smaller, in-frame deletion of 16 amino acids from positions 101 through 116 has also recently been shown not to impair virus growth (23).

Our results also indicate that deletion of the region of 29 amino acids from positions 121 through 150 of the 289R product severely impairs both the transactivation function and the ability to complement the *ras* gene in transformation. The same mutation, independently constructed in a 13S cDNA product and prepared as purified protein from an *E. coli* expression vector, has recently been shown to be deficient in the transactivation function, measured as the ability to complement Ad5dl312 in an E1A protein microinjection assay in Vero cells (29). This report also demonstrated that deletion or alteration of amino acids from position 222 to the carboxy terminus barely reduces the ability of the 13S product to complement Ad5dl312, while

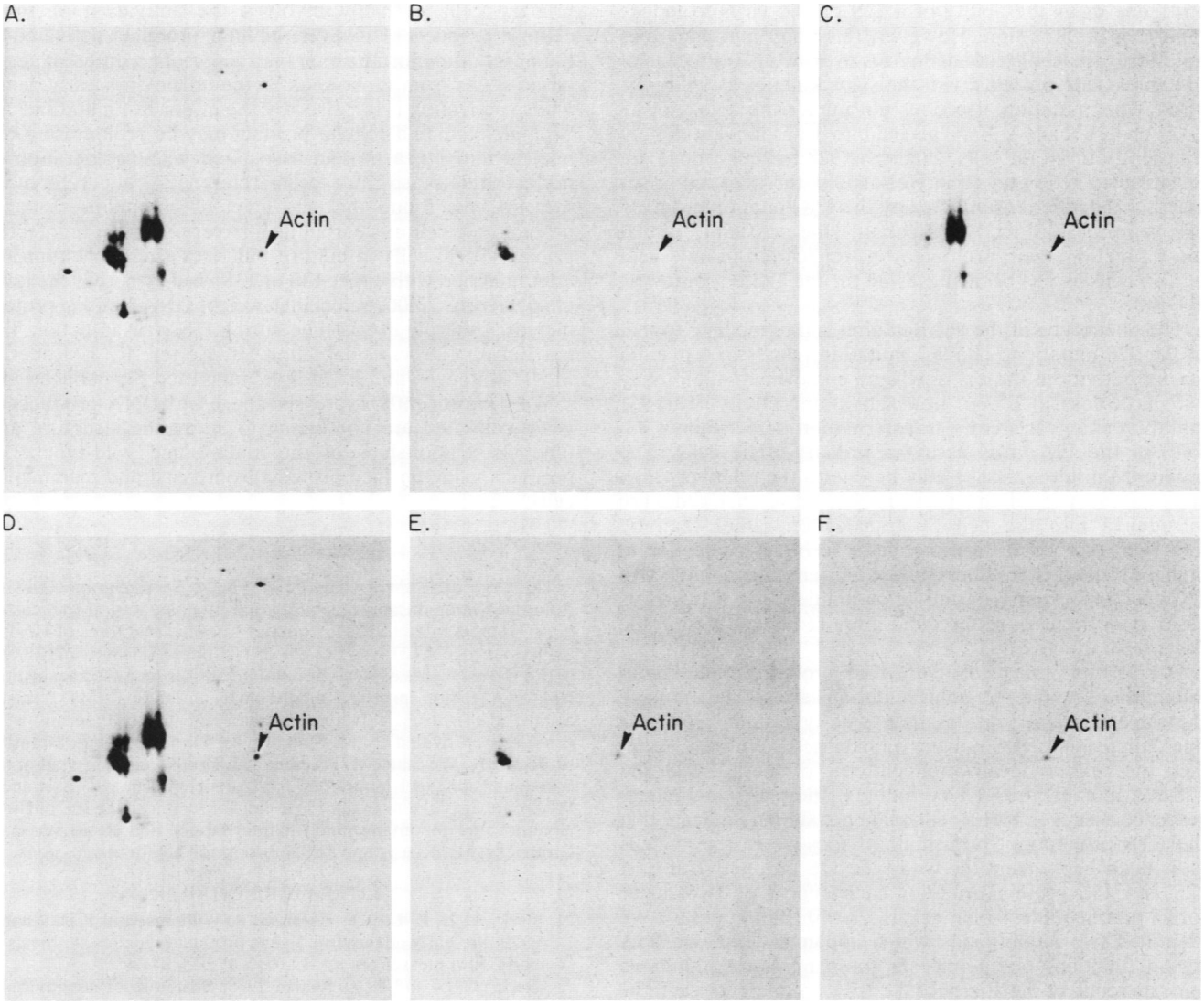

FIG. 7. Two-dimensional gel analysis of mutant E1A proteins. Immune complexes prepared as described in the legend to Fig. 6 were separated on gels containing pH 3.5 to 10 ampholytes in the first dimension and 10% acrylamide in the second dimension. Only the E1A product region of the gels is illustrated. (A) Ad5dl309; (B) 12S-WT; (C) 13S-WT; (D) E1A-928; (E) 12S-928; (F) mock.

deletion or alteration of amino acids from position 150 to the carboxy terminus markedly impairs complementation of Ad5dl312, further supporting the implication that the region of amino acids from positions 121 through 185 includes the functional domain for the early adenovirus gene transactivation activity. Three missense mutations have recently been identified, occurring toward the carboxy end of this region (at amino acid positions 173, 176, and 185), which severely impair the transactivation function (13, 22). We reconstructed two of these mutations into 13S cDNA plasmids and demonstrated that these missense mutations do not impair the ability of these plasmids to induce immortalized foci when cotransfected with the *ras* oncogene into primary BRK cells.

The results presented here further distinguish the areas of function toward the N terminus of the region extending from positions 121 through 185. Interestingly, a degree of homology was noted between the amino acid sequences of other genes associated with immortalization and the sequence of the E1A products in the region approximately bounded by positions 121 to 150. Besides being highly conserved among different adenovirus serotypes (56), this region shows some degree of homology with the *myc* oncogene (40) and with polyomavirus large T antigens (50). The cellular tumor antigen p53 and the *myc* oncogene also have homologous sequences which overlap the region of homology between *myc* and E1A (3).

