Lytic and Transforming Functions of Individual Products of the Adenovirus E1A Gene

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To distinguish the individual roles of the 13S, 12S, and 9S adenovirus E1A gene products, we isolated the corresponding cDNA clones and recombined them into both plasmids and viruses. Only the expected E1A mRNA products were made from the corresponding 12S and 13S viruses. The 9S mRNA was detected when the 9S virus was coinfecting with the 13S virus but not when either virus was infected alone. The 13S virus formed plaques equally well in 293 cells, HeLa cells, and A549 cells, a human lung oat cell carcinoma line. Plaque titers of the 12S virus were much reduced in HeLa and A549 cells compared with 293 cells, although the 12S virus is multiplicity-dependent leaky in both HeLa and A549 cells. A549 cells were significantly more permissive than HeLa cells for growth of the 12S virus. In A549 cells even at low multiplicities of infection the final yield of 12S virus eventually approached the maximum yield from 293 cells. Expression from the adenovirus early region 2 and early region 3 promoters in HeLa cells was activated in the presence of a 13S cDNA E1A region but not in the presence of a 12S E1A cDNA region. Although defective for lytic growth in HeLa cells, the 12S virus immortalized BRK cells at very high efficiency, whereas infection of these cells with 13S virus, as with wild-type E1A virus, resulted mainly in cell death. The 13S product does have an immortalization function, however, revealed in the absence of adenovirus lytic functions when a plasmid containing the E1A 13S cDNA region was transfected into BRK cells. The 9S virus failed to immortalize infected BRK cells or to interfere with focus formation when coinfecting with the 12S virus.

The E1A gene of adenovirus types 2 and 5 (Ad2 and Ad5) is capable of producing a partially transformed phenotype in rat cells (26, 44) and together with the E1B gene is sufficient for complete transformation of rodent cells (8, 17, 18, 35, 47, 60). In addition to the transformation of nonpermissive or semipermissive rodent cells, E1A products are involved in the regulation of expression of other adenovirus gene products during productive infection in human cells. In the absence of E1A products, expression of other early genes and transcription from the major late promoter is reduced (4, 29, 33, 39, 63). E1A gene products can also regulate the expression of various other eucaryotic promoters (15, 21, 38, 52, 54, 58).

At early times after infection, two E1A mRNAs are produced (5, 9, 31, 41). The 12S and 13S E1A messages (Fig. 1) are predicted to encode proteins 243 and 289 amino acids long, respectively, although the apparent sizes of the E1A proteins on sodium dodecyl sulfate-polyacrylamide gels are larger than expected from the amino acid length of the proteins (10, 12, 46). The predicted 12S and 13S proteins differ only by the presence of an additional 46 amino acids at positions 140 through 185 in the 13S product. Later in infection a 9S E1A mRNA accumulates (9, 48). The protein encoded by the 9S mRNA sequence (62) is predicted to contain the same 26 amino-terminal amino acids as the 12S and 13S products; however, the second exon of the 9S mRNA is in a different frame from that shared by the 12S and 13S mRNAs, so the carboxy-terminal 29 amino acids of the 9S product are unique to the 9S polypeptide.

The relationship between the various E1A products and the known biological activities of the E1A region has been the subject of much recent investigation. The properties of an E1A mutant, Ad5hr-1 (24), suggested that the 13S product

is required for productive infection. This mutant, which produces a normal 12S product and a mutant 13S product (12, 42), is defective for growth in HeLa cells and cold sensitive for transformation in rodent cells (2, 25), but it is more efficient than wild type for transformation at 37°C (2, 17, 25). These studies did not determine whether the 12S product contributes to productive infection or where the transformation function of E1A resides. Use of various other E1A mutants has confirmed that it is the 13S product which stimulates early transcription in a lytic infection (7, 12, 17, 37, 42).

Studies with adenoviruses containing E1A mutations which selectively destroy splice sites showed that a virus that only produces a 13S mRNA (37) is as competent as wild-type virus for productive infection in HeLa cells, although its yield is reduced compared with wild-type virus in growth-arrested human cells (36, 50). Two splice-site mutant viruses that produce only 12S mRNA (23, 36) are defective for growth in HeLa cells and fail to stimulate expression from other adenovirus early promoters. In addition, a virus which produces only 12S mRNA does not stimulate expression of a preproinsulin gene carried on a coinfecting virus, while viruses containing a wild-type E1A region or producing only 13S mRNA do (36). The viruses that produce only 12S E1A mRNA established foci on rat cells at a higher frequency than wild-type virus, but the foci did not have a fully transformed phenotype. Both studies reported cold-sensitive characteristics in transformation with viruses which only make the 12S mRNA product and partial transforming activity in viruses which only make the 13S mRNA product.

