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Dissection of overlapping functions within the adenovirus type 5 E1A gene

Gösta Winberg[1] and Thomas Shenk

Department of Microbiology, Health Sciences Center, State University of New York, Stony Brook, NY 11794, USA

[1]Present address: Department of Tumor Biology, Karolinska Institute, Soluavagen 1, S-10401 Stockholm, Sweden

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The adenovirus E1A gene encodes multiple, overlapping mRNAs whose products function both to regulate mRNA levels during the lytic cycle of the virus and to facilitate transformation of non-permissive cells. To assign specific functions to the E1A gene products, two adenovirus type 5 variants have been constructed. Mutants *dl*347 and 348 carry cloned segments corresponding to the E1A 12 and 13S mRNAs, respectively, in place of the normal E1A gene. The variants produced the predicted E1A-specific mRNAs and polypeptides. Both viruses grew efficiently in HeLa cells. Although the 13S mRNA products were more effective, the products of either mRNA species could stimulate the accumulation of mRNAs from additional transcription units. Both viruses could induce the formation of transformed foci in an established rat cell line. Neither virus could transform primary rat embryo cells at normal frequency, and the *dl*347 foci which were induced were incomplete or abortive transformants. Thus, functions encoded by both 12S and 13S mRNAs are required for efficient and complete transformation of primary rat cells.

Key words: mutants/cDNAs/transcriptional regulation/ transformation

Introduction

The early region 1A (E1A) transcription unit lies at the extreme left end of the adenovirus chromosome and is apparently the first gene to be expressed subsequent to infection. It encodes three mRNAs which have common 5′ and 3′ ends but differ in their splicing patterns (Berk and Sharp, 1978; Perricaudet *et al.*, 1979; Baker and Ziff, 1981). The three mRNAs (designated 13, 12 and 9S, see Figure 1B) contain potential coding regions to specify 31.9, 26.5 and 13.3-kd polypeptides, respectively (Van Ormondt *et al.*, 1978; Gingeras *et al.*, 1982). The 12 and 13S mRNAs are expressed early after infection and encode a family of four to six polypeptides *in vivo* with apparent mol. wts. of 40–60 kd (Smart *et al.*, 1981; Yee *et al.*, 1983; Feldman and Nevins, 1983) which are localized in both nucleus and cytoplasm of the infected cell. The reasons for the heterogeneity and slower-than-predicted electrophoretic mobility are not yet clear. The 9S mRNA is expressed only later after infection.

E1A gene products are required both for normal expression of viral genes during the lytic growth cycle (Berk *et al.*, 1979; Jones and Shenk, 1979a; Nevins, 1981) and for transformation of non-permissive cells (Van der Eb *et al.*, 1977; Graham *et al.*, 1978; Jones and Shenk, 1979b; Houweling *et al.*, 1980). To differentiate functions of the overlapping E1A-specific gene products, we have constructed two adenovirus type 5 (Ad5) variants, each of which produces only one of the early E1A mRNAs. Mutant *dl*347 carries a cloned DNA segment which corresponds to the 12S mRNA in place of the normal E1A gene, and *dl*348 contains a copy of the 13S mRNA. Mutant *dl*348 generates near wild-type yields in productively infected HeLa cells, while the yield of *dl*347 is only 5-fold reduced. Neither virus can transform primary rat embryo cells as efficiently as the wild-type parent.

Results

Construction and growth properties of viral mutants

Rearranged E1A genes were initially prepared in recombinant plasmids. The parental plasmid used for these constructions was pE1A-WT which contains the left-end 1339 bp of the Ad5 chromosome including the majority of the E1A gene (Figure 1A). DNA segments corresponding to mature 12 and 13S mRNAs were excised from cDNA clones 133 and 131, respectively (Perricaudet *et al.*, 1979), and inserted into pE1A-WT, replacing the wild-type sequence (Figure 1A). Based on their DNA sequences, the modified E1A genes should produce either a 12S or 13S mRNA, but not both.

The rearranged E1A genes were rebuilt into viral chromosomes using the method of Stow (1981). In this procedure, plasmid DNA is linearized at the Ad5 *Xba*I cleavage site (sequence position 1339) and ligated to a right-end Ad5 fragment derived from a viable variant, *dl*309, which carries only one *Xba*I cleavage site at sequence position 1339 (Jones and Shenk, 1979b). The recombined molecules are then used to transfect permissive cells. The resulting viruses contain a normal left end, having lost pBR322 sequences, presumably by copying the right-end terminal repeat during replication or through recombination. The variants are shown in Figure 1B. *dl*347 should produce only 12S mRNA, and *dl*348 only 13S mRNA.

