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Adenovirus E1a *ras* Cooperation Activity Is Separate from Its Positive and Negative Transcription Regulatory Functions

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The E1a gene of adenovirus encodes two proteins, 289 and 243 amino acids long, which have positive (transactivator) and negative (enhancer repressor) RNA polymerase II transcriptional regulatory properties and cell transformation activities including cooperation with an activated *ras* gene. The E1a transforming functions more closely correlate with the repressor property than with transactivation in that both E1a proteins express the repressor and transformation functions while only the 289-amino-acid protein is an efficient transactivator. To understand whether the transcriptional regulatory activities of E1a are related to its *ras* cooperation activity, we generated a series of mutant E1a expression vectors by linker insertion mutagenesis of the 289-amino-acid protein. Here we describe a new class of mutants which although defective for enhancer repression still can cooperate with the *ras* oncogene in cell transformation. The mutants are also defective in transcription transactivation. Our data suggest that enhancer repression and transformation via *ras* cooperation are separate E1a functions and that cooperation with *ras* does not rely on either of the RNA polymerase II transcription regulatory functions of E1a. We also show that mutations which inactivate enhancer repression are not confirmed to a single critical domain necessary for repression. We therefore propose that the integrity of the overall configuration of the E1a proteins is important for the repression activity.

The E1a region of adenovirus has an essential role in activating the expression of early viral transcription units during a lytic infection (reviewed in reference 1). In addition, E1a can activate transcription from several cellular genes (1) and repress transcription dependent on either viral or cellular enhancer sequences (3, 9, 28, 32, 34). Two mRNAs which are 13S and 12S species, respectively, are produced by the E1a gene via differential splicing (21) and encode two proteins 289 and 243 amino acids long, respectively. These two proteins have both common and distinct biological functions (reviewed in reference 2). The transcription transactivation function is unique to the 289-amino-acid protein (15, 30), while both proteins can repress enhancer activity (3, 34). In addition to providing the transcriptional regulatory properties, the E1a region can exert profound effects on the growth properties of cells (reviewed in reference 4). Both proteins can immortalize primary cells (17, 23). This establishment function appears to be related to the ability of E1a to cooperate with other oncogenes such as an activated *ras* gene in primary cell transformation (36). Furthermore, during viral infections, both E1a products can induce cell host proliferation (26, 27), while only the 243-amino-acid protein facilitates viral DNA replication in quiescent cells (26).

The 289- and 243-amino-acid proteins differ by the presence of an additional 46 amino acids encoded in the 13S mRNA product (21). This region, referred to as domain 3, is unique to the 13S mRNA product and is highly conserved among several adenovirus serotypes (10). Two other regions, domains 1 and 2, are also conserved and are common to the two E1a proteins (10). The presence of these highly conserved sequences has suggested that E1a proteins are composed of several different domains, each of which contributes a subset of the multiple E1a functions (18). Indeed, mutational analysis of the E1a gene supports a model in which distinct biological activities of E1a are provided by specific conserved domains. Domain 3 is responsible for the transactivation function (8, 13, 14, 17, 19, 24), while functions provided by domains 1 and 2 are necessary for cell transformation (12–14, 17, 19, 24) and possibly transcriptional repression as well (13, 14, 24). The possibility that each domain functions independently is further strengthened by the recent report of Lillie et al. (14) that a synthetic peptide corresponding to domain 3 of the E1a protein is sufficient to transactivate an early viral promoter.

A critical issue in establishing the mechanism of cell transformation by nuclear oncogene proteins such as E1a is whether these proteins mediate the transformation process by regulating transcription, for example, by modulating the activity of cellular genes that control growth. To define the relationship between the transcriptional regulatory functions of E1a and its transformation activity, we undertook a genetic analysis of the E1a gene, comparing wild-type and mutant E1a vectors for their ability to transactivate early viral promoters, to repress enhancer-dependent transcription, and to transform REF52 rat embryo fibroblast cells in cooperation with the *ras* oncogene. In this report, we describe a new class of E1a mutants which can transform via *ras* cooperation although they are defective for the enhancer repression activity. Our data thus support the novel finding that enhancer repression and transformation via *ras* cooperation are separate E1a functions. Indeed, these mutants are capable of *ras* cooperation with near wild-type efficiency although they lack both the positive and negative transcription regulatory functions. Furthermore, the structural alterations in these mutants do not map in either domain 1 or 2, the regions previously indicated as important for repression as well as *ras* cooperation. We discuss a model in which single E1a domains may function as independent units for transactivation and transformation, while in contrast, the integrity of the greater part of the E1a protein is required for the enhancer repression function.