Competence for productive infection of HeLa cells and partial transforming activity has also been demonstrated in a 13S cDNA virus (27). However, another study reported that both 13S and 12S cDNA viruses are competent for produc-

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FIG. 1. Schematic representation of the differentially spliced mRNA products of the E1A region. Positions of the transcription start site (3) as well as of selected restriction endonuclease cleavage sites are indicated. The numbers indicate base pairs beginning at the left end of the Ad2 genome. The arrows indicate the direction of transcription of the mRNAs. Open boxes indicate the exons of the 13S, 12S, and 9S mRNAs; carets indicate the introns removed by splicing. The nucleotide positions of the splice sites are indicated above the exons. The location of the splice sites for the E1A 13S and 12S mRNAs are from reference 41; the position of the E1A 9S mRNA splice junction is from reference 62.

tive infection in HeLa cells and that the 12S cDNA virus is less efficient than wild-type virus for transformation of rat cells (66). Furthermore, in a transient expression assay with E1A cDNA plasmids, a 12S cDNA plasmid was found to stimulate transcription of the adenovirus early region 2A (E2A) and early region 3 (E3) genes (32). In a different transient expression study, a plasmid expressing the 13S mRNA stimulated transcription of adenovirus genes as well as of the rabbit β -globin gene, while the E1A 12S mRNA had no detectable stimulatory effect on either cellular or viral genes (54).

To examine the roles of the individual E1A products, we separated the 12S and 13S products by isolating the corresponding cDNAs. We also used synthetic oligonucleotides to construct a cDNA whose sequence corresponds to that of the 9S mRNA product (62). The cDNAs were reconstructed into both plasmids and viruses, and the properties of the E1A products were studied during infection and transfection of human and rat cells. Our results confirm that the 13S product is sufficient for productive infection in HeLa cells and that this product activates expression of other adenovirus early genes. Neither the 12S nor the 9S cDNA product showed transactivation activity under the same conditions. We also found that both the 13S and the 12S products can establish primary baby rat kidney (BRK) cells in culture but that the 9S product is unable to do so.

MATERIALS AND METHODS

Cells and viruses. Monolayer cultures of cell lines 293, HeLa, and A549 were grown in Dulbecco modified Eagle medium supplemented with 10% calf serum, 100 μ g of streptomycin per ml, and 100 μ g of penicillin per ml. Ad5, Ad2, and Ad5d/309 were propagated on HeLa monolayers. Ad5d/312 and the 9S, 12S, and 13S E1A cDNA viruses were propagated on 293 cells. Ad5d/309 and Ad5d/312 were kindly provided by T. Shenk. Viral infections and titrations were performed essentially as described previously (65), except that $MgCl_2$ was omitted for plaque assays on 293 cells.

Construction of E1A cDNA plasmids. A cDNA library made from early mRNA purified from Ad2-infected cells (14) was probed with a nick-translated Ad2 *Hpa*I E fragment (0 to 1.5 kilobases). Plasmids from positive colonies were screened for characteristic 12S or 13S restriction enzyme digestion patterns and sequenced across the splice junction

from the *Xba*I site at base pair 1336 to either the upstream terminus of the E1A sequence or the *Pvu*II site at base pair 622. A 9S cDNA (MTEB9S; B. Zerler, B. Moran, K. Maruyama, J. Moomaw, T. Grodzicker, and H. E. Ruley, Mol. Cell. Biol., in press) was constructed in vitro, using synthetic oligonucleotides to span the splice junction. The cDNA sequences were inserted into pLA1 (55), which contains the *Bgl*II D fragment of Ad5 DNA, 0 to 3.3 kilobases (black bar, Fig. 2), cloned into *Eco*RI-*Bam*HI-digested pAT153. pLE13S and pLE12S contain the *Cla*I to *Xba*I fragment of the corresponding Ad2 cDNA cloned into *Cla*I-*Xba*I-digested pLA1. The 9S cDNA sequence was inserted into *Bst*XI-*Xba*I-digested pLA1. The restriction fragments containing the cDNA sequences are indicated by the dotted regions in Fig. 2. All restriction enzymes were obtained from New England BioLabs, Inc. (Beverly, Mass.).

Isolation of viruses. Viruses were constructed essentially as described previously (53). Briefly, the adenoviral *Eco*RI-*Xba*I fragment (map unit position, 0 to 3.8) excised from pLE13S, pLE12S, or pLE9S was ligated to the large *Xba*I A fragment (map unit position, 3.8 to 100) of Ad5d/309 (30). Viruses were isolated and plaque purified after transfection of the ligated DNA into 293 cells (20). Stocks were grown on 293 cells and titrated on 293 and HeLa cells.

Growth properties of viruses. Monolayer cultures of HeLa, 293, or A549 cells were infected with virus at different multiplicities of infection (MOIs), and cells were incubated at 38.5°C for various times. Cells were harvested by scraping with a rubber policeman and collected along with the medium. Virus was released by freezing and thawing the cells, followed by sonication. The resulting lysates were assayed by titration on 293 cells.

