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The Amino-Terminal Region of the Adenovirus Serotype 5 Ela Protein Performs Two Separate Functions when Expressed in Primary Baby Rat Kidney Cells

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Adenovirus serotype 5 Ela proteins immortalize primary cells and in cooperation with products of a second oncogene, such as adenovirus serotype 5 Elb or EJ *ras*, produce full transformation. Ela also activates transcription of specific viral and cellular promoters, represses enhancer-dependent genes, and induces cellular DNA synthesis in quiescent cells. Comparison of different adenovirus serotypes has identified three conserved regions in the Ela protein sequence. We have analyzed Ela mutants with deletions-linker insertions in or preceding the first conserved region, region 1 (amino acids 40 through 77 of adenovirus serotype 5 Ela). Ela mutants which have in-frame deletions-substitutions in region 1 or pre-region 1 sequences were reconstructed into adenovirus to yield a total of 14 mutant viruses. All the mutant viruses showed wild-type growth in HeLa cells, confirming that region 1 is nonessential in these cells. However, we show that region 1 provides two distinct functions in infected primary rodent cells. One function is essential for induction of cell DNA synthesis, and the other is essential for focus formation. In addition, our results are consistent with a requirement for the DNA induction function in focus formation.

Protein products of the Ela transcription unit of adenovirus serotype 5 are expressed from the earliest stages of adenovirus infection and regulate a variety of viral and cellular processes. Ela proteins *trans*-activate viral early promoters, as well as promoters of some cellular genes, and repress transcription of enhancer-dependent viral and cellular genes. Additional properties of Ela can be observed when the virus infects rodent cells that are semipermissive or nonpermissive for viral growth. When quiescent cells are infected with adenovirus in the absence of exogenous growth factors, virus-encoded Ela proteins induce the synthesis of cellular DNA (1–3, 27, 28, 38). When integrated into rodent cells, viral sequences corresponding to the Ela and Elb transcription units can have a profound effect on cell growth properties and morphology. Although the exact changes induced by El products depend on the cell type under study, the changes can include loss of contact inhibition, growth to high saturation densities, growth in reduced serum, and transition to anchorage independence (8, 9, 15, 22, 23). Cells transformed by Ela and Elb form characteristic foci of small, densely packed, round or cuboidal cells (7). Primary cells transfected with the Ela gene alone can escape senescence and grow as immortal cell lines (13, 34, 36), but these cells are not fully transformed.

At early times of infection, 13S and 12S mRNAs, produced by differential splicing of a single primary Ela transcript, encode proteins of 289 and 243 amino acids, respectively. Both Ela proteins have identical amino and carboxy termini but differ by 46 internal amino acids unique to the larger protein. Only the 289-amino-acid Ela protein is capable of transcription *trans*-activation (11, 14, 17, 23–25, 31, 32, 34, 41, 44, 46). Comparison of amino acid sequences of Ela proteins from different strains reveals three regions which are highly conserved (16). The *trans*-activation function maps to the third of these domains, which consists of the 46 amino acids present exclusively in the 289-amino-acid protein plus 3 adjacent amino acids common to both the 289- and 243-amino-acid proteins (20).

Expression of both the 289- and 243-amino-acid proteins is required to obtain complete transformation, although each protein alone is sufficient to induce a partially transformed phenotype (11, 23, 25). This suggests that transformation by Ela relies on functions other than *trans*-activation. Conserved region 2, which is common to both the 289- and 243-amino-acid proteins, is required for cell immortalization and *ras* cooperation and provides functions required for induction of cell proliferation. The latter include a function necessary to induce cell DNA synthesis (21, 26) and a second function required for progression of the cells through mitosis (47).

We have investigated the functions of the most amino terminal of the conserved regions, region 1. Viruses with in-frame deletion-linker insertion mutations were assayed for growth in HeLa cells, induction of cell DNA synthesis in quiescent rodent cell lines and in primary baby rat kidney (BRK) cells, and induction of transformed foci in both established and primary cells. We demonstrate that sequences within and preceding region 1 of Ela provide two separate functions in primary BRK cells, one essential for induction of cell DNA synthesis and the other essential for focus formation. Our results suggest that the ability to induce cell DNA synthesis is also a prerequisite for transformation of primary cells by Ela. In contrast, pre-region 1 and region 1 sequences which have been deleted from our mutant Ela products are not essential for growth of the virus in HeLa cells.

MATERIALS AND METHODS

Plasmids, cell culture, and antisera. Plasmids were generously provided as follows: p28S from Norman Arnheim and

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E1a, E2, E4, L5, and VAI constructs in M13 from Bob Schneider. Cells and viruses were originally obtained as follows: *dl*312, *dl*309, *dl*347, and *dl*348 from Tom Shenk, cell line 293 from Bob Schneider, NIH 3T3 cells from P. D'Eustacio, and CREF cells from Paul Fisher. Monoclonal antibody to E1a, M73, was provided by Ed Harlow.

Cells were maintained in Dulbecco minimal essential medium (DMEM; GIBCO Laboratories) supplemented with antibiotics (100 μg of streptomycin and 100 μg of penicillin per ml; Sigma Chemical Co.) and either 5% fetal calf serum for HeLa, 293, CREF, and primary BRK cells or 10% calf serum for NIH 3T3 cells.

BRK cells were prepared as described previously (34) by digestion with 0.025% collagenase (Boehringer Mannheim Biochemicals) and 2.5% dispase (Boehringer) of kidneys from 2- to 6-day-old Fisher rats (Taconic Farms). Cells from each kidney were used to seed 10 to 20 6-cm-diameter dishes. In some experiments, cells were plated in or transferred to K1 medium (30), which consists of equal parts of F12 (GIBCO) and DMEM supplemented with hydrocortisone (40 ng/ml), insulin (5 μg/ml), prostaglandin E1 (250 ng/ml), selenium (10^{-8} M), transferrin (5 μg/ml), and triiodothyronine (4 ng/ml) (Sigma) and antibiotics as described above.