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MATERIALS AND METHODS

Plasmids. The wild-type E1a expression vectors have been described previously (34). Vector pSVE1a contains the genomic sequence of adenovirus type 5 DNA (nucleotides 1 to 1830) cloned in the plasmid pSVod, while pSVF12 and pSVN20 are the intronless forms of pSVE1a, reconstructed with sequences from, respectively, the 12S and 13S cDNAs. Mutated forms of the 13S vector were constructed by *Xho*I linker insertion mutagenesis as described in reference 25. PyE92-1 and pβGSV, gifts from Jean de Villiers, contain the genomic form of the rabbit β-globin gene without the promoter linked to the polyomavirus and simian virus 40 (SV40) enhancer, respectively (5). pT24neo (7), a gift from E. Ruley, contains the *ras* oncogene isolated from T24 human bladder carcinoma cells cloned in the same vector with the *neo* gene, which confers resistance to G418.

Cell culture and DNA transfection. HeLa and REF52 cells were maintained by standard procedures. Transfections were performed by the $CaPO_4$ precipitate method as described in reference 35. In all the transfection experiments, the final DNA concentration per plate was kept constant at 15 μg by the addition of pBR322 as carrier DNA.

Immunofluorescence, transactivation, and transformation assays. In the immunofluorescence assay for E1a protein expression, HeLa cells were transfected with 1 μg of the E1a expression vectors. Forty-eight hours later, cells were fixed with cold (−20°C) methanol for 15 min, washed with phosphate-buffered saline, and incubated at 37°C for 1 h with the first antibody, a mouse monoclonal antibody. Monoclonal antibodies were chosen such that the epitopes they recognized were retained in the E1a deletion mutant protein to be analyzed. Monoclonal antibody M73, which recognizes an epitope proximal to the carboxy terminus of the E1a polypeptide (29), was used to detect E1a protein in mock-transfected cells and in cells transfected with pSVF12 and pSVXL105. Monoclonal antibody M37, which recognizes an epitope proximal to the amino terminus of E1a protein (29), was used to detect E1a protein in cells transfected with plasmids pSVN20, pSVXL124, and pSVXL214. M73 and M37 antibodies were kindly provided by Ed Harlow. After several washes with phosphate-buffered saline, cells were counterstained with fluorescein isothiocyanate-conjugated rabbit anti-mouse antibody for 1 h at 37°C and then washed many times with phosphate-buffered saline. The transactivation assay has been described in reference 34. Briefly, HeLa cells transfected with the E1a expression vectors were infected at a low multiplicity (20 PFU per cell) with *dl*312, a mutant virus which expresses no E1a product; 48 h later, cells were fixed and stained to detect the adenovirus type 5 DNA-binding protein by an indirect immunofluorescence assay. Transactivation was measured as the percentage of HeLa cell nuclei immunopositive for DNA-binding protein. The transformation activity was measured by the *ras* cooperation assay in REF52 cells, according to reference 7. The various E1a plasmids (1 μg) were transfected with 1 μg of pT24neo DNA along with 15 μg of high-molecular-weight REF52 cell DNA as the carrier. At 48 h after transfection, each group of transfected cells (1- by 100-mm dish) was passaged to 6- by 100-mm dishes in medium containing the selective antibiotic G418. Colonies were fixed and counted 15 to 20 days later, and the number of morphologically transformed colonies was expressed as a percentage of the total number of G418-resistant colonies.

RNA isolation and analysis. Total cytoplasmic RNA was isolated 48 h after transfection and subjected to Northern (RNA) blot analysis according to reference 34 with minor modifications. Total cytoplasmic RNA (10 to 15 μg) electrophoresed on a 1.1 M formaldehyde-agarose gel was hybridized in situ in the dried gel. Prehybridization, hybridization, and washing conditions were those described in reference 34.

RESULTS

We determined the transcription regulation and *ras* cooperation phenotypes of a series of E1a vectors which express wild-type or mutated forms of the 13S mRNA product (Fig. 1). The 13S mRNA product has an ability to repress enhancer-dependent transcription (3, 34) and to cooperate with the *ras* oncogene in transformation of rat embryo fibroblasts (36) comparable to that of the 12S mRNA product. In addition, it is the major transcriptional activator (15, 30). Because the mutations were studied in the 13S background, we were able to test the multiple biological functions of E1a with the same plasmid. The E1a mutations, which consist of small in-frame deletions and truncations, affect both regions of E1a which are highly conserved among adenovirus serotypes (10) and nonconserved regions.