Transient expression assays. Transfections were carried out by a modified calcium phosphate coprecipitation technique (19, 64). HeLa cells at approximately 75% confluence were fed with Dulbecco modified Eagle medium containing penicillin (100 μ g/ml), streptomycin (100 μ g/ml), and 5% fetal calf serum approximately 4 h before transfection. Plasmid DNA (5 μ g of pKCAT23 plus 5 μ g of pLA1, pLE13S, pLE12S, pLE9S, or pAT153) and high-molecular-weight salmon sperm DNA to a total of 20 μ g were suspended in 250 μ l of 2 $\times$ HEPES-buffered saline containing (grams per liter): HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 10; NaCl, 16; KCl, 0.74; Na_2HPO_4 , 0.25; and dextrose, 2 (pH 7.05). An equal volume of 0.25 M

FIG. 2. Construction of E1A cDNA plasmids. pLA1 (55) contains the *Bgl*II D fragment of Ad5 DNA (nucleotides 1 to 3332) represented by the black bar in the figure, cloned between the *Eco*RI and *Bam*HI sites of pAT153. pLE13S and pLE12S contain the *Cla*I to *Xba*I fragment of the respective Ad2 cDNAs in place of the corresponding *Cla*I to *Xba*I fragment of pLA1. pLE9S contains 9S cDNA sequences constructed by using synthetic oligonucleotides to span the splice junction (Zerler et al., in press), cloned into *Bst*XI-*Xba*I-digested pLA1. The subcloned cDNA fragments are indicated by the dotted regions in the figure. Positions of restriction endonuclease cleavage sites are illustrated in Fig. 1.

CaCl₂ was added drop by drop, and the mixture was allowed to form a precipitate for 10 min at room temperature. The entire precipitate was added to a 6-cm plate containing 2 ml of fresh medium. Plates were incubated at room temperature for 20 min, at which time the medium was brought back up to 5 ml, and the plates were placed at 37°C. Four hours later the medium was removed, and the plates were covered for 1 min with 2 ml of fresh medium containing 15% glycerol, washed with phosphate-buffered saline, and fed with 5 ml of fresh medium. Cells were harvested 36 to 48 h later and assayed for chloramphenicol acetyltransferase (CAT) activity (see reference 16, as modified in reference 63) with the

further modification that half of the lysate resulting from a 6-cm plate was incubated with 0.1 μ Ci of [¹⁴C]chloramphenicol (approximately 40 mCi/mmol; New England Nuclear Corp., Boston, Mass.), 48 μ l of distilled water, and 1 μ l 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.

Western analysis of viral proteins. Protein extracts were prepared 17 h postinfection from monolayer cultures of HeLa cells infected at a multiplicity of 20 PFU per cell. Infected cells were lysed in RIPA buffer (1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris [pH 7.4], 150 mM NaCl). RIPA lysate supernatants corresponding to approximately 5×10^5 cells were separated in 10% sodium dodecyl sulfate-polyacrylamide gels, electrophoretically transferred to nitrocellulose (57), and incubated with either anti-*trpE*-E1A fusion protein antibodies (51) or with anti-Ad5 72,000-dalton DNA-binding protein (72K DBP) antibodies. The immune complexes were detected with ¹²⁵I-labeled protein A (New England Nuclear Corp.). Protein levels were quantitated by excising the radioactive bands and counting with a gamma counter.

Viral RNA preparation. For the experiments shown in Fig. 4, 5, and 6, total cytoplasmic RNA was prepared as described previously (1) from suspension cultures of HeLa cells infected at a multiplicity of 10 PFU per cell. Virus stock titers, determined by plaque assay on 293 cells, were also checked by measuring the level of late protein synthesis in infected 293 cells. Cells were concentrated to a density of 5×10^6 cells per ml before virus infection. After absorption for 1 h, infected cells were diluted to approximately 5×10^5 cells per ml with Joklik modified minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 5% calf serum (Armour). RNA was harvested at 8 h postinfection from untreated cells and from cells treated with cycloheximide (25 μ g/ml; Sigma) from 3 to 8 h postinfection. For the experiment shown in Fig. 9, total cytoplasmic RNA was prepared as above from the same infections used for viral protein preparation.

Northern analysis of viral mRNAs. Polyadenylated RNA was selected from total cytoplasmic RNA by oligo(dT)-cellulose (type 3; Collaborative Research, Inc., Waltham, Mass.) chromatography. The poly(A)⁺ RNA was fractionated by electrophoresis through a 0.9% formaldehyde-agarose gel and transferred to GeneScreen Plus (New England Nuclear Corp.) (56). E1A- and E2A-specific RNAs were detected by hybridization to ³²P-labeled RNA probes generated by SP6 polymerase. The E1A probe extended from the *Xba*I site at base pair 1336 to the *Nae*I site at base pair 810. The E2A probe extended from the *Bgl*II site at base pair 23,086 to the *Xho*I site at base pair 23,924. For the

TABLE 1. Plaque-forming ability of cDNA viruses

Virus	Infectivity (PFU/ml)		
	293	HeLa	A549
12S	1×10^9	5×10^5	1×10^6
13S	2×10^9	5×10^9	2×10^9
9S	4×10^8	2×10^5	ND ^a
Ad5dl309	2×10^{10}	3×10^{10}	ND
Ad5dl312	4×10^8	4×10^4	ND

^a ND, Not done.

FIG. 3. Growth curves of cDNA viruses. HeLa (○), A549 (△), and 293 (□) cells were infected with virus, incubated at 38.5°C, and harvested at the time points indicated, and virus yields were titrated on 293 cells. (A) 13S virus, MOI = 1. (B) 12S virus, MOI = 1. (C) 12S virus, MOI = 10. The relative yields of virus in these experiments are listed in Table 2.

experiment shown in Fig. 9, the bands were cut out and counted for radioactivity.