It has been demonstrated previously (20, 25, 34, 63) that deletion of amino acids from positions 140 through 185 (the difference between the 13S and 12S cDNA products) leaves intact at least some aspects of the transformation functions. We show here that deletion of amino acids 121 through 150 impairs the transformation functions as well as the transactivation functions, but we also show that it is possible to make a specific missense mutation in this region which impairs the transformation function while having no discernable effect on the transactivation function. In particular, the 928 point mutation, changing the cysteine at position 124 to glycine, is markedly defective in transformation activity when measured either as the ability to complement the *ras*

oncogene or as the ability of a 12S cDNA virus to induce rapid cell proliferation. However, this point mutation does not impair the ability of adenovirus to grow in HeLa cells or to express late proteins with the same kinetics as wild-type virus. This mutation also does not impair the ability of an E1A or 13S cDNA E1A plasmid to transactivate E3-*cat* transiently in HeLa cells. Since the 928 mutation does not impair virus lytic growth in HeLa cells, the question arises whether the functions affected by the 928 mutation normally play a role in productive infection. One possibility is that these functions help promote viral replication in quiescent human cells as has been suggested for the 12S E1A product (34, 48).

The phenotype of the 961 mutation is intermediate to that of the 928 mutation. The 961 mutation results in a fivefold loss of activity in the *ras* oncogene complementation assay with little loss in E3-*cat* transactivation activity. The 961 mutation shows a greater transformation defect (about 45-fold) in the 12S virus assay in primary BRK cells. The difference in these assays may be due to the relatively high copy number per cell that presumably occurs when genes are introduced into the cell by transfection rather than by infection; the 12S-961 virus appears to be less defective at higher MOIs. It is possible that the NCdl mutation might also show a more extensive transformation defect in a 12S virus assay than the two- to threefold more reduction seen in the *ras* cooperation assay.