No single domain is responsible for enhancer repression activity. To test the effects of deletions in the E1a gene on the enhancer repression function, we assayed the ability of the mutant E1a plasmids to repress expression from the recombinant PyE92-1 (5), a plasmid with which transcription of the rabbit β-globin gene is activated by the polyomavirus enhancer. Plasmids pSVE1a, pSVN20, and pSVF12, which express wild-type E1a proteins, efficiently decreased the β-globin mRNA level between 10- and 15-fold (Fig. 2). Mutations in domain 1 had mixed effects on enhancer repression. Deletions preceding or within domain 1 partially reduced the repression function, conferring on mutants pSVXL185 and pSVXL132 an intermediate repressor phenotype. Surprisingly, pSVXL105, with a deletion entering domain 1, had a wild-type phenotype, as we observed previously (34). This deletion was flanked by the mutations

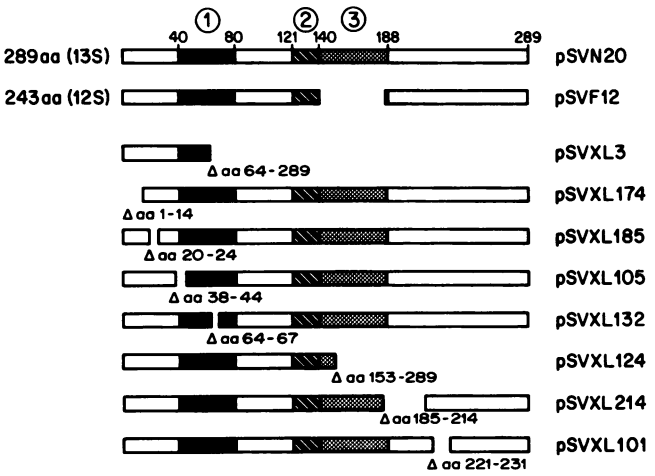

FIG. 1. Predicted E1a polypeptide structures encoded by mutant E1a vectors. The 289-amino-acid (aa) wild-type protein encoded by the 13S mRNA is represented at the top. The regions which are highly conserved among the adenovirus serotypes are boxed and indicated as domains 1, 2, and 3. Below are illustrated the predicted protein structures encoded by the 12S cDNA and the 13S cDNA E1a mutant vectors studied in this report. Also marked are the wild-type amino acid residues deleted in each mutant.