Transformation of primary baby rat kidney cells. Primary baby rat kidney cells were prepared by collagenase-dispase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) treatment (44) of kidneys from 6-day-old Fisher rats. About 3×10^5 to 5×10^5 cells per 60-mm dish were plated 2 days before infection. Cells were approximately 70% confluent at the time of infection and were infected in the same manner as for HeLa cells. Cultures were maintained at 37°C in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum, 100 µg of penicillin per ml, and 100 µg of streptomycin per ml. Cultures were fed twice weekly with the same medium. Cells were fixed with methanol-acetone (1:1, vol/vol) and stained with 10% Giemsa stain 3 to 4 weeks postinfection.

RESULTS

Isolation of E1A cDNAs. The adenovirus E1A gene encodes three known mRNAs, referred to as 9S, 12S, and 13S, specifying partially overlapping polypeptides (Fig. 1). Plasmids containing 12S and 13S sequences were isolated from a cDNA library prepared from early viral mRNA (14). The 12S- and 13S-specific segments were substituted for the corresponding E1A segment in plasmids containing the entire E1A region under the control of either the mouse metallothionein-I promoter (11) or the authentic viral E1A promoter (see Materials and Methods). The 9S cDNA was constructed (Zerler et al., in press) by making use of synthetic oligonucleotides corresponding to the 9S RNA splice junction sequence (62). The 9S cDNA sequence was also placed under the control of both the E1A and metallothionein promoters. The structure of the different E1A cDNA plasmids (Fig. 2) (metallothionein-promoted cDNA plasmids are illustrated separately; Zerler et al., in press) was confirmed by restriction nuclease digestion and by direct sequence analysis (data not shown). Further proof of the structures is provided by the demonstration that baby rat kidney cell lines established with the 12S and 13S clones contain the expected 12S and 13S E1A mRNAs, respectively (Zerler et al., in press).

Virus construction. To construct viruses containing the 9S, 12S, or 13S cDNA sequences (referred to here as 9S, 12S, and 13S viruses) in place of the E1A gene, the adenovirus left-end fragment was excised from the plasmids and ligated to the *Xba*I A fragment of Ad5dl309 (30, 53). Virus was isolated after transfection of the ligated DNA into 293 cells (20) which contain and express the Ad5 E1A and E1B genes which would complement potential E1A defects. The structure of resultant viruses was verified by restriction digests of viral DNA (data not shown) and by examination of the E1A mRNAs produced in HeLa cells infected with the 12S and 13S viruses (see Fig. 4 and 5).

Growth properties of the cDNA viruses. The plaque-forming efficiency of the 9S, 12S, and 13S viruses was determined on 293 cells and on HeLa cells. In addition, the plaque-forming efficiency of the 12S and 13S viruses was measured on A549 cells, which are derived from a human lung oat cell carcinoma and support the growth of Ad9, a serotype that grows poorly on HeLa and 293 cells (W. Topp, unpublished data). Table 1 shows that the 13S virus, like the nondefective virus Ad5dl309, formed plaques equally well on all three cell lines, while the 12S virus gave approximately 5,000-fold-fewer plaques on HeLa cells and 1,000-fold-fewer plaques on A549 cells than on 293 cells. The 9S virus, which produced 2,000-fold-fewer plaques on HeLa cells than on

TABLE 2. Growth of cDNA viruses in different cell lines

Virus	MOI	Time after infection (h)	Relative yields of virus	
			293-HeLa ^a	293-A549 ^a
12S	10	24	75	30
		48	28	10
		72	40	1.6
		96	3.6	1.3
12S	1	24	1,500	200
		48	3,000	100
		72	1,000	10
		96	250	1
13S	1	24	3.5	1
		48	0.5	1
		72	0.5	0.3
		96	0.7	0.7

^a 293, HeLa, or A549 cells were infected at an MOI of 10 or 1 PFU per cell and incubated at 38.5°C for the indicated time. Virus yields were titrated on 293 cells. The ratios of the yields (PFU per milliliter) from different cell lines are given.

293 cells, was similarly defective for plaque formation. The Ad5 mutant *d1312*, which lacks almost all E1A sequences, produced 10,000-fold-fewer plaques on HeLa cells than on 293 cells.

For an alternative measure of virus production, infected cultures of each cell line were harvested at various times after infection, and the virus yield from each was determined by titration on 293 cells. This experiment was conducted by using the 13S virus at an MOI of 1 PFU per cell (Fig. 3A) and the 12S virus at MOIs of 1 and 10 PFU per cell (Fig. 3B and C). The kinetics of 13S virus production, like those of wild-type Ad2 and Ad5 virus and Ad5*d1309* (data not shown), were essentially the same in all three cell lines. At low MOI the 12S virus grew very poorly on HeLa cells, as expected from the plaque titration data, but in A549 cells the yield was greater, and as infection progressed the yield approached that obtained in 293 cells. It appears that the A549 cells, though lacking E1A sequences, can partially compensate for

the missing 13S function. A similar partial compensation is also seen at high MOIs: the yield of 12S virus improved dramatically in both HeLa cells and A549 cells relative to 293 cells when the cells were infected with 10-fold-more virus (Table 2). At even lower MOIs, the ratio of the yields of 12S virus on 293 cells versus HeLa or A549 cells were even higher (data not shown).