Our results indicate that the E1A protein products containing the 928 and 961 point mutations are stable and have the same migration rate as their wild-type counterparts in one-dimensional SDS-polyacrylamide gels. We also examined the cellular localization patterns of the 12S-928 and 12S-961 mutant virus E1A products. Indirect immunofluorescence assays in HeLa cells (data not shown) indicate that the E1A products of both mutants localize to the nucleus indistinguishably from the wild-type 12S product. The E1A 12S and 13S protein products each appear as two major forms on one-dimensional gels (8, 9, 45) (Fig. 5 and 6). We identified two amino acids which impair at least one E1A region function, but neither of these missense mutations appears to affect the ability of the E1A products to reach the higher-apparent-molecular-weight forms. The predicted deletions in the NCdl and CXdl 13S products (35 and 29 amino acids, respectively) are less than the difference between the 12S and 13S products (46 amino acids), yet the largest forms of both the NCdl and CXdl products migrate faster than the largest form of the wild-type 12S product, and the CXdl product migrates faster than the largest NCdl product. Perhaps amino acids between positions 85 and 150 are required for formation of the higher-molecular-weight products, but in this case formation of the higher-molecular-weight products is not required for the functions expressed by the NCdl mutant. The difference in migration rates between the NCdl and CXdl products cannot be explained by a greater proline composition in the smaller deletion, as 9 of the 35 amino acids deleted in the NCdl mutation are proline residues while proline accounts for only 3 of the 29 amino acids deleted in the CXdl mutation.

The existence of 13S E1A products such as 13S-928 and 13S-hr3 with single point mutations which selectively impair either the transformation functions assayed in the *ras* oncogene complementation assay or the ability to transactivate other early adenovirus genes argues that these are independent activities with different domains in the 13S product. Both activities may involve transactivation, possibly via transcriptional control, since immortalization of primary cells certainly involves the activation of some cellular products. However, cellular products activated at the onset of immortalization may represent a different set of target genes from those such as the adenovirus early genes whose activation is impaired by the hr3 or hr4 mutation. An example of such a cellular product may be proliferating cell nuclear antigen, a protein associated with proliferation of normal and transformed cells (reference 4 and references therein). We found that the level of immunoprecipitable proliferating cell nuclear antigen is very small in uninfected primary BRK cells in culture but rises sharply within 9 h after infection with either 12S or 13S virus, whereas the level of adenovirus 72,000-molecular-weight DNA-binding protein increases sharply in this time during 13S virus infection, but not during 12S virus infection (B. Zerler, R. J. Roberts, M. B. Mathews, and E. Moran, submitted for publication).

We have recently expressed the E1A cDNA products in yeast cells and are continuing to study the structure and function relationships of the mutant and wild-type E1A products using purified proteins from yeast and mammalian cells.

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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. **Berk, A. J., and P. A. Sharp.** 1978. Structure of adenovirus 2 early mRNAs. Cell **14**:695–711.
3. **Bienz, B., R. Zakut-Houri, D. Givol, and M. Oren.** 1984. Analysis of the gene coding for the murine cellular tumour antigen p53. EMBO J. **3**:2179–2183.
4. **Bravo, R., and H. Macdonald-Bravo.** 1985. Changes in the nuclear distribution of cyclin (PCNA) but not its synthesis depend on DNA replication. EMBO J. **4**:655–661.
5. **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.
6. **Chinnadurai, G.** 1982. Adenovirus 2 lp^+ locus codes for a 19 kd tumor antigen that plays an essential role in cell transformation. Cell **33**:759–766.
7. **Chow, L. T., T. R. Broker, and J. B. Lewis.** 1979. Complex splicing patterns of RNAs from the early regions of adenovirus-2. J. Mol. Biol. **134**:265–303.
8. **Downey, J. F., C. M. Evelegh, P. E. Branton, and S. T. Bayley.** 1984. Peptide maps and N-terminal sequences of polypeptides from early region 1A of human adenovirus 5. J. Virol. **50**:30–37.
9. **Esche, H., M. B. Mathews, and J. B. Lewis.** 1980. Proteins and messenger RNAs of the transforming region of wild-type and mutant adenoviruses. J. Mol. Biol. **142**:399–417.
10. **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.
11. **Ferguson, B., B. Krippl, N. Jones, J. Richter, H. Westphal, and M. Rosenberg.** 1985. Functional characterization of purified