Vector construction. E1a mutants were constructed by deletion and linker insertion mutagenesis of pJN20 (37). This created the pXL series of plasmid vectors. The fragments released from these plasmids by *Eco*RI and *Pst*I were ligated to the *Eco*RI-*Pst*I fragment of pSVod to generate the pSVXL series of vectors. Additional mutants were constructed by ligating an *Eco*RI-to-*Xho*I fragment (encoding the 5′ end of E1a) from one pSVXL series plasmid to an *Xho*I-to-*Eco*RI fragment (encoding the 3′ end of E1a) from a second mutant vector. Each mutation was also inserted into a 12S cDNA vector by ligating a 916-nucleotide *Eco*RI-to-*Cla*I fragment from a mutant pXL plasmid to the corresponding sites in the pJF12 vector.

Viruses. Construction of the mutant viruses was by a strategy similar to that used by Stow (40). Plasmid vectors encoding mutant E1a from the pXL series, a 12S cDNA version of the pXL series, or the pSVXL series were linearized with *Eco*RI. *dl*309 DNA was cleaved with *Xba*I to generate a viral fragment extending from 3.8 to 100 map units. The DNA was further cut with *Cla*I, which cuts only at 2.5 map units to ensure that no intact parental viral DNA remained. The large *dl*309 fragment, extending from map units 3.8 to 100, was purified on a sucrose gradient and cotransfected with linearized E1a plasmid into cell line 293. Well-separated, individual plaques were picked and grown in cell line 293. Restriction digest analysis of the viral DNAs verified, for each virus, the presence of a novel *Xho*I site at the expected distance from the left end of the virus.

*dl*309 and *dl*348 were propagated in HeLa cells; all other viruses were propagated in cell line 293. To prepare viral stocks free from growth factors, viral lysates were banded twice in succession in CsCl, dialyzed extensively against 10 mM Tris hydrochloride (pH 8)–80 mM NaCl–2 mM $MgCl_2$ to remove the CsCl, and diluted with 4 volumes of 0.1% bovine serum albumin–10 mM Tris hydrochloride (pH 8)–80 mM NaCl–2 mM $MgCl_2$–50% glycerol. The concentration of viral particles was determined from the optical density at 260 nm (1.0 A_{260} unit = 1.0×10^{12} particles per ml). The concentration of infectious viral particles, PFU, was determined by titration on cell line 293. The particle-to-PFU ratio was approximately 30 for all of the viral stocks used.

Immunofluorescence assay. For the immunofluorescence assays of E1a protein, cells were fixed with 3.7% formaldehyde and permeabilized with Triton X-100 as described previously (10). Cells were incubated with M73 for 1 h at 37°C, washed three times with phosphate-buffered saline, incubated with fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin G for 1 h at 37°C, and washed three times with phosphate-buffered saline.

Immunoblotting. HeLa cells infected at 20 PFU per cell were harvested at 18 h postinfection (p.i.) by lysis in 250 mM NaCl–0.1% Nonidet P-40–1% aprotinin (Sigma)–50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7). Lysates were centrifuged for 15 min to remove insoluble material, and samples of 100 μg of total protein were analyzed on a 10% polyacrylamide gel (18). Proteins were electrophoretically transferred to nitrocellulose (Schleicher & Schuell, Inc.) for 16 h at 0.1 A with a Bio-Rad electroblot apparatus. The filter was soaked in 3% bovine serum albumin in TBST (150 mM NaCl, 0.05% Tween 20, 50 mM Tris hydrochloride [pH 7.5]) for at least 1 h to block remaining protein sites. The filter was incubated for 1 h with M73 supernatant, rinsed with TBST, and then incubated for 1 h with 10^6 cpm of ^{125}I-protein A (Dupont, NEN Research Products) and 3% bovine serum albumin in TBST. The filter was washed with three changes of TBST over 20 min and exposed to XAR-5 film.

Transformation assays. Transformation assays in CREF cells were carried out essentially as described previously (6). CREF cells were seeded at a density of 8×10^5 cells per 60-mm-diameter dish, after 1 day were infected with virus at 20 PFU per cell, and 3 to 6 h after infection were split 1:5 onto 10-cm-diameter dishes. Two days after infection and replating, cultures were shifted to DMEM which contained reduced (0.1 mM) Ca^{2+}, 2% fetal calf serum, 5% calf serum, plus antibiotics. Cells were refed with medium with low Ca^{2+} twice a week, and transformed foci were scored after 6 weeks. For focus formation assays, BRK cells were infected at 5 to 20 PFU per cell 2 days after plating onto 35-mm-diameter dishes and were refed twice a week. Foci were scored 3 to 4 weeks later.

Cell DNA synthesis. NIH 3T3 cells were plated at 1.4×10^5 cells per 35-mm-diameter dish and 3 days later, after the cells had reached confluence, the medium was changed to DMEM containing 0.2% calf serum, 10 mM HEPES, plus antibiotics. After 24 h in low serum, cells were infected at 100 or 200 PFU per cell. BRK cells were infected at 5 or 10 PFU per cell 2 days after plating in the indicated medium. Cultures were incubated with [^{3}H]thymidine (12 μCi; Dupont) per ml of medium. NIH 3T3 cells were labeled 24 to 48 h p.i.; BRK cells were labeled either 0 to 24, 24 to 48, or 48 to 72 h p.i. At the end of the labeling period, cells were washed three times with phosphate-buffered saline and exposed to emulsion (Kodak NTB2) for 3 days at 4°C. The autoradiograms were developed in D19 (Kodak) for 3 min.

RESULTS

To determine the functions of E1a provided by the amino-terminal region of the E1a protein, including the highly conserved sequences of region 1, we analyzed E1a mutants with in-frame deletions-substitutions within or preceding region 1. The E1a mutants which we have analyzed include several which were constructed previously (37), as well as additional in-frame region 1 mutants (see Materials and Methods) with more extensive deletions, 25 to 50 amino acid residues long, in the E1a coding sequences.