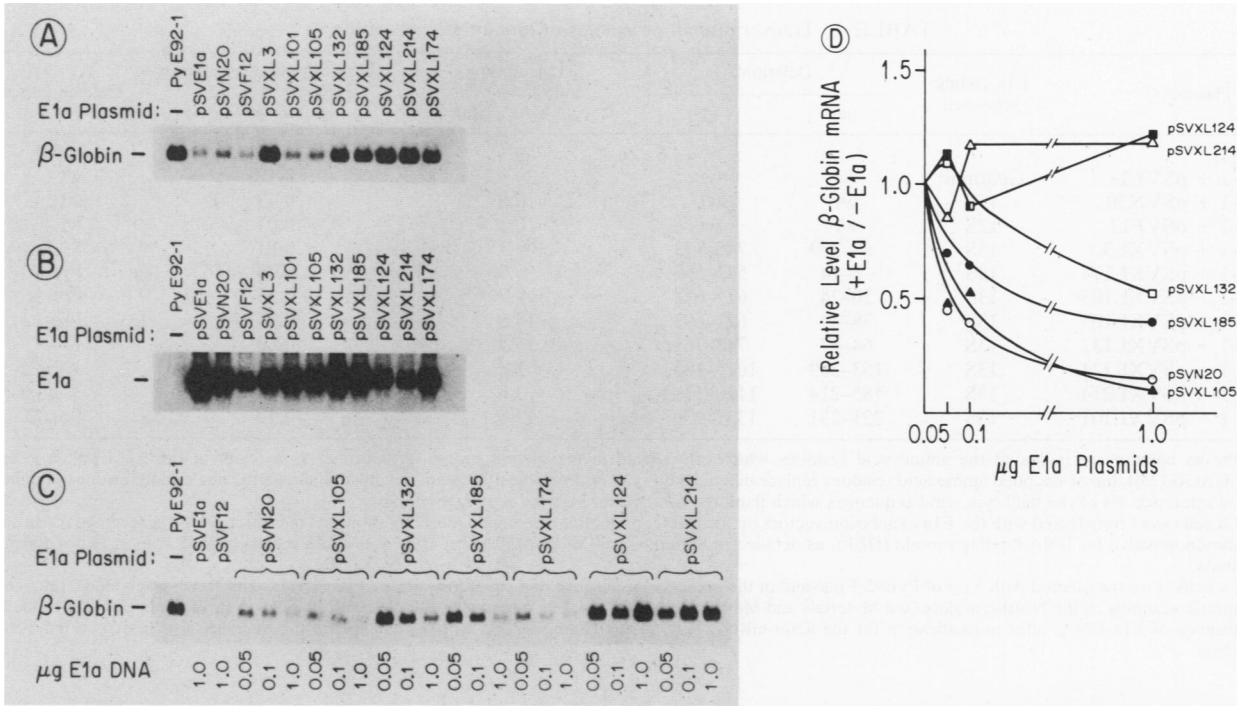

FIG. 2. Analysis of the repression activity of the mutant E1a plasmids. (A) Northern blot analysis of β-globin mRNA obtained from HeLa cells transfected with 5 μg of PyE92-1 plus or minus 5 μg of E1a plasmids. Total cytoplasmic RNA was analyzed as described in Materials and Methods. The β-globin mRNA was detected with a randomly primed labeled (6) *Pvu*II fragment isolated from PyE92-1. In the lane marked pSVXL174, a greater amount of mRNA was loaded, elevating the β-globin mRNA signal. For repression values obtained after normalization to the actin mRNA content in each sample, see Table 1. (B) Level of E1a mRNA expressed in HeLa cells transfected as in panel A. E1a mRNA was detected with a purified *Eco*RI-*Pst*I double-digested fragment isolated from pSVE1a. (C and D) Titration analyses of mutant E1a vectors with intermediate repression phenotypes. PyE92-1 (5 μg) was transfected with increasing amounts (0.05, 0.1, 1 μg) of the indicated E1a plasmids (pSVN20, pSVXL105, pSVXL132, pSVXL174, pSVXL124, pSVXL214). As controls, 1 μg of pSVE1a and pSVF12, respectively, were included in the transfection. (C) Northern blot analysis performed as described for panel A. (D) Repression activities of pSVN20, pSVXL105, pSVXL185, pSVXL132, pSVXL124, and pSVXL214 are expressed as the ratio of the level of β-globin mRNA in the presence of the indicated E1a plasmids to the level in their absence as a function of increasing amount of E1a DNA.

present in pSVXL185 and pSVXL132, which are partially deficient in repression. Inspection of the amino acid sequence showed that the pSVXL105 deletion overlaps a proline-rich region which may have an extended structure by virtue of its proline content. Thus, the sequence mutagenized in pSVXL105 may constitute a linker region joining two domains deleted by the mutations in pSVXL185 and pSVXL132. Mutants pSVXL124 and pSVXL214 which lack, respectively, the entirety of exon 2 or its beginning, were completely defective for repression (Fig. 2A). The phenotypes of these two mutants thus suggest that the amino-terminal portion of exon 2, which is common to both 13S and 12S mRNA products and conserved among adenovirus serotypes (10), contains a novel domain important for the E1a repression function. This region of the E1a protein could play a direct role in repression. Alternatively, the mutations in pSVXL124 and pSVXL214 could act indirectly and alter the protein conformation of other, neighboring functional residues so as to block the repressor activity. The second interpretation is consistent with the fact that mutations in other regions of the gene, as indicated by mutants pSVXL132 and pSVXL185, affect the repression function. Thus, repression appears to be sensitive to mutations at many positions within the gene (see Discussion).

The differences between the repression activities of the mutant E1a plasmids are not due simply to variations in E1a expression by these plasmids. E1a mRNA expression is shown to be similar for these plasmids in Fig. 2B. In fact, pSVXL124 and pSVXL214, which failed to repress (Table 1), synthesized levels of E1a mRNA, as quantitated by densitometric scanning, that were somewhat greater than that of pSVF12, which represses efficiently.

To analyze further the repressor activities of those mutants which show intermediate phenotypes, we performed a titration experiment in which repression was measured in the presence of increasing amounts of E1a plasmid DNA. As shown in Fig. 2C and quantitated by densitometric scanning in Fig. 2D, the control plasmid pSVN20 gives half of the maximal repression value with as low as 0.05 μg of plasmid DNA and maximal repression activity at 1 μg of DNA. The same behavior is observed for pSVXL105 and pSVXL174. Mutants pSVXL132 and pSVXL185, which displayed intermediate phenotypes at the maximal DNA concentration tested (5 μg, Table 1), showed no or very little activity at 0.05 μg of DNA and reached their maximal repression activity at 1 μg of DNA. Further increase of mutant DNA did not increase the repression activity (compare values in Table 1 with those of the graph in Fig. 2D), suggesting that the partial deficiency of the mutant protein cannot be compensated by increasing the amount of the protein in the cell. Furthermore, pSVXL124 and pSVXL214, which are defective for the repression function at 5 μg of plasmid DNA (Fig. 2A), failed to repress transcription from the plasmid PyE92-1 at any DNA concentration tested (Fig. 2C and 2D). These mutants also failed to repress plasmid pβGSV (5) in which β-globin expression is driven by the SV40 enhancer, even at