Initially, the mutant viruses were propagated on 293 cells (human embryonic kidney cells which contain and express the Ad5 E1 genes, Graham *et al.*, 1977) which would complement potential E1A defects. They were then tested for growth on HeLa cells (Figure 2). The rate of growth and final yield of *dl*348 (E1A-13S) was indistinguishable from the parental virus, *dl*309. *dl*347 (E1A-12S) replicated at a reduced rate, and generated a 5-fold reduced yield by 72 h after infection. Thus, both mutant viruses are viable. The polypeptides encoded by the 13S mRNA are sufficient for normal lytic growth in cultured HeLa cells. The 12S mRNA products are only slightly less effective in promoting viral growth.

The variants synthesize E1A-specific products predicted by their structures

E1A-specific mRNAs produced by the mutant viruses were assayed by the S1 endonuclease mapping procedure (Figure 3). RNA encoded by the parental virus, *dl*309, generated three fragments. The 613 and 475 nucleotide fragments corre-

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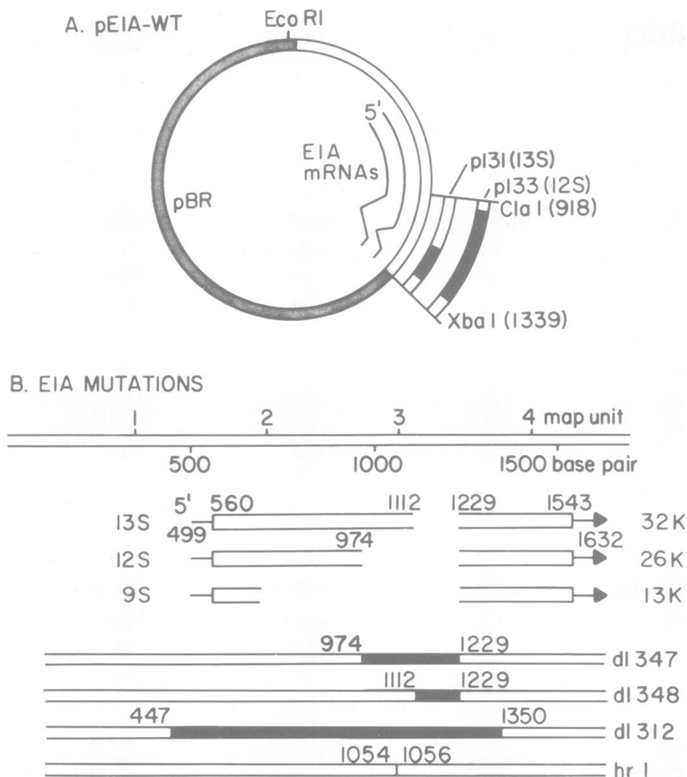

Fig. 1. Diagrams of pE1A-WT, the E1A transcription unit, and the deletion mutations. **(A)** Representation of pE1A-WT. Sequences derived from pBR322 are shaded. Ad5 sequences are from the extreme left end of the viral chromosome. Positions within the Ad5 segment are located by their nucleotide sequence numbers (Van Ormondt *et al.*, 1978). The E1A 12S and 13S mRNAs are indicated with intervening sequences represented as carat symbols. The sequences encoding the 3′ ends of the mRNAs are not present in the plasmid. The segments of p131 and 133 which were substituted into pE1A-WT to generate *dl*347 and 348 are indicated. **(B)** Representation of the Ad5 E1A transcription unit and deletion mutations studied in this report. The top of the figure positions the map in terms of map units and sequence position relative to the left end of the viral chromosome. mRNAs are designated by lines, intervening sequences by spaces and polypeptide coding regions by open boxes. Deletion mutations are represented by closed bars. The first and last nucleotides which are present bracketing the deletions are designated by their nucleotide sequence numbers.