E1a mutants in plasmid form were reconstructed into viral chromosomes by standard methodologies (see Materials and

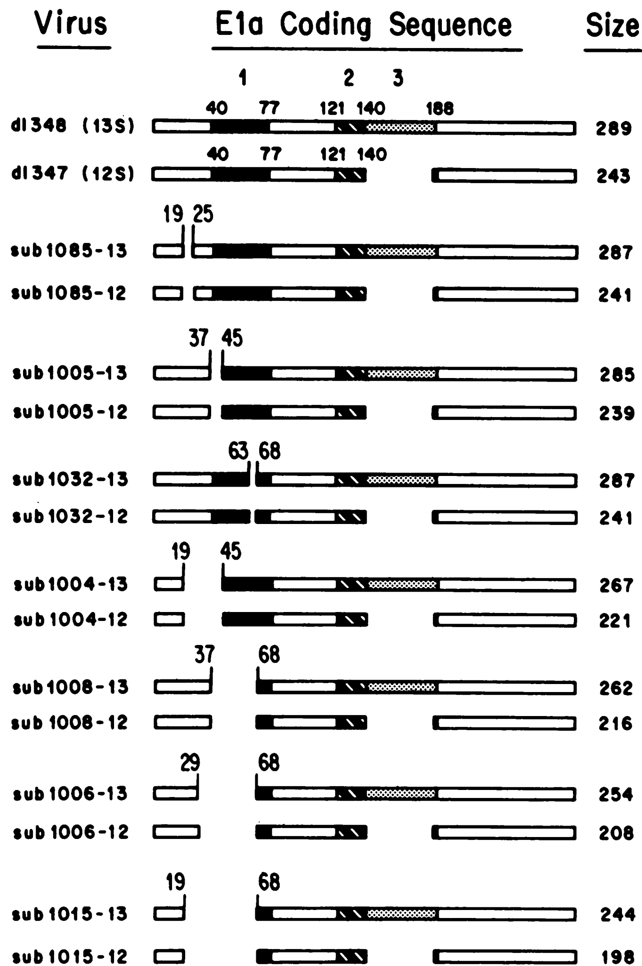

FIG. 1. Structures of the E1a proteins encoded by wild-type and mutant forms of adenovirus serotype 5. Coding sequences of the E1a proteins with regions of highly conserved sequence (■, ▒, ▧) are shown. Numbers immediately above sequences refer to amino acid residues within the sequence of the wild-type proteins and, for the mutants, indicate the last wild-type amino acid on either side of the deleted amino acid. The 2- to 3-amino-acid insertion encoded by the *Xho*I linker sequence is not shown. Size is given as total number of amino acid residues in the protein.

Methods). This yielded a series of mutant 13S cDNA viruses, with protein products diagrammed in Fig. 1. Figure 2 shows the actual amino acid sequences deleted from each mutant. The mutant *sub*1085-13 lacks five amino acids preceding region 1, including a leucine residue highly conserved among human and simian adenoviruses. All the other mutants affect region 1 directly. In most cases, the deleted sequences were replaced by the linker-encoded tripeptide, Ser-Ser-Arg, or, for *sub*1032-13, by Ser-Arg. Each mutation was also introduced into a 12S cDNA vector and reconstructed into virus to produce a series of mutant 12S cDNA viruses with E1a protein products also diagrammed in Fig. 1.

Infectious cycle of mutants in HeLa cells. The viruses were first assayed for growth in a cell line permissive for adenovirus infection, HeLa cells. *dl*309 and *dl*348, which encode wild-type or 13S E1a products, respectively, reached titers of 5×10^9 PFU/ml in HeLa cells by 48 h p.i. (Fig. 3A). This yield was an increase of 20,000-fold over the input virus. The 13S mutant viruses showed a similar increase in titer, indicating that the viruses were fully competent for growth in HeLa cells. Growth curves for the 12S viruses in HeLa cells are shown in Fig. 3B. In contrast to the 13S viruses, the wild-type 12S cDNA virus, *dl*347, showed little growth for at least 24 h and then reached a titer of 8×10^6 PFU/ml by 48 h p.i. Also, the viral yield for *dl*347 at 48 h p.i. was 1,000-fold lower than that for *dl*309 or *dl*348. The 12S mutant viruses had similar growth kinetics to the 12S wild-type virus, although they yielded even lower titers at 48 h p.i.

The wild-type growth rates of the 13S mutant viruses suggested that the mutants produced functional levels of E1a protein. To confirm this and to show that E1a protein was also produced by the 12S mutant viruses, infected Hela cells were analyzed by immunoblotting. Figure 4A, lane 2, shows wild-type E1a proteins from a *dl*309 infection. The lower two bands correspond to the 30,000-molecular-weight (30K) and 35K products of the 10S and 11S mRNAs, respectively (39), while the upper group of bands represent multiple protein species produced from the 12S and 13S mRNAs. *dl*348 gave rise to two distinct protein bands (lane 3). The 13S mutants (lanes 4 to 10) yielded levels of E1a protein comparable with that of the wild-type 13S virus. In addition, several of the mutants yielded a doublet of bands (see lanes 4 and 5 and lanes 9 and 10). However, three of the mutants, *sub*1006-13, *sub*1008-13, and *sub*1015-13, produced a single protein band. This loss of E1a protein heterogeneity was observed only with the three mutants that have deletions removing a major portion of region 1 (Fig. 2). We conclude that sequences responsible for producing the heterogeneity in the E1a protein reside between amino acids 45 and 63 of the wild-type 289-amino-acid protein. This region could either contain a site which receives a posttranslational modification or could direct the binding of a cellular protein responsible for modification of E1a at a distant site.

An immunoblot of the 12S viruses (Fig. 4B) shows that the 12S mutant viruses (lanes 2 through 8) gave equal or higher levels of E1a protein than the 12S wild-type virus (lane 1). In this experiment, we were not able to clearly resolve the wild-type or mutant forms of the 12S protein product into multiple species, so no conclusion can be reached concerning heterogeneity of the mutant proteins.