TABLE 1. Transcriptional properties of mutant E1a plasmids

Plasmid(s)	E1a coding sequence	Deletion[a]		Transcription transactivation % cells DBP positive[b]	Transcription repression relative level of β-globin mRNA[c]	Deleted domain(s)
		aa	nt			
PyE92-1				<0.1	1.00	
PyE92-1 + pSVE1a	Genomic	wt	wt	24.9	0.12	wt
PyE92-1 + pSVN20	13S	wt	wt	16.8	0.13	wt
PyE92-1 + pSVF12	12S	wt	wt	<0.1	0.07	wt
PyE92-1 + pSVXL3	13S	64–289	746–753	<0.1	0.81	1, 2, 3
PyE92-1 + pSVXL174	13S	1–14	542–567	6.9	0.24	Pre-1
PyE92-1 + pSVXL185	13S	20–24	617–632	9.7	0.55	Pre-1
PyE92-1 + pSVXL105	13S	38–44	671–692	18.6	0.26	Pre-1, 1
PyE92-1 + pSVXL132	13S	64–67	746–761	15.5	0.60	1
PyE92-1 + pSVXL124	13S	153–289	1015–1032	1.8	0.90	3, post-3
PyE92-1 + pSVXL214	13S	185–214	1110–1319	3.0	0.85	3, post-3
PyE92-1 + pSVXL101	13S	221–231	1337–1367	17.9	0.12	Post-3

[a] In the aa column are indicated the amino acid residues which are deleted in the mutant protein structures. As a result of the *Xho*I linker insertion (pCCTCGAGG [25]), linker-encoded amino acid residues replace deleted wild-type (wt) residues. In the nt column are shown the nucleotide residues (numbering system in reference 31) of the wild-type viral sequences which flank the *Xho*I linker inserted into each mutant.

[b] HeLa cells were transfected with the E1a expression vectors by the $CaPO_4$ precipitate method. Transactivation was measured as the percentage of HeLa cell nuclei immunopositive for DNA-binding protein (DBP), as detailed in Materials and Methods. The reported values are the average of at least two independent experiments.

[c] HeLa cells were transfected with 5 μg of PyE92-1 plasmid in the presence or absence of 5 μg of the various Ela vectors. The repression values, obtained by densitometric scanning of the Northern blots (see Materials and Methods) are expressed as the ratio of β-globin mRNA level in the presence of E1a to the level in the absence of E1a DNA, after normalization for the actin mRNA level in each sample. The reported values are the average of at least two independent experiments.

5 μg per assay (Fig. 3). Thus, both the polyomavirus and SV40 enhancers, the enhancers which were initially used to define the E1a repressor function (3, 34), are unaffected by these two mutants.

The failure of pSVXL124 and PSVXL214 to repress either the polyomavirus or the SV40 enhancers could possibly be due to instability of the mutant proteins or their failure to localize in the nucleus. To test these possibilities, we analyzed HeLa cells transfected by wild-type or mutant plasmids by indirect immunofluorescence for the level and localization of E1a protein. The wild-type 13S and 12S plasmids, pSVN20 and pSVF12, respectively, as well as the mutant pSVXL105 gave predominantly nuclear staining (Fig. 4A to D). The two mutants pSVXL124 and pSVXL214, which lack the repression function, produced similar levels of proteins which were also predominantly localized in the nucleus (Fig. 4E and F). With the mutants, however, some cytoplasmic staining, most intense adjacent to the nucleus, was also observed. These transfections were performed with 1 μg of vector plasmid per dish, a quantity which gave maximal repression by the wild-type vectors and no repression by the two mutants (Fig. 2D). Transfection of 5 μg of

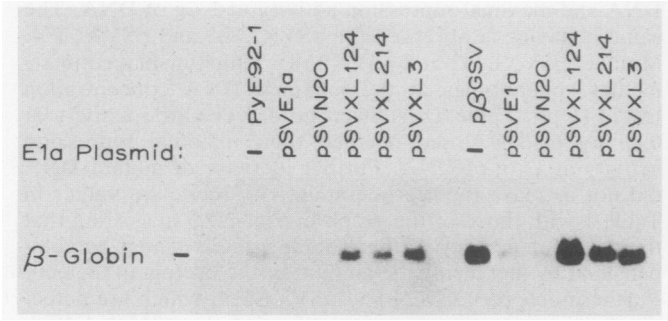

FIG. 3. Comparison of repression of polyomavirus and SV40 enhancers by mutant E1a plasmids. Shown is Northern blot analysis of β-globin mRNA obtained from HeLa cells transfected with 5 μg of the indicated E1a plasmids. Total cytoplasmic RNA was analyzed as described in the legend to Fig. 2.