These data show that the 13S virus grows as well as wild-type adenovirus in both HeLa cells and A549 cells, suggesting that the 13S function is the only E1A function required under these circumstances. The 12S virus shares the growth defects and multiplicity-dependent leakiness phenotype of E1A deletion mutants such as Ad5*d1312* and Ad5hr-1 (43, 45). These results imply that the 12S and 9S products play no essential role during lytic infection of HeLa cells.

Regulation of other adenovirus early genes by E1A gene products. Since E1A mutants are deficient in activating other early viral genes (4, 29, 33, 39, 63), we examined the expression of early viral RNA and protein in HeLa cells infected with the cDNA viruses. Figure 4 illustrates the results obtained from Northern analysis of mRNA isolated 8 h after infection of HeLa cells with the cDNA viruses. Protein synthesis was blocked by cycloheximide from 3 to 8 h postinfection. Under these conditions, the 13S E1A mRNA produced by the 13S cDNA virus and the 12S E1A mRNA produced by the 12S cDNA virus were present at levels similar to the levels of each found in wild-type virus infections. The 9S E1A mRNA was detected when the 9S virus was coinfecting with the 13S cDNA virus but not when either virus was infected alone. This result suggests a possible role for the 13S product in the enhancement of E1A 9S expression. Figure 5 illustrates the results obtained from Northern analysis of mRNA isolated 8 h after infection of HeLa cells when protein synthesis was not blocked. While the 13S E1A mRNA produced by the 13S cDNA virus was present at a level similar to that found in wild-type virus infections, the E1A mRNAs produced by the 9S and 12S cDNA viruses were not detected. The 9S mRNA again could be detected when the 9S virus was coinfecting with the 13S virus but was still not detected during infection with either virus alone.

FIG. 4. Northern analysis of E1A mRNA in cDNA virus-infected HeLa cells blocked with cycloheximide. RNA was isolated at 8 h postinfection from HeLa cells treated with cycloheximide from 3 to 8 h postinfection. The poly(A)⁺ fraction from 200 µg of cytoplasmic RNA was fractionated on a formaldehyde-agarose gel and transferred to GeneScreen Plus. E1A RNAs were detected by hybridization to an RNA probe generated by SP6 polymerase. The probe extends from the *Xba*I site (base pair 1336) to the *Nae*I site (base pair 810). Lanes: 1, 13S and 9S virus coinfection; 2, Ad5*d1312*; 3, Ad5*d1309*; 4, 13S virus; 5, 12S virus; 6, 9S virus.

FIG. 5. Northern analysis of E1A RNA in cDNA virus-infected HeLa cells harvested at 8 h postinfection. Cells were not cycloheximide treated, but in other respects experimental procedures were as described in the legend to Fig. 4. The autoradiogram was overexposed to reveal any 12S or 9S RNA bands; separate 13S, 12S, and 9S bands can be detected in Ad5*d1309* RNA preparations at shorter exposure times. Lanes: 1, 13S and 9S virus coinfection; 2, Ad5*d1312*; 3, Ad5*d1309*; 4, 13S virus; 5, 12S virus; 6, 9S virus.

The mRNA samples prepared in the absence of cycloheximide were also probed for the presence of mRNA transcribed from the E2 72K DBP gene. This mRNA was present in approximately equal amounts in wild-type and 13S cDNA virus-infected cells but was not detected in 9S, 12S, or Ad5dl312 virus-infected cells (Fig. 6).

Maximal E1A protein concentrations occur between 14 and 18 h postinfection (49). Therefore, we probed again for the presence of the 72K DBP mRNA at a later time. We compared the 72K DBP mRNA levels with the E1A protein levels directly because RNA levels may not be a good indication of the amount of E1A protein products present in infected HeLa cells, as the half-life of the protein product of the 12S mRNA appears to be more than twice as long as the half-life of the protein product of the 13S mRNA (49). At 17 h postinfection virus-infected HeLa cells were divided into portions used to prepare extracts for RNA and protein analysis. Figure 7 illustrates the results of a Western blot probed with antibodies made against a *trpE*-E1A fusion protein product (51). E1A protein products were detected in extracts from 12S, 13S, and wild-type virus-infected HeLa cells but not in Ad5dl312-infected cell extracts. The 9S protein product was not identified in infected cells. Another Western blot of the infected-cell extracts was probed with anti-72K DBP antiserum. The 72K DBP product was detected in 13S and wild-type virus-infected HeLa cell extracts but not in extracts from cells infected with 12S or Ad5dl312 virus (Fig. 8). Figure 9 illustrates the results of Northern analysis of the virus-infected cells analyzed in Fig. 7 and 8. The Northern blot was probed for the presence of mRNA from the 72K DBP gene. This mRNA was detected in extracts from wild-type and 13S virus-infected cells but was not detected in extracts of 9S, 12S, or Ad5dl312 virus-infected cells.

The ability of the separate 12S and 13S products to

FIG. 6. Northern analysis of 72K DBP mRNA in cDNA virus-infected HeLa cells harvested at 8 h postinfection. Cells were not cycloheximide treated, but in other respects experimental procedures were as described in the legend to Fig. 4. The E2A RNA probe extends from the *Bgl*II site (base pair 23,086) to the *Xho*I site (base pair 23,924). The arrow indicates the position of the 72K DBP mRNA. Lanes 1, Ad5dl312; 2, Ad5dl309; 3, 13S virus; 4, 12S virus; 5, 9S virus.