Stimulation of cell DNA synthesis in quiescent NIH 3T3 cells. Infection of HeLa cells by adenovirus leads rapidly to cell lysis and death. The stimulatory effects of the viruses on cell growth can be detected only in semipermissive rodent cells when viral replication is absent or delayed. We have found that the wild-type 12S cDNA virus, *dl*347, does not replicate in growth-arrested NIH 3T3 cells (H. Wong and E. Ziff, unpublished observation). We therefore measured the ability of the wild-type and mutant 12S cDNA viruses to induce cell DNA synthesis in NIH 3T3 cells that had been growth-arrested in medium with low serum (Table 1). After infection with *dl*312, which encodes no E1a products, virtually no stimulation of cell DNA synthesis was detected, as less than 0.1% of the nuclei incorporated labeled thymidine. After infection with *dl*347, 27% of the nuclei were labeled, a 500-fold stimulation above the level seen with *dl*312. Among the 12S mutants, *sub*1085-12 and *sub*1032-12 induced NIH 3T3 cell DNA synthesis at levels comparable with that of the wild type. However, all the mutants with more extensive deletions, *sub*1004-12, *sub*1006-12, *sub*1008-12, and *sub*1015-12, were unable to induce cell DNA synthesis above background levels. In addition, one small-deletion mutant, *sub*1005-12, had completely lost the DNA induction function. These results demonstrate that region 1 of E1a is required by adenovirus serotype 5 for stimulation of cell DNA synthesis in quiescent NIH 3T3 cells.

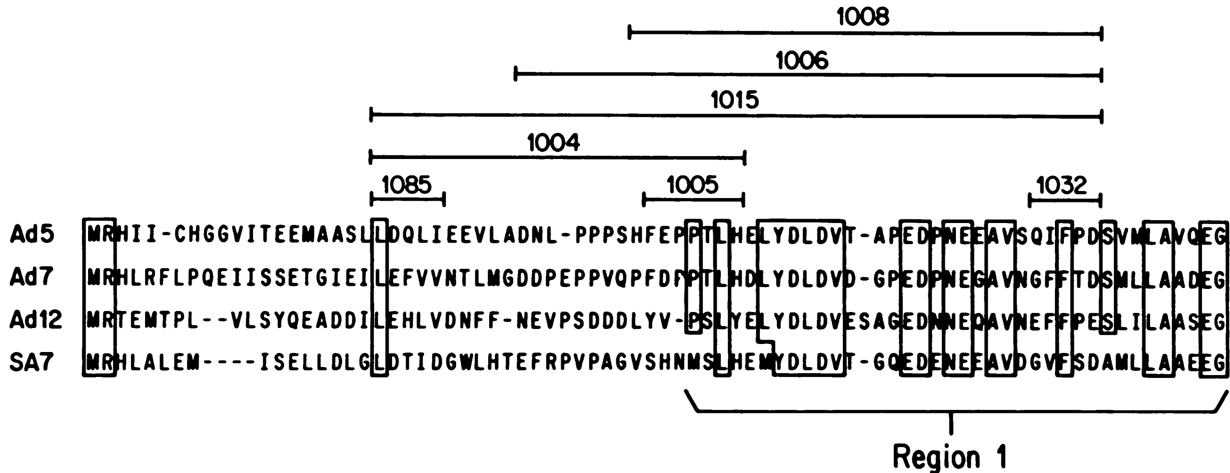

FIG. 2. Sequences deleted in the Ela mutant viruses. The N-terminal amino acid sequences of wild-type adenovirus serotype 5 (Ad5), adenovirus serotype 7 (Ad7), adenovirus serotype 12 (Ad12), and simian adenovirus 7 (SA7) Ela proteins are given in the single-letter code (16), with conserved sequences boxed. Bars at the top of the figure indicate sequences deleted in each of the Ela mutants (*sub*1008-13 indicated by 1008, etc).

Focus formation in CREF cells. Focus formation by the viruses was assayed in CREF cells, a cell line developed for its high efficiency of transformation by Ela (6). Infection of CREF cells with *dl*309 yielded an average of 56 foci per dish (Table 1). In agreement with previous reports (11, 23, 25), we found that the wild-type cDNA viruses produced foci which differ in both number and morphology from foci yielded by genomic wild-type virus. The foci produced by the 13S virus, *dl*348, were morphologically similar to foci produced by wild-type virus. However, the efficiency of focus formation by *dl*348 was only 15% that of *dl*309. It was clear, however, that the four mutants with large deletions, *sub*1004-13, *sub*1006-13, *sub*1008-13, and *sub*1015-13, did not transform CREF cells (data not shown). Because the transformation efficiency of the wild-type 13S virus was low, it was not possible to determine unambiguously whether certain other 13S mutants had reduced transforming abilities and we present only the results with the 12S cDNA viruses. The wild-type 12S virus consistently yielded greater numbers of foci than *dl*309 (Table 1), but the foci were smaller and the cells were less refractile than with wild-type virus (data not shown). As with the 13S mutants, the 12S versions of the large-deletion mutants did not induce focus formation on CREF cells. In addition, two of the small-deletion mutants, *sub*1085-12 and *sub*1005-12, did not yield foci. *sub*1032-12 was the only mutant among the 12S viruses which retained the ability to form foci with CREF cells.