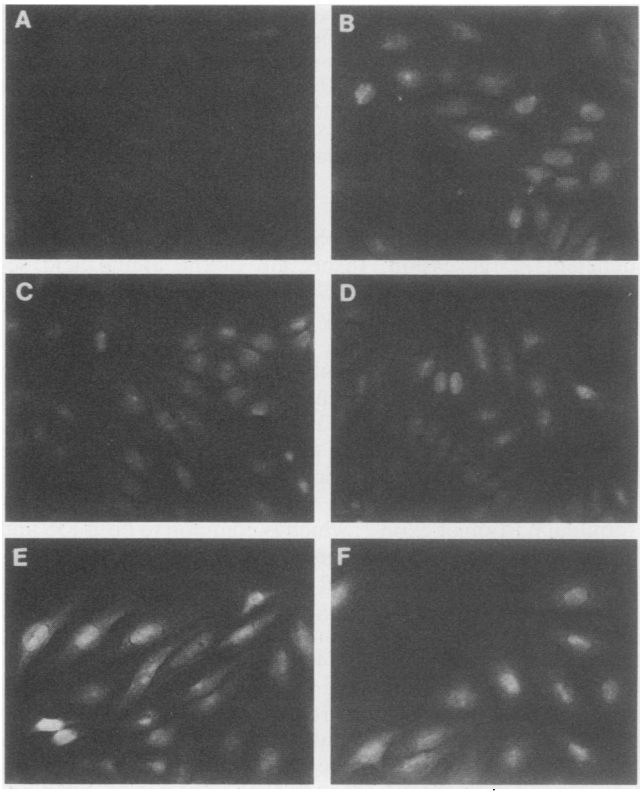

FIG. 4. Levels of E1a protein in transfected HeLa cells. Immunofluorescence micrographs are shown of HeLa cells transfected with plasmid PyE92-1 alone (A) or with this plasmid along with wild-type E1a plasmids pSVN20 and pSVF12 (B and C) or E1a mutant plasmids PSVXL105, pSVXL124, and pSVXL214 (D, E, and F). Cells were transfected, fixed, and stained as described in Materials and Methods.

plasmid vector, which was expected to induce yet higher levels of E1a protein than shown in Fig. 4, also failed to repress (Fig. 2A). These data taken together indicate that the inability of pSVXL124 and pSVXL214 to repress is not the result of the production of insufficient levels of E1a protein, a conclusion also indicated by the ability of these two mutants to cooperate with *ras* in transforming REF52 cells (see below).

***Ras* cooperation and enhancer repression are two distinct functions of E1a.** The mutant E1a plasmids were next assayed for their ability to cooperate with the activated *ras* oncogene to transform REF52 cells (7). This cell line resembles primary rodent cells in that it is not transformed by the *ras* oncogene alone but is transformed by *ras* in cooperation with E1a. Previous studies (36) have suggested that the E1a functions which collaborate with the *ras* oncogene are related to those which facilitate establishment of primary cells. Furthermore, in the *ras* cooperation assay the transforming activity of E1a can be assessed independently from the transactivation activity which is otherwise required, such as when transformation by E1a in cooperation with the viral E1b gene is measured. REF52 cells were cotransfected with an E1a plasmid and plasmid pT24neo (7), a T24 *ras-neo* plasmid. Cells were selected for G418 resistance, and surviving colonies were scored for morphological transformants. Several points are evident from the results of the transformation assay (Table 2).

Most significantly, two mutants, pSVXL124 and pSVXL 214, which were completely deficient for the repressor function (Fig. 2A, C, and D; Table 1), retained *ras* cooperation activity. Indeed, in this assay, the transformation efficiency of mutant pSVXL214 was equivalent to that of the parent wild-type 13S cDNA vector, pSVN20. pSVXL124 retained approximately one-half of this activity, a level comparable to that of other mutants (e.g., pSVXL105, -101) which retain an intact enhancer repression function. Thus, the phenotypes of pSVXL124 and pSVXL214 separate the repression and transformation functions of E1a. The fact that these mutants transform (Table 2) argues against the possibility that their lack of repression activity results from the synthesis of a very unstable E1a protein and is in agreement with the detection of mutant protein by immunofluorescence in Fig. 4.