sponded to the 13S and 12S mRNA 5′ exons, respectively. The 375 nucleotide fragment was derived from the identical 13 and 12S 3′ exons. RNA prepared from *dl*348-infected cells produced large amounts of the 613 and 375 nucleotide fragments derived from 13S mRNA. Since the 13S intron is no longer present in RNAs coded by *dl*348, there is clearly no requirement for splicing *per se* for the efficient production of the 13S mRNA. A faint 475 nucleotide band was also evident. This corresponds to the 12S mRNA 5′ exon. Apparently, the 12S mRNA splice donor site present in the 13S sequence is joined to the 13S splice junction with low efficiency. Assuming that the sequence UCUACAG/U acts as splice acceptor (it shows good homology to the consensus acceptor $^{\mathrm{UUU}}_{\mathrm{CCC}}\mathrm{N}^{\mathrm{C}}_{\mathrm{U}}\mathrm{AG/G}$, Mount, 1982), the minor 12S species would carry normal coding sequences to the 5′ side of the splice junction, but the reading frame following the junction would be shifted. As a result, the minor 12S mRNA would produce an abnormal polypeptide which would be truncated shortly after crossing the novel splice junction. RNA prepared from *dl*347-infected cells produced the 475 and 375 nucleotide fragments derived from the 12S mRNA. Curiously, these frag-

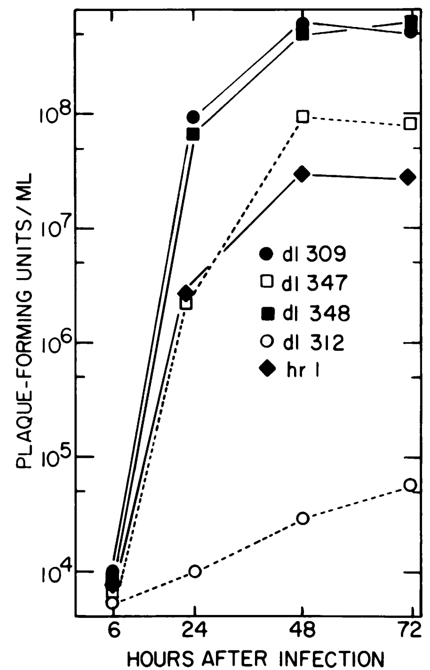

Fig. 2. Growth kinetics of mutant and parental viruses. HeLa cells were infected at a multiplicity of 1 p.f.u./cell, and the virus yield was assayed at the times indicated by plaque assay on 293 cells. Symbols: ●, *dl*309; □, *dl*347; ■, *dl*348; ○, *dl*312; ◆, *hr*1.

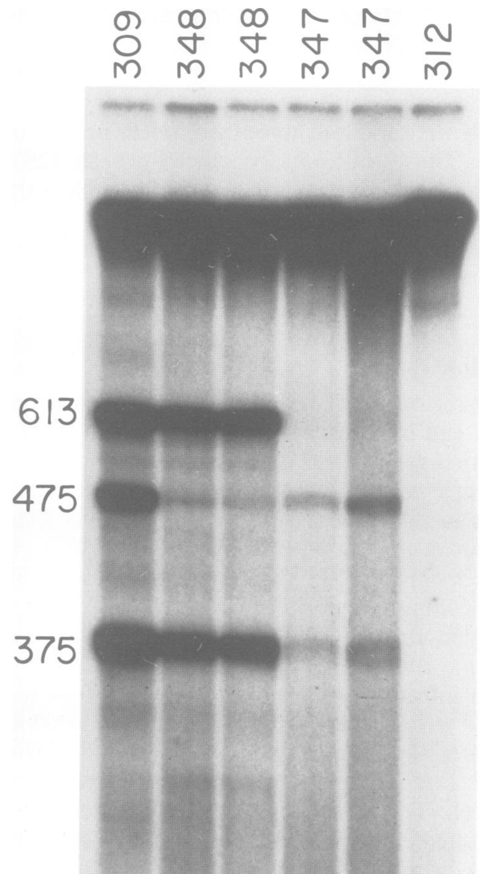

Fig. 3. S1 endonuclease analysis of E1A-specific mRNAs produced in HeLa cells infected with mutant or parental viruses. RNAs were prepared from HeLa cells at 5 h after infection and subjected to S1 endonuclease analysis using M13-E1A single-stranded DNA as probe. S1-treated products were resolved by electrophoresis in a 5% polyacrylamide gel containing 8 M urea in a Tris-borate buffer. Bands corresponding to 5′ and 3′ exons are identified.