These results suggest that region 1 is required both for

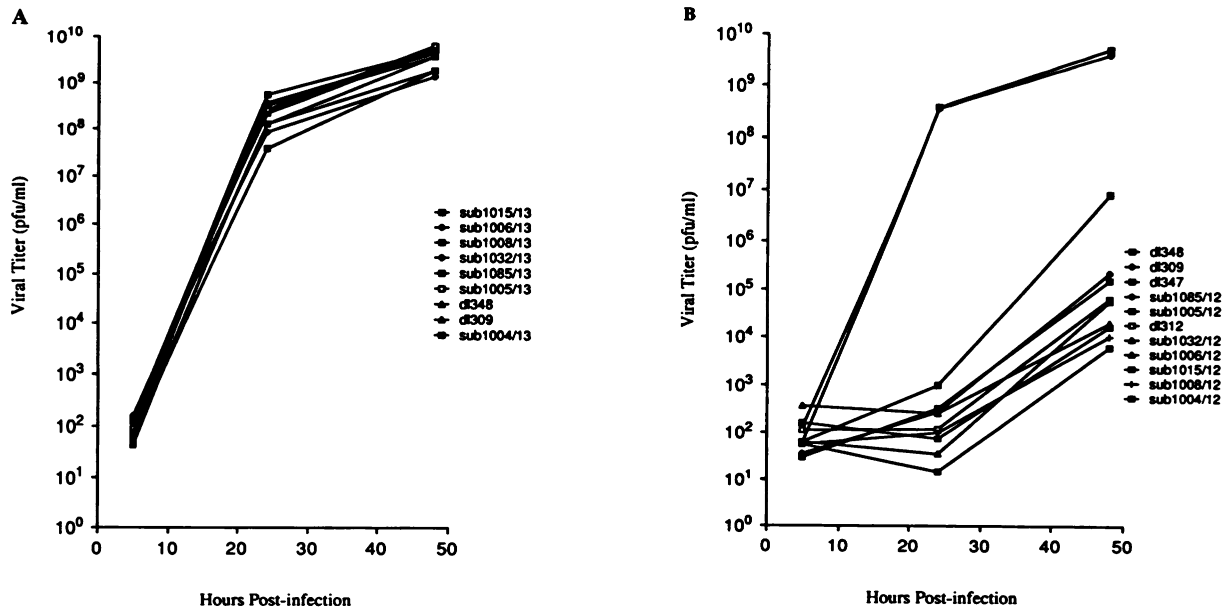

FIG. 3. Growth kinetics of mutant 13S and 12S viruses. The growth curves for mutant 13S (A) and 12S (B) viruses are shown. The growth curve for *dl*309, a phenotypically wild-type virus, is also shown for comparison. HeLa cells were infected at a multiplicity of 2 PFU per cell, and viral lysates were prepared at the indicated times and titers were determined on cell line 293.

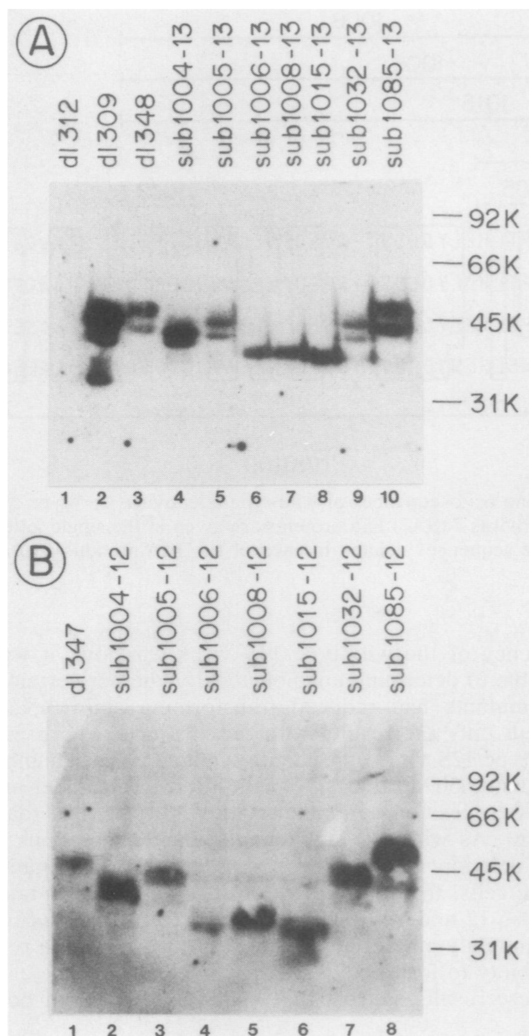

FIG. 4. Immunoblot analysis of Ela protein encoded by mutant 13S and 12S viruses. HeLa cells were infected with either mutant 13S (A) or mutant 12S (B) viruses. Protein extracts prepared at 18 h p.i. were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% gels), transferred to nitrocellulose, and probed with M73, a monoclonal antibody which recognizes an epitope encoded by the second exon of the Ela transcription unit (12). The antibodies were localized by reacting with ^{125}I-labeled protein A. Molecular weight (in thousands [K]) is shown to the right of the gel.

induction of cell DNA and for focus formation. To establish whether these two functions are linked, we assayed both functions in a single cell type, primary BRK cells.

Induction of cell DNA synthesis and focus formation in BRK cells. Primary cultures of BRK cells consist of a mixture of fibroblasts and epithelial cells. They can be maintained in DMEM with 5% fetal calf serum, which supports the growth of the fibroblasts, or in a serum-free, defined medium, K1, which is inhibitory to fibroblast growth. In either medium, the epithelial cell population does not survive beyond 3 to 4 days. Infection of primary BRK cells with a 12S virus, however, can induce epithelial cell DNA synthesis within 12 h p.i. and yield foci of immortalized cells after 3 to 4 weeks (15, 25, 30). Only the 12S viruses can be assayed in this system because BRK cells are permissive for growth of the 13S and wild-type viruses (25, 30).

TABLE 1. Viral functions assayed in established cell lines

Virus	Deletion[a]	NIH 3T3 cell DNA synthesis[b]	CREF cell foci[c]
*dl*312	All of Ela	<0.1 (12)	0.5 (12)
*dl*309 (genomic wild type)			56 (12)
*dl*347 (wild-type 12S)		27 (7)	79 (12)
*sub*1085-12	20–24	26 (5)	0.4 (7)
*sub*1005-12	38–44	<0.1 (6)	0.1 (8)
*sub*1032-12	64–67	65 (5)	110 (8)
*sub*1004-12	20–44	<0.1 (6)	0.2 (7)
*sub*1006-12	30–67	<0.1 (4)	0.5 (8)
*sub*1008-12	38–67	<0.1 (6)	0.5 (8)
*sub*1015-12	20–67	<0.1 (4)	0.3 (8)

[a] Amino acid residues deleted from the mutant Ela protein.