FIG. 7. Western analysis of E1A proteins in cDNA virus-infected HeLa cells harvested at 17 h. Protein extracts were prepared 17 h postinfection, separated in 10% sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose, and incubated with anti-*trpE*-E1A fusion protein antibodies (51). Lanes: 1, Ad5dl312; 2, Ad5dl309; 3, 12S virus; 4, 13S virus. Quantitation of the Western analysis indicated that the level of E1A protein product detected during 12S virus infection was about 40% of the level of protein detected during 13S virus infection.

stimulate expression from another adenovirus early promoter was studied in the absence of virus infection by using a transient expression assay. HeLa cells were cotransfected with a plasmid containing E1A sequences together with a plasmid carrying the bacterial CAT gene under the control of the adenovirus E3 promoter (pKCAT23; 63). In the absence of the E3-CAT gene no enzyme activity was detected in extracts of transfected cells (data not shown). Transfection of the E3-CAT plasmid together with the plasmid pAT153 (59), the derivative of pBR322 used as the vector for the E1A constructs, resulted in the appearance of a low level of CAT activity (Fig. 10). Cotransfection of the E3-CAT plasmid together with a plasmid containing the E1A 12S cDNA under the control of the E1A promoter (pLE12S) showed no increase in CAT activity detectable by liquid scintillation spectroscopy of the appropriate regions of the thin-layer chromatography plate. Cotransfection of the E3-CAT plasmid with plasmids containing either the E1A gene itself (pLA1) or the E1A 13S cDNA region (pLE13S) increased the level of CAT activity by a factor of approximately 10. Cotransfection of the E3-CAT plasmid together with a plasmid containing the E1A 9S cDNA (pLE9S) resulted in an

FIG. 8. Western analysis of the 72K DBP in cDNA virus-infected HeLa cells harvested at 17 h. Samples of the same extracts described in the legend to Fig. 7 were probed in the same way, except that these were incubated with anti-72K DBP antiserum. Lanes: 1, Ad5dl312; 2, Ad5dl309; 3, 12S virus; 4, 13S virus. Quantitation of the Western analysis indicated that the level of 72K DBP product detected during 12S virus infection was about 6.5% of the level of DBP detected during 13S virus infection.

FIG. 9. Northern analysis of the 72K DBP mRNA in cDNA virus-infected HeLa cells harvested at 17 h. RNA isolated from extracts of the infected cells as described in the legend to Fig. 7 was analyzed by the procedures described in the legend to Fig. 4. The probe is described in the legend to Fig. 6. The arrow indicates the position of the 72K DBP mRNA. Lanes: 1, Ad5dl312; 2, 12S virus; 3, 13S virus; 4, Ad5dl309; 5, 9S virus. Quantitation of the Northern analysis indicated that the level of 72K DBP mRNA detected during 12S virus infection was approximately 6% of the level detected during 13S virus infection. The amount of 72K DBP mRNA detected in extracts of 9S virus-infected cells was about 8% of the level found during 13S virus infection.

occasional small increase, about two- to threefold, in the level of CAT activity. These results support the conclusion that the 13S, but not the 12S, E1A product can efficiently activate other adenovirus early genes. These experiments employed plasmids which contained most of the adenovirus E1B gene in addition to E1A sequences (Fig. 2), but similar results were obtained with E1A cDNA plasmids containing

less than 100 base pairs of E1B coding sequence (data not shown), indicating that the E1B sequences are probably not involved in the response. Furthermore, as shown elsewhere (Zerler et al., in press), expression of the E3-CAT gene was also stimulated by the E1A 13S cDNA and genomic E1A coding sequences under control of the metallothionein promoter (in plasmids pMT13S and pMTEBE1A), but not by the corresponding 12S or 9S cDNA constructs (pMTEB12S and pMTEB9S); pMTEB9S, like pLE9S, occasionally induced a small increase in CAT activity when cotransfected with E3-CAT. Since the metallothionein promoter is expected to promote similar levels of expression of the cDNA products, we conclude that the failure of the 12S and 9S E1A products to activate E3-CAT efficiently does not result specifically from differential expression of the E1A cDNAs from the E1A promoter.

Transformation functions of E1A gene products. The transforming properties of the 9S, 12S, and 13S viruses were studied by infection of primary baby rat kidney (BRK) cells. While defective for lytic growth in HeLa cells, the 12S virus immortalized BRK cells with an efficiency about 50 times greater than wild-type virus (Fig. 11), although the immortalized foci which arose were not fully transformed. Increasing the MOI of 12S virus led to the appearance of larger numbers of immortalized foci. Wild-type virus killed the cells when infected at high multiplicity. No foci were observed with the 13S virus over the entire range of MOIs tested; with this virus the cytopathic effect predominated and infected cells were efficiently killed. However, in the absence of lytic functions, the E1A 13S gene is able to reveal its establishment potential. Like plasmids containing E1A 12S sequences, plasmids containing the E1A 13S region can establish BRK cells in culture; moreover, cotransfection of BRK cells with either 12S or 13S plasmids and the *ras* oncogene give stably transformed cell lines (Zerler et al., in press). The BRK cells immortalized by the 12S virus have the same morphology and plating efficiency as the BRK cells established by transfection with the 12S plasmid (Zerler et al., in press). The 9S virus failed to produce foci on BRK cells, and the E1A 9S plasmids also failed to establish foci