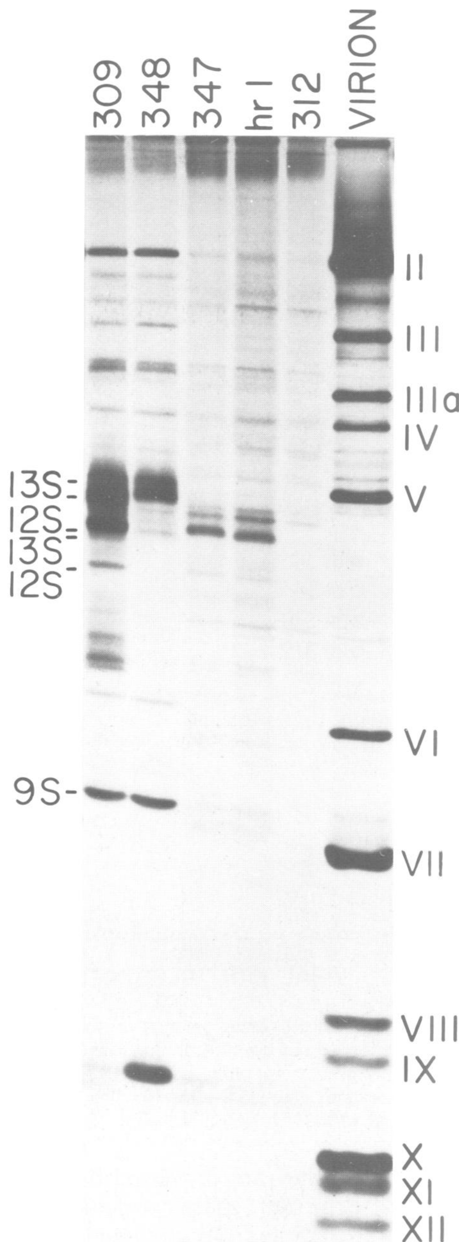

Fig. 4. Electrophoretic analysis of E1A-specific polypeptides produced in HeLa cells infected with mutant or parental viruses. Cells were labeled for 1 h with [^{35}S]methionine at 12 h after infection. Extracts were prepared and immunoprecipitations carried out using an Ad5 E1A-specific antibody. Electrophoresis was in a 15% SDS-containing polyacrylamide gel. E1A-specific polypeptides are identified by designating the mRNA on which they are coded. Ad5 virion polypeptides are included as size markers.

ments were present in reduced amounts as compared with those derived from *dl*309-specific RNA. The reason for the reduced level of 12S mRNA in *dl*347-infected cells is not clear.

E1A-specific polypeptides were monitored by immunoprecipitation of infected cell extracts using an antibody specific for the C-terminal region of E1A products. The parental virus, *dl*309, carrying a wild-type E1A gene produced a series of polypeptides specifically precipitated by the antiserum. *dl*347 and 348 each produced a subset of the 309 peptides. In addition to peptides encoded by the early E1A mRNAs, *dl*309 and *dl*348-infected cells also contain an E1A-specific polypeptide which migrates at the position predicted for the product of the late E1A 9S mRNA. *dl*348 could possibly utilize the 13S mRNA splice junction as an acceptor site to generate a 9S mRNA as discussed above. Two additional mutants were analyzed as controls. Mutant *hr*1 contains a 1-bp deletion which disturbs the 13S but not 12S mRNA coding region (Figure 1B). As expected, *hr*1 generated precisely the same series of polypeptides as *dl*347. *dl*312 lacks a large segment of the E1A gene and does not produce E1A products. Comparison of this mutant with *dl*309 permitted positive identification of E1A-specific polypeptides.

We conclude that *dl*347 and 348 produce the subsets of E1A-specific mRNAs and polypeptides predicted by their DNA structures.

Accumulation of early mRNAs in dl347 and 348-infected cells

The E1A gene is known to produce a product which stimulates accumulation of mRNAs encoded by the other early viral transcription units (Berk *et al.*, 1979; Jones and Shenk, 1979a; Nevins, 1981). Polypeptides encoded by the E1A 12 and 13S mRNAs were tested for their ability to perform this function. Figure 5 displays an experiment in which cytoplasmic RNA was prepared from infected cells, and subjected to S1 endonuclease analysis using probe DNAs specific for regions E1A, E2A or E4. Bars immediately above the gel tracks mark groups of three RNAs prepared at 5, 9 and 13 h after infection. *dl*309 and 348 (E1A-13S)-infected cells contained similar levels of E2A and E4 mRNAs at each time assayed suggesting that products encoded by the 13S mRNA can efficiently stimulate accumulation of viral mRNAs. *dl*347 (E1A-12S)-infected cells contained reduced levels of the test mRNAs. However, *dl*347-infected cells contained substantially more of the early mRNAs than cells infected with *dl*312 which lacks the E1A gene. Thus, the products encoded by the 12S mRNA can also stimulate the accumulation of early mRNAs, albeit less efficiently than the polypeptides encoded by the 13S species.