adenovirus E1A proteins expressed in *Escherichia coli*, p. 265–274. *In* J. Feramisco, B. Ozanne, and C. Stiles (ed.), Cancer cells 3: Growth factors and transformation. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

12. **Garrels, J. I.** 1983. Quantitative two-dimensional gel electrophoresis of proteins. Methods Enzymol. **100**:411–439.
13. **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.
14. **Goldfarb, M., K. Shimizu, M. Perucho, and M. Wigler.** 1982. Isolation and preliminary characterization of a human transforming gene from T24 bladder carcinoma cells. Nature (London) **296**:404–409.
15. **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.
16. **Graham, F., T. Harrison, and J. Williams.** 1978. Defective transforming capacity of adenovirus type 5 host range mutants. Virology **86**:10–21.
17. **Graham, F. L., P. J. Abrahams, C. Mulder, H. L. Heijneker, S. O. Warnaar, F. A. J. DeVries, W. Friers, and A. J. van der Eb.** 1974. Studies on *in vitro* transformation by DNA and DNA fragments of human adenoviruses and simian virus 40. Cold Spring Harbor Symp. Quant. Biol. **39**:637–650.
18. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
19. **Graham, F. O., J. Smiley, W. Russell, and R. Nairn.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36**:59–72.
20. **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.
21. **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.
22. **Harrison, T., F. Graham, and J. Williams.** 1977. Host-range mutants of adenovirus type 5 defective for growth in HeLa cells. Virology **77**:319–329.
23. **Hearing, P., and T. Shenk.** 1985. Sequence-independent autoregulation of the adenovirus type 5 E1A transcription unit. Mol. Cell. Biol. **5**:3214–3221.
24. **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.
25. **Hurwitz, D. R., and G. Chinnadurai.** 1985. Evidence that a second tumor antigen coded by adenovirus early gene region E1a is required for efficient cell transformation. Proc. Natl. Acad. Sci. USA **82**:163–167.
26. **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.
27. **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.
28. **Kitchingman, G. R., and H. Westphal.** 1980. The structure of adenovirus 2 early nuclear and cytoplasmic RNAs. J. Mol. Biol. **137**:23–48.
29. **Krippl, B., B. Ferguson, N. Jones, M. Rossenberg, and H. Westphal.** 1985. Mapping of functional domains in adenovirus E1A proteins. Proc. Natl. Acad. Sci. USA **82**:7480–7484.
30. **Land, H., L. F. Parada, and R. A. Weinberg.** 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature (London) **304**:596–602.
31. **Leff, T., R. Elkaim, C. R. Goding, P. Jalinot, P. Sassone-Corse, M. Perricaudet, C. Kedinger, and P. Chambon.** 1984. Individual products for the adenovirus 12S and 13S E1a mRNAs stimulate viral E11a and E111 expression at the transcriptional level. Proc. Natl. Acad. Sci. USA **81**:4381–4385.
32. **Lewis, J. B., and M. B. Mathews.** 1980. Control of adenovirus early gene expression: a class of immediate early products. Cell **21**:303–313.
33. **McKinnon, R. D., S. Baccheti, and F. L. Graham.** 1982. Tn5 mutagenesis of the transforming genes of human adenovirus type 5. Gene **19**:33–42.
34. **Montell, C., G. Courtois, C. Eng, and A. Berk.** 1984. Complete transformation by adenovirus 2 requires both E1a proteins. Cell **36**:951–961.
35. **Montell, C., E. F. Fisher, M. H. Caruthers, and A. J. Berk.** 1982. Resolving the functions of overlapping viral genes by site-specific mutagenesis at a mRNA splice site. Nature (London) **295**:380–384.
36. **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.
37. **Nevins, J. R.** 1981. Mechanism of activation of early viral transcription by the adenovirus E1A gene product. Cell **26**:213–220.
38. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
39. **Pericaudet, 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.
40. **Ralston, R., and J. M. Bishop.** 1983. The protein products of the *myc* and *myb* oncogenes and adenovirus E1a are structurally related. Nature (London) **306**:803–806.
41. **Ricciardi, R. P., R. L. Jones, C. L. Cepko, P. A. Sharp, and B. E. Roberts.** 1981. Expression of early adenovirus genes requires a viral encoded acidic polypeptide. Proc. Natl. Acad. Sci. USA **78**:6121–6125.
42. **Rowe, D. T., S. P. Yee, J. Otis, F. L. Graham, and P. E. Branton.** 1983. Characterization of human adenovirus type 5 early region 1A polypeptides using antitumor sera and an antiserum specific for the carboxy terminus. Virology **127**:253–271.
43. **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.
44. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
45. **Smart, J., J. Lewis, M. Mathews, M. Harter, and C. Anderson.** 1981. Adenovirus type 2 early proteins: assignment of the early region 1A proteins synthesized *in vivo* and *in vitro* to specific mRNAs. Virology **112**:703–713.
46. **Solnick, D.** 1981. An adenovirus mutant defective in splicing from early region 1A. Nature (London) **291**:508–510.
47. **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.
48. **Spindler, K. R., C. Y. Eng, and A. J. Berk.** 1985. An adenovirus early region 1A protein is required for maximal viral DNA replication in growth-arrested human cells. J. Virol. **53**:742–750.
49. **Spindler, K. R., D. S. E. Rosser, and A. J. Berk.** 1984. Analysis of adenovirus transforming proteins from early regions 1A and 1B with antisera to inducible fusion antigens produced in *Escherichia coli*. J. Virol. **49**:132–141.
50. **Stabel, S., P. Argos, and L. Philipson.** 1985. The release of growth arrest by microinjection of adenovirus E1A DNA. EMBO J. **4**:2329–2336.
51. **Stow, N. D.** 1981. Cloning of a DNA fragment from the left-hand terminus of the adenovirus type 2 genome of its use in site-directed mutagenesis. J. Virol. **37**:171–180.
52. **Svensson, C., and G. Akusjarvi.** 1984. Adenovirus 2 early region 1A stimulates expression of both viral and cellular genes. EMBO J. **3**:789–794.
53. **Tamanoi, F., and B. W. Stillman.** 1982. Function of adenovirus terminal protein in the initiation of DNA replication. Proc. Natl. Acad. Sci. USA **79**:2221–2225.
54. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose

sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.

55. **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.
56. **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.
57. **Weeks, W. 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.
58. **Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub, and L. Chasin.** 1979. DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. Proc. Natl. Acad. Sci. USA **76**:1373–1376.
59. **Williams, J. F.** 1970. Enhancement of adenovirus plaque formation on HeLa cells by magnesium chloride. J. Gen. Virol. **9**:251–255.
60. **Winberg, G., and T. Shenk.** 1984. Dissection of overlapping functions within the adenovirus type 5 E1A gene. EMBO J. **3**:1907–1912.
61. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.
62. **Yee, S.-P., D. T. Rowe, M. L. Tremblay, M. McDermott, and P. E. Branton.** 1983. Identification of human adenovirus early region 1 products by using antisera against synthetic peptides corresponding to the predicted carboxy termini. J. Virol. **46**:1003–1013.
63. **Zerler, B., B. Moran, K. Maruyama, J. Moomaw, T. Grodzicker, and H. E. Ruley.** 1986. Analysis of adenovirus E1A coding sequences which enable *ras* and *pmt* oncogenes to transform cultured primary cells. Mol. Cell. Biol. **6**:887–899.
64. **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.