FIG. 10. Transactivation by E1A of an adenovirus E3-promoted CAT gene. HeLa cells were cotransfected with pKAT23 (63) and pAT153 (lane 1), pLA1 (lanes 2 and 3), pLE9S (lanes 4 and 5), pLE12S (lanes 6 and 7), or pLE13S (lanes 8 and 9). Cell lysates were incubated with [14 C]chloramphenicol and acetyl coenzyme A and analyzed by thin-layer chromatography to separate chloramphenicol (CM) from its acetylated forms (AC). The extent of conversion of chloramphenicol to its acetylated forms was assayed by liquid scintillation spectroscopy. In the averages of three separate experiments, including the ones illustrated, CAT activity measured after cotransfection with the E1A-containing plasmids compared with activity after cotransfection with pAT153 was 10.4-fold, 2.8-fold, 0.7-fold, and 13.1-fold higher than pAT153 for pLA1, pLE9S, pLE12S, and pLE13S, respectively.

FIG. 11. Immortalization of primary BRK cells by cDNA virus infection. Primary BRK cells from 6-day-old rats were infected with wild-type Ad5dl309, 13S cDNA virus, or 12S cDNA 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 a week, and plates were stained with 10% Giemsa stain at 3 weeks postinfection.

when transfected either alone or together with the *ras* gene. The E1A 9S plasmids also failed to prevent focus formation when cotransfected with either E1A 12S or E1A 13S plasmids alone or together with *ras*. Similarly, the 9S virus did not interfere with focus formation when coinfecting with the 12S virus in BRK cells in culture.

DISCUSSION

Two functions have been attributed to the adenovirus E1A gene, a transforming function and a transactivation function, and three differently spliced E1A mRNAs have been described. To examine the roles of the individual E1A mRNAs, we cloned their corresponding cDNAs as plasmids and rebuilt them separately into viruses. The properties of the three E1A products were studied during productive infection of human cells and during the abortive, transforming infection of rat cells, as well as during transient expression of plasmids in HeLa cells.

The 12S and 9S products may be insufficient for normal expression from the E1A promoter itself since the E1A mRNA levels detected at 8 h postinfection in 12S or 9S virus-infected HeLa cells not treated with cycloheximide were much lower than those detected in wild-type or 13S virus-infected cells. However, the 12S protein product level detected later during 12S virus infection was more nearly comparable to the level of 12S product detected during wild-type infection.

The kinetics of expression of the 9S mRNA in 9S cDNA virus-infected HeLa cells are interesting since the 9S mRNA normally does not appear at appreciable levels until late in

wild-type infection. It is noteworthy that the level of 9S mRNA in HeLa cells increased when the 9S virus was coinfecting with the 13S virus, suggesting a possible role for the 13S RNA or protein products in 9S expression. The enhancement of the 9S mRNA level occurred even in the presence of cycloheximide, but the mechanism of action of cycloheximide on mRNA levels in virus-infected cells is not certain. Transcription of other early genes dependent on E1A is readily detectable in cycloheximide-treated cells (40) and may depend on residual protein synthesis. It is also interesting that 293 cells do not normally express 9S mRNA from the endogenous E1A sequences they contain (34) but that 9S mRNA is expressed during adenovirus infection of 293 cells. We have observed the production of 9S mRNA during infection of 293 cells with the 9S cDNA virus (data not shown). We are continuing to investigate the regulation of 9S mRNA expression.

In an infection of HeLa cells, our 13S cDNA virus, like other 13S cDNA viruses (27, 66) and viruses which do not express the 12S product owing to mutations at the appropriate splice sites (23, 36, 37), appeared to grow as well as wild-type virus, suggesting that the products of the 13S mRNA are sufficient for productive infection. On the other hand, growth of both the 12S and 9S viruses in HeLa cells was depressed by a factor of more than 1,000, approximating the defect observed with the E1A deletion mutant Ad5dl312. These results agree with results obtained with viruses which express a normal 12S product but which do not express the 13S product owing to splice-site mutations (23, 36). We conclude that the products of the 12S and 9S mRNAs are dispensable for the productive infection of HeLa cells.