[b] Given as percent positive nuclei. Quiescent NIH 3T3 cells were infected at 100 PFU per cell and labeled with [^{3}H]thymidine from 24 to 48 h p.i. The total number of dishes scored is indicated in parentheses. Between 400 and 2,000 cells were counted for each dish. For dishes with less than 1% positive cells, all of the positive nuclei on the dish were counted and divided by the total number of cells on the dish.

[c] Average number of foci per 10-cm-diameter dish. CREF cells were infected at 20 PFU per cell as described in Materials and Methods, and 6 weeks later, foci were scored. The total number of dishes counted is indicated in parentheses.

Table 2 shows the results of an assay of the induction of cell DNA synthesis following virus infection of BRK cells. Virus *dl*347 induced substantial levels of cell DNA synthesis compared with *dl*312 or mock-infected cells. The three mutant viruses with small deletions, *sub*1085-12, *sub*1005-12, and *sub*1032-12, were comparable with the wild type in their ability to stimulate BRK cell DNA synthesis, while all of the mutants with large deletions of region 1 were entirely inactive. This pattern of induction was established by 24 h p.i. and maintained for at least 3 days after infection for all the viruses (data not shown). In addition, the same result was found for cells grown in either DMEM or K1 medium.

At 3 to 4 weeks p.i., infected BRK cell cultures were scored for focus formation. *dl*347 efficiently induced foci in BRK cell cultures (Table 2). Region 1 mutants with small deletions, i.e., *sub*1085-12, *sub*1005-12, and *sub*1032-12, as

TABLE 2. Viral functions assayed in primary BRK cells

Virus	Deletion[a]	Cell DNA synthesis[b]		Foci[c]	
		DMEM[d]	K1[e]	DMEM	K1
Mock		3 (2)	12 (2)	0 (18)	ND[f]
*dl*312	All of Ela	0.7 (3)	9 (3)	0 (23)	0.1 (14)
*dl*347 (wild-type 12S)		78 (3)	60 (3)	27 (23)	20 (13)
*sub*1085-12	20–24	30 (3)	47 (2)	0 (23)	0 (25)
*sub*1005-12	38–44	21 (3)	44 (3)	0.5 (23)	0.1 (13)
*sub*1032-12	64–67	47 (3)	58 (3)	0 (23)	0 (25)
*sub*1008-12	38–67	2 (3)	10 (3)	0 (23)	0 (25)

[a] Amino acid residues deleted from the mutant Ela protein.

[b] Given as percent positive nuclei. Cells were infected at 5 to 10 PFU per cell and then labeled with [^{3}H]thymidine from 24 to 48 h p.i. The total number of dishes scored is indicated in parentheses. Between 600 and 1,500 cells were counted on each dish.

[c] Average number of foci per 6-cm-diameter dish. Cells were infected with between 5 and 20 PFU per cell, and 3 to 4 weeks later, foci were scored. The total number of dishes counted is indicated in parentheses.

[d] Cells were plated and maintained in DMEM with 5% fetal calf serum (see Materials and Methods).

[e] Cells were plated and maintained in K1, a defined medium, in the absence of serum (see Materials and Methods).

[f] ND, Not determined.

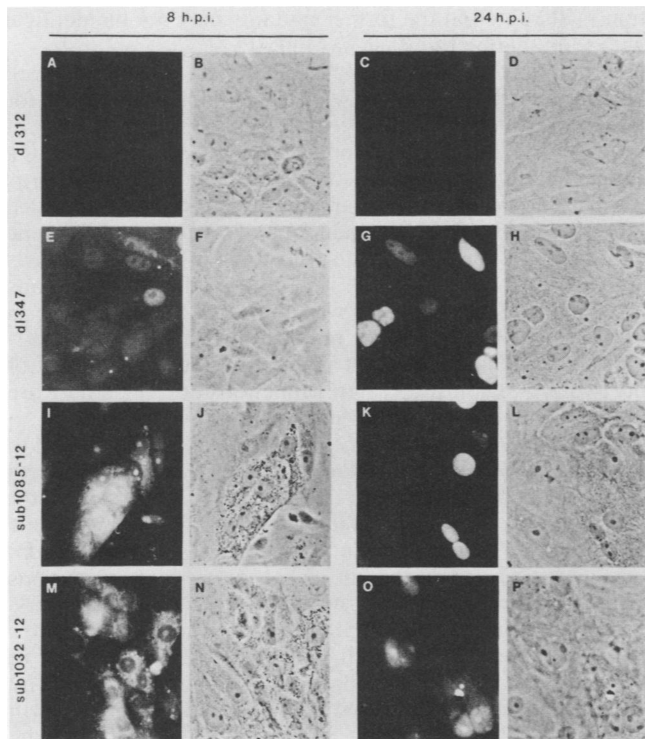

FIG. 5. Localization of E1a protein in infected BRK cells. Phase-contrast and immunofluorescence micrographs of BRK cells 8 or 24 h p.i. (h.p.i.) with wild-type or mutant 12S cDNA viruses or *dl*312 are shown. Phase-contrast micrographs (first and third columns from the left) and the corresponding immunofluorescence micrographs (second and fourth columns) of given fields are paired.

well as a mutant with a large deletion, *sub*1008-12, were all defective for focus formation in these primary cells. The other three large-deletion mutants were not assayed for focus formation in BRK cells.

These results indicate that, in primary rodent cells as well as in established cell lines, both induction of cell DNA synthesis and focus formation require the amino-terminal sequences of E1a. However, we find that in BRK cells the DNA induction and focus formation functions are not tightly linked, suggesting that two distinct functions are provided by the amino-terminal region of E1a. This conclusion is based on the observation that the three small-deletion mutants are incapable of focus formation but still retain the ability to induce cell DNA synthesis.