Our data also suggest that the inverse of the above result is true, i.e., that mutants with impaired *ras* cooperation activity may still repress. The deletion in mutant pSVXL132, which overlaps amino acids 64 to 67, reduces the transformation activity to background levels (Table 2), although repression activity remains at an intermediate level, about half that of the wild type under saturating conditions of plasmid DNA transfection (Table 1; Fig. 2A). Likewise, mutant pSVXL174, which encodes a product that lacks the first 14 amino acids of E1a, had a substantially reduced transforming activity (Table 2) but repressed with an efficiency that approached wild-type levels (Table 1). Recently, Kuppuswamy and Chinnadurai (12) have reported other mutants with similar phenotypes. In addition to separating repression and *ras* cooperation, the phenotypes of pSVXL132 and pSVXL174 also imply a role for the predomain 1 and domain 1 sequences in the *ras* cooperation mechanism. Reports from other laboratories have previously demonstrated a role for domain 1 in transformation by E1a (14, 24).

TABLE 2. *ras* cooperation assay in REF52 cells[a]

Plasmid(s)	% morphological transformants[b]	% of control (pSVN20)
pT24neo (*ras*)	0.6 ± 0.4	2.6
pSVE1a + pT24neo	32.1 ± 12.3	133.8
pSVN20 + pT24neo	24.0 ± 6.8	100.0
pSVF12 + pT24neo	24.7 ± 5.7	102.9
pSVXL3 + pT24neo	0.4 ± 0.4	1.8
pSVXL174 + pT24neo	6.2 ± 3.4	25.8
pSVXL185 + pT24neo	15.0 ± 6.3	62.5
pSVXL105 + pT24neo	17.8 ± 5.5	74.2
pSVXL132 + pT24neo	2.1 ± 1.7	8.8
pSVXL124 + pT24neo	12.5 ± 4.4	52.1
pSVXL214 + pT24neo	23.5 ± 7.6	97.9
pSVXL101 + pT24neo	16.0 ± 4.6	66.7

[a] Different E1a plasmids (1 μg) were transfected with 1 μg of pT24neo according to reference 7 as detailed in Material and Methods.

[b] Cells were fixed and colonies were counted 15 to 20 days after transfection. The number of morphologically transformed colonies is expressed as the average percentage of the total number of G418-resistant colonies from four independent experiments with the standard error for each sample given.

Transactivation and transformation are independent functions. We next analyzed the abilities of the mutant E1a plasmids to transactivate the adenovirus type 5 early promoter in a transient complementation assay. In agreement with previous reports (13, 14, 17, 19, 24), our results (Table 1) showed that mutations outside domain 3 do not have a great influence on transactivation. Mutations within or preceding domain 1 (pSVXL174, -185, -105, -132) did not affect or only partially affected the ability of E1a to transactivate the E2a promoter. Conversely, the plasmids pSVF12, pSVXL3, and pSVXL124, in which domain 3 is partially or totally removed, lost the transactivation function. In mutant pSVXL214, the removal of the last residue of the domain unique to the 13S mRNA protein resulted in loss of the transactivation function, in agreement with previous reports (8, 13, 24). When these data are considered together with the results of the transformation assay (Table 2), it is evident that a mutant may loose either transactivation or transformation while retaining the other function. Thus, pSVXL132 transactivates without transforming, while pSVF12 (the wild-type 12S cDNA construct) transforms without transactivating (the latter in agreement with previous results from several laboratories [12–14, 17, 19, 24]).

DISCUSSION

We used deletion mutagenesis of the adenovirus type 5 E1a gene to examine the relationship between the *ras* cooperation and enhancer repression functions of E1a. This relationship is of interest since increasing evidence implicates viral and cellular oncogenes in control of transcription (11), which in turn raises the possibility that nuclear oncogenes bring about cell transformation through alterations of the expression of cellular regulatory genes. The E1a gene of adenovirus is particularly interesting because it exerts well-established positive and negative transcription regulatory functions (reviewed in references 1 and 2) and furthermore can cooperate with the *ras* oncogene in imparting full morphological cell transformation (23, 36). Both the E1a 243- and 289-amino-acid proteins are capable of repressing enhancer-dependent transcription, while only the 289-amino-acid 13S mRNA product exerts the transactivation function. Thus, the repression property more closely correlates with the transformation function, which is also provided by both E1a products. On this basis, we (33, 34) and others (13, 14, 24) have proposed that the enhancer repression function could provide the biochemical basis for *ras* cooperation and cell transformation by E1a.