Since the 13S virus is fully competent for productive infection, it must harbor the ability to activate other essential early viral genes. The 12S and 9S viruses may fail to grow efficiently in HeLa cells because the cellular level of the 12S and 9S products is low compared with that of the 13S products. Alternatively, the protein products of the 12S and 9S sequences may have relatively little promoter-stimulating activity compared with the 13S protein. Both interpretations are consistent with our inability to detect viral products apart from those of E1A in cells infected with the 12S or 9S virus. However, the demonstration that during 12S virus infection the 72K DBP protein and mRNA products are barely detectable above the levels expressed during Ad5d/312 infection, although the protein product of the 12S mRNA is present at nearly half the level of that of the 13S protein, argues that the 12S protein intrinsically has less ability than the 13S protein to stimulate expression from the adenovirus E2 promoter. The promoter-stimulating activity of E1A products was studied directly by analysis of the ability of the E1A cDNA plasmids to activate a heterologous gene (CAT) under the control of the E3 promoter. The 13S cDNA plasmid was as active as the wild-type E1A plasmid in this assay, whereas the 12S cDNA plasmid had no detectable activity even when its coding sequences were expressed from the mouse metallothionein-I promoter, which was used to avoid possible autoregulatory effects on the E1A promoter. Whether the 12S product retains any transactivation function is uncertain, but such activity, if it exists, is insufficient to activate the E2 or E3 promoters efficiently or to promote effective virus multiplication. However, the level of expression of the 12S product is sufficient to induce immortalization of BRK cells as discussed below.

The requirement for the E1A activator function can be circumvented by a number of routes. At high MOIs, the 12S virus grows very much more efficiently in HeLa cells. This phenomenon, multiplicity-dependent leakiness, has been noted repeatedly with viruses mutant in E1A (43, 45). It may be that when a cell contains a large number of viral DNA molecules, the viral promoters compete more effectively for limiting components of the transcription machinery. A second means whereby the requirement may be bypassed is shown here: the A549 cell line is considerably more permissive than HeLa cells for the 12S virus. In this cell line the concentration of available transcription factors might be greater than in HeLa cells; alternatively, another activator, comparable to the pseudorabies virus immediate-early gene product, which can complement Ad5d/312 (13) might be present in A549 cells. Another indication that A549 cells contain a compensating function for E1A transactivation is the demonstration that the 72K DBP is expressed in these cells during Ad5d/312 infection (28). While the requirement for the E1A products is clearly not absolute, the existence of compensating mechanisms such as these argues that such transactivating proteins may be widespread.

Although the 12S product is unable to promote the efficient growth of the 12S cDNA virus in human cells, it retains an active establishment function. Infection of BRK cells with the 12S virus led to the appearance of foci of immortalized cells with a frequency much greater than that seen with wild-type virus. While the 12S cDNA product does not appear to be efficient in activating early adenovirus genes, the establishment activity presumably results from the ability to promote or suppress expression of some cellular products. There is no information allowing a distinction to be made between these two possibilities at present, but it may be relevant that the 12S product has been reported to

suppress expression from the late promoter of the adenovirus E2A gene (22) and that both the 12S and 13S products have been reported to suppress the action of the simian virus 40 enhancer (6, 61).

The 13S product was also able to establish primary BRK cells in culture, but in a virus infection this activity was obscured by the cytopathic effects of the virus. The 13S plasmid is able both to cooperate with the *ras* oncogene to induce stable transformation of BRK cells and to establish these cells when transfected alone (Zerler et al., in press).

Hitherto, investigation of the roles of individual E1A products has relied heavily on the use of mutants which fail to make one or more of the E1A proteins. In some cases novel fused or truncated versions of the E1A proteins are generated as a result of the mutation. We attempted to extend and clarify these studies through the use of specific cDNAs to introduce single E1A products into cells. Our transformation results are in general agreement with those obtained with mutants which fail to express either the 12S or the 13S mRNAs as a result of small changes at splice junction sites (36, 37). Both of these mutant viruses presumably remain capable of producing the 9S mRNA in addition to the 13S or 12S species. These authors also reported that the virus which is unable to make the 12S mRNA is defective for multiplication in growth-arrested human cells. The missing 12S function could correspond to the transforming function discussed above. Many of our conclusions on the transforming activities of the E1A products also concur with those drawn from work on E1A deletion mutants (23). Earlier studies with mutants with insertions or deletions located within the 13S-specific region had suggested that the 12S product is not effective for the activation of other viral early genes (7, 12, 42). Although a study with E1A cDNA plasmids in a transient expression assay reached the opposite conclusion (32), our results with both viruses and plasmids sustain the earlier conclusion, as does a different transient expression assay (54) and the work of Montell et al. (36, 37) with viruses. Perhaps differences in cell lines used may be partly responsible for the differences in results.

An analysis of independently derived E1A cDNA viruses reported results comparable to ours with respect to the 13S virus (27, 66) but differing sharply with respect to the 12S virus (66). The latter authors reported that the 12S virus transformed CREF or primary rat embryo cells at a lower frequency than wild-type virus, whereas our 12S virus was at least 50-fold more active than wild type in transforming baby rat kidney cells. Moreover, these authors reported that their 12S virus was only about fivefold reduced in growth yield in HeLa cells and displayed significant ability to activate the adenovirus E2A and E4 genes. At a comparable MOI our 12S virus was about 1,000-fold reduced in growth yield in HeLa cells and exhibited no detectable transactivation function. The reasons for the discrepancies are unknown but could be related in part to multiplicity dependence effects.

Our results indicate that although the 12S product has little of the early adenovirus gene activating function found in the 13S product, it does have a very active immortalization function. However, it is not clear whether the transactivation activity of the 13S product is dependent on its establishment activity. To distinguish further the functions of the E1A products, we have made selected point mutations in the E1A region and in the cDNA plasmids and viruses. Using these mutants, we hope to distinguish whether the active sites for the transactivation function and the establishment function are linked.

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