Localization of mutant E1a protein in infected BRK cells. As wild-type E1a proteins are primarily nuclear (4, 33, 35, 45), one possible explanation for loss of function by the E1a mutants would be failure of the mutant proteins to translocate to the nucleus. To examine this, infected BRK cells were assayed for localization of E1a protein by indirect immunofluorescence. Wild-type 12S virus, *dl*347, showed a predominantly nuclear fluorescence with, at most, faint diffuse staining of the cytoplasm (Fig. 5E through H). This agrees with previous reports of E1a localization (4, 33, 35, 45). All of the mutant 12S E1a products accumulated in the nucleus even more slowly than the wild-type protein. At 8 h p.i., cells infected with mutant virus gave strong cytoplasmic fluorescence with little, if any, staining of the nucleus outside of the nucleolus (Fig. 5I, J, M, and N; data not shown). This pattern was followed by the gradual appearance of E1a in the nucleus. By 24 h p.i., the majority of E1a protein was localized in the nucleus (Fig. 5K, L, O, and P; data not shown). The slowest nuclear accumulation was observed for *sub*1032-12, which also yielded the faintest nuclear fluorescence at 24 h p.i. Nevertheless, this virus was able to stimulate cell DNA synthesis by 24 h p.i. (see above). Taken together, these results suggest that loss of function observed for mutants other than *sub*1032-12 is not due to insufficient levels of E1a protein in the nucleus.

DISCUSSION

We have investigated the functions of the amino-terminal 70 residues of the E1a proteins. This segment contains most of region 1, a peptide sequence of nearly 40 amino acids, whose structure is highly conserved among E1a proteins from different adenovirus serotypes. We have also investigated the function of the less highly conserved residues which precede region 1.

The major conclusion of our study is that the amino-terminal segment of E1a contributes two distinct functions when expressed in primary BRK cells. This conclusion is drawn from the phenotypes of two groups of viruses which have mutations in region 1 and pre-region 1 sequences of E1a. Only the 12S versions of these mutants are studied in BRK cells, as 13S and wild-type viruses are cytopathic for these cells.

One group of viruses, *sub*1004-12, *sub*1006-12, *sub*1008-12, and *sub*1015-12, consists of mutants with large deletions of 25 to 50 amino acid residues, within or preceding region 1. These mutants also have linker-encoded insertions of two to three amino acids at the same site. The large-deletion mutants are completely incapable of either inducing cell DNA synthesis or producing foci on BRK cells. (Focus formation was assayed only for *sub*1008-12.) This class of mutants is similar to other previously described region 1 mutants (20, 26) that are defective for both induction of cell DNA synthesis and focus formation. In other studies (21, 36, 39, 42), it was also reported that plasmids or viruses expressing mutant E1a proteins with large deletions in region 1 fail to produce foci, but induction of cell DNA synthesis was not assayed.

The second group of mutants, which has smaller deletions of four to seven residues (again with linker substitutions), exhibits a novel phenotype for E1a region 1 mutants. This group consists of *sub*1085-12, *sub*1005-12, and *sub*1032-12. Each of these viruses induces BRK cell DNA synthesis with near-wild-type efficiency, yet is incapable of focus formation in BRK cells. The phenotype of this second group of mutants establishes that mutations in the amino-terminal region of E1a which leave intact the ability to induce cell DNA synthesis may still impair focus formation.

We conclude from these results that the region 1 and pre-region 1 sequences of E1a provide two distinct functions in BRK cells. One function, which is lost by both the first group of mutants (those with large deletions) and the second group of mutants (those with small deletions), is strongly required for focus formation in BRK cells but is dispensable for induction of cell DNA synthesis. The second function, lost by the large-deletion mutants but retained by the small-deletion mutants, is essential for induction of cell DNA synthesis in BRK cells. Since no case was found in which a virus could form foci without the capacity to induce cell DNA synthesis, this DNA induction function may also be required for BRK cell focus formation. We have also assayed the phenotypes of the mutants in established rodent cell lines. It was anticipated that assay in established cells,

rather than primary cells, might lead to differences in the viral phenotypes. Furthermore, in the BRK cell experiments, a single preparation of primary cells was used to assay both induction of cell DNA synthesis and focus formation. In contrast, the assays in established cells employed two separate cell lines. NIH 3T3 cells, growth-arrested in medium with low serum, were used to assay DNA induction, and CREF cells were used to assay focus formation. We expected that the use of two different cell lines might also influence the viral phenotypes. Nonetheless, the pattern of phenotypes found in BRK cells was exactly repeated in the cell lines for the 12S versions of the large-deletion mutants. Both the cell DNA induction and focus formation functions were lost. One 12S mutant with a small deletion, *sub*1085-12, also maintained the same phenotype as in BRK cells. The phenotypes in the cell lines of the two remaining small-deletion mutants varied from the BRK results. Mutant *sub*1005-12 was deficient in both cell DNA induction and focus formation, while mutant *sub*1032-12 was active for both functions. Thus, for both of these E1a mutants, the two region 1 functions cannot be resolved in the cell lines we have used. Interestingly, *sub*1032-12, which was completely incapable of focus formation in BRK cells, produced foci on CREF cells as efficiently as *dl*347, the wild-type 12S virus. This phenotype raises the possibility that the mutant lacks a function that is required for focus formation in the primary BRK cells but is dispensable in the cell line. It is possible that the viral mutation is complemented by a cellular function expressed in the established line of CREF cells. In no case was a mutant found to be defective for cell DNA induction but capable of focus formation. This is consistent with the suggestion (see above) that the DNA function is required for focus formation.

Lillie et al. (20) have described four point mutations within region 1 of E1a which bracket the mutation in *sub*1032-12. Unlike *sub*1032-12, which is able to induce cell DNA synthesis at wild-type levels, the mutant E1a proteins of Lillie et al. were two- to threefold less efficient than the wild type for the DNA induction function. This difference may be due to the different cell types used in the assays or may reflect the fact that individual amino acids within a functional domain may or may not be essential for a given function.