Here we report the identification of a new class of E1a mutants which retain their ability to cooperate with *ras* in transformation of REF52 cells but lack the enhancer repression function. The phenotypes of two mutants, pSVXL124 and pSVXL214, show that an E1a gene which is incapable of enhancer repression can nonetheless cooperate with an activated *ras* gene with an efficiency approaching or equaling that of the wild-type gene. These results suggest that enhancer repression is not the basis for *ras* cooperation. Recently, Kuppuswamy and Chinnadurai (12) described a mutation in domain 2 of E1a which abolishes the transformation activity but does not alter either the repression or the transactivation functions. This mutant establishes a different but related feature of E1a: enhancer repression and transactivation are not sufficient for *ras* and E1b cooperation. In agreement, our mutants pSVXL174 and pSVXL132 from which, respectively, amino acid residues 1 to 14 in predomain 1 and 64 to 67 in domain 1 were deleted lost the transformation function although they retained a low but detectable repression activity (Tables 1 and 2; Fig. 2A, C, and D). In agreement with the results of Kuppuswamy and Chinnadurai (12), these mutants demonstrate that the repression activity is not sufficient for transformation. The present study further separates these E1a properties by demonstrating that the repressor function is not necessary for *ras* cooperation and suggests that repression does not play a direct role in the *ras* cooperation mechanism.

The mutations in pSVXL124 and pSVXL214, which lack the repression activity, affect domain 3 of E1a and exon 2 as well. Domain 3 is, however, not essential for repression as shown by the ability of the 12S mRNA product to repress enhancer activity at least as efficiently as the 13S mRNA product. Also, mutations in the unique region of the 289-amino-acid protein which abrogate transactivation leave unchanged the repression activity (13, 24). Furthermore, the integrity of exon 2 is not essential for repression as demonstrated by pSVXL101. Thus, the alterations in pSVXL124 and pSVXL214 do not identify a particular conserved domain which is sufficient for repression. The absence of a specific repressor domain is further strengthened by the fact that two other mutants which have a reduced ability to repress the polyomavirus enhancer have deletions which either precede domain 1 (pSVXL185) or map within domain 1 (pSVXL132). Furthermore, several mutants have been described with alterations in domain 2 which eliminate the enhancer repression function (12–14, 24). The repression function therefore seems to be sensitive to mutations scattered in the E1a protein and may not be contributed by a discrete domain of the protein. Indeed, the repression function may be particularly sensitive to alterations in the overall configuration of the protein as well as to mutation of specific functional residues. This interpretation is consistent with the findings of Schneider et al. (24) that a mutant which deletes amino acids 185 to 289 still maintains a residual enhancer repression function.

The apparent lack of a single domain which is responsible for repression is in contrast with the transactivation function of E1a, which depends almost exclusively upon domain 3 (8, 13, 14, 17, 19, 24). The transformation activity, too, seems to map to distinct conserved regions and requires intact domains 1 and 2 (12–14, 17, 19, 24). It has been suggested that the E1a protein is a mosaic structure in which specific domains function as distinct entities (14, 18). Recently, Lillie et al. (14) have reported that a peptide corresponding to the unique region of the 13S mRNA product plus the first three amino acids of exon 2 are sufficient to transactivate an early viral promoter. Domain 2 has been previously shown to be required for cooperation with *ras* (12, 13, 17–19, 24). It is possible that domain 1 and 2, which are both required for cell transformation, function in the cell as distinct entities on separate molecules. Thus, although the mosaic structure model seems to be consistent with the transactivation and possibly the transformation functions, it does not accommodate the mutational analysis for the repression activity presented here. Indeed, our data suggest that repression depends on the integrity of the overall protein configuration.

In addition to domain 1, the sequences which precede domain 1 may also function in transformation. This is indicated by the phenotype of pSVX174, which lacks residues 1 to 14 and has reduced *ras* cooperation activity. The amino terminus of the E1a protein is required for the association of E1a with a specific cellular protein(s) (Ed Harlow, personal communication). Were this association important for the transformation process, it would explain the reduced transforming efficiency of pSVXL174. We note, however, that the terminal residues are not required in a different transformation assay in which E1a cooperates with E1b to transform primary BRK cells (20).

In conclusion, the data of this report demonstrate for E1a the independence of *ras* cooperation from enhancer repression. When taken together with the previously established separation of transactivation from transformation (13, 14, 19, 24), also reported here, these data indicate that neither the positive nor the negative classical RNA polymerase II transcription regulatory functions provide the basis for *ras* cooperation by E1a. Our conclusions do not exclude that transactivation and repression activities may play a role in *ras* cooperation or transformation under certain circumstances when the E1a transforming functions are assayed in different systems. Indeed, altered transformed phenotypes are obtained when E1a mutants cooperate with the viral E1b gene (16, 22). Furthermore, mutations in the E1a gene which do not affect either the establishment or the *ras* cooperation function abolish the ability of E1a to cooperate with polyomavirus middle T antigen (36). Thus, E1a cooperation with different oncogenes may take place by different mechanisms. With these considerations in mind, it will be of interest to determine whether new properties of the E1a proteins in addition to transactivation and enhancer repression functions provide the basis for the mechanism of *ras* cooperation and transformation by E1a.

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