A clue to the identity of the amino acids from the terminal sequences of E1a which are essential for the focus formation function is provided by a recent study of human papillomavirus type 16 (HPV-16) by Phelps et al. (29). These researchers found that HPV-16 has a transforming function which, like E1a, can cooperate with activated *ras* to induce foci in BRK cells. The HPV-16 transforming function is encoded by the E7 gene, which has two short stretches of sequence similarity to E1a, one of which overlaps conserved region 1 of E1a. The sequence similarity between E7 and E1a extends from amino acid 37 to 49 of E1a and thus includes the seven amino acids deleted from mutant *sub*1005-12. We have shown that mutant *sub*1005-12 fails to induce focus formation in BRK cells. It is interesting to speculate that the amino acids deleted from *sub*1005-12 and the corresponding residues of the HPV-16 E7 protein are directly involved in providing the focus formation or *ras* cooperation function. The deletions in *sub*1085-12 and *sub*1032-12 do not overlap the deletion in *sub*1005, but these viruses have a similar phenotype to that of *sub*1005-12 in BRK cells. Perhaps the former two deletions indirectly affect the function provided by the residues conserved in E7. However, because *sub*1085-12 and *sub*1032-12 each have a phenotype in established cell lines distinctive from *sub*1005-12, the E1a function(s) disrupted in the former mutants may not be identical with the function(s) lost in *sub*1005-12.

An implication of our study is that the ability to induce cell DNA synthesis is a necessary but not sufficient condition for BRK cell transformation by the virus. Previous studies of the *ras* T24 bladder carcinoma oncogene suggest that a similar situation exists with *ras*. Microinjection of active *ras* protein into quiescent primary rat kidney cells induces cell DNA synthesis (5), yet introduction of an activated *ras* gene on its own is not sufficient to transform primary BRK cells (19, 34). In this connection, it is noteworthy that functions provided by E1a cooperate with activated *ras* to transform primary cells and that some region 1 mutants fail to cooperate (20, 26, 43). It will be of interest to determine which of the two pre-region 1 or region 1 functions described here are necessary for *ras* cooperation.

The ability of E1a to induce cellular DNA synthesis and to transform cells is reminiscent of the actions of growth factors. Binding of a growth factor to a cell surface receptor can trigger multiple intracellular events, which may include activation of protein kinase C, tyrosine phosphorylation, Ca^{2+} release, and induction of the expression of various cellular proteins, such as c-*fos*, actin, *myc*, p53, and others. Transformation of cells by E1a greatly reduces or eliminates the requirements for growth factors in the proliferation of cells. Thus, it is likely that some of the functions provided by the E1a proteins are related to those induced by growth factors and other mitogens. Since growth factors stimulate multiple cellular pathways, it is possible that E1a also provides multiple functions to reproduce growth factor action. The existence of multiple E1a activities which stimulate growth is supported by the identification of two such functions in the present study and also in previous reports. For example, Zerler et al. (47) have characterized a region 2 E1a mutant which fails to induce foci in BRK cells as a consequence of a failure of the cells to progress through G_2 into mitosis. It seems unlikely that the region 1 function, described in the present report, that is lacking in the small-deletion viruses and required for BRK focus formation is the same as the region 2-dependent function of Zerler et al. (47) required for induction of mitosis. This conclusion is based on our observation that in contrast to the region 2 mutants, the small-deletion viruses were able to induce proliferation of BRK cells, detected by increases in total cell numbers during the period of 3 to 6 days following infection (data not shown). Thus, it appears that E1a provides at least three functions required for cell proliferation and transformation, the two functions described here and a function provided exclusively by region 2. In contrast, the *trans*-activation function provided by region 3 appears to be unrelated to cell proliferation, in that the 12S cDNA constructs efficiently induce cell DNA synthesis and focus formation although they lack region 3.

Although the functions provided by the amino-terminal 70 amino acids of E1a are essential for cell DNA induction and focus formation, they are not necessary for viral growth in HeLa cells so long as region 3 is intact. All of the 13S mutant viruses displayed wild-type growth curves in infections of HeLa cells (Fig. 3). Consistent with this, the 13S mutants also activate normal or near-normal levels of transcription from viral promoters (D. Smith, unpublished data). This suggests that the mutations in region 1 do not indirectly interfere with the proper functioning of other regions of the protein, i.e., region 3. However, none of our mutations deletes the carboxy-terminal third of region 1. Recently, viruses encoding variants of E1a which lack all of region 1

were found to retain the *trans*-activation function but to be defective for growth in permissive cells (26, 39, 42). The reason for this difference with the results of the present report is not known but suggests even greater loss of function for E1a when all of region 1 is absent.

Both the 289- and 243-amino-acid E1a proteins localize in the nucleus of an infected cell (4, 33, 35, 45). We considered the possibility that the loss of DNA induction and focus formation functions by the large-deletion mutant 12S viruses in BRK cells was the result of a failure of the mutant E1a proteins to localize to the nucleus. However, indirect immunofluorescence experiments showed that all the 12S mutant-encoded E1a proteins were detectable at normal levels within the nucleus of BRK cells by 24 h p.i. (Fig. 5; data not shown). It is of interest, however, that all of these mutant E1a proteins exhibited a significantly lower rate of nuclear accumulation than did the wild-type 243-amino-acid protein. Very little mutant E1a protein was detected in the nucleus at 8 h p.i., whereas the majority of the wild-type 243-amino-acid protein was nuclear at this time. The basis for this difference is not evident.

Another question raised by our results is the nature of the mechanism by which pre-region 1 or region 1 sequences, which encompass one highly conserved protein domain, provide two separate functions. In this connection, our finding that the loss of amino acid residues 45 through 63, within region 1, reduced the heterogeneity of the E1a protein is intriguing. The loss of heterogeneity suggests that region 1 sequences may be required, directly or indirectly, for modification of a subset of the E1a protein in the cell. Conceivably, multiple forms of the E1a protein, differing in extent or type of posttranslational modification, could carry out the different functions, identified here, which rely on region 1. Another possibility is that the amino-terminal sequences of E1a bind two or more cellular factors which could mediate different E1a functions. In this regard, it is significant that two distinct cellular proteins of 300K and 105K are found to bind to the amino-terminal region of E1a (Peter Whyte and Ed Harlow, personal communication). These two possibilities, protein heterogeneity and a multiplicity of cellular E1a-binding proteins, are not mutually exclusive means by which E1a could provide multiple functions. Further studies will be necessary to test these possibilities.

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