Transformation of rat cells

The variants were tested for their ability to induce transformed foci subsequent to infection of either primary rat embryo cells or an established rat cell line (CREF cells, Fisher *et al.*, 1982). In CREF cell assays, *dl*348 (E1A-13S) produced nearly the same number of transformed foci as the wild-type parent, *dl*309, and *dl*347 (E1A-12S) produced about half as many foci (Figure 6B). *dl*312 (no E1A products) produced very few foci and *hr*1 (E1A-12S) generated slightly more foci than *dl*309. An increased transformation frequency by *hr*1 in CREF cells has been reported previously (Babiss *et al.*, 1983). Cell lines were readily established from *dl*309, 347 and 348-induced foci. *dl*347-transformed cell lines displayed a fibroblastic morphology while *dl*309 and 348-induced lines were epitheloid. Although the 13S products appear to function with somewhat greater efficiency, these data indicate that the polypeptides coded by either the 12 or 13S mRNAs can function in the transformation of established rat cells.

Experiments with primary rat embryo cells produced quite different results. *dl*347, 348 and *hr*1 each produced 4- to 5-fold fewer transformed foci than *dl*309 (Figure 6A). Further, the *dl*347 and *hr*1-induced foci appeared quite different from *dl*309 or 348 foci (Figure 7). Although transformed cell lines were readily established from *dl*309 or 348 foci, most *dl*347 and *hr*1-induced foci failed to generate a cell line. Usually, the cells would grow well for several passages, but then gradually cease dividing. The rare *dl*347 and *hr*1-induced

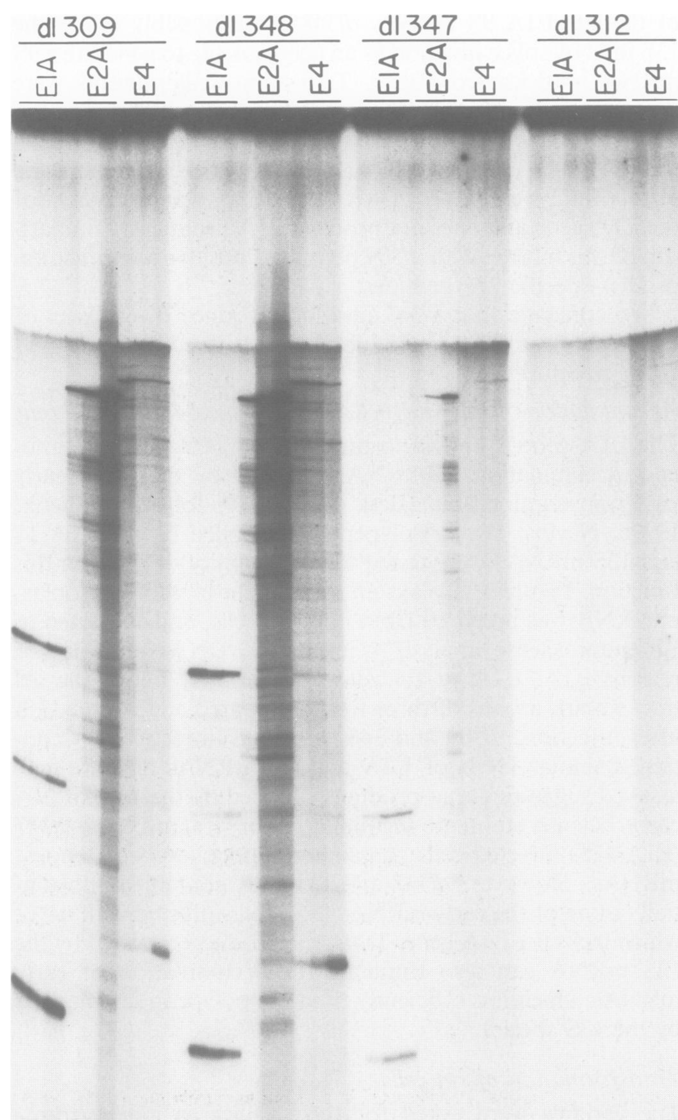

Fig. 5. S1 endonuclease analysis of early mRNAs produced in HeLa cells infected with mutant or parental viruses. RNAs were prepared from HeLa cells at 5, 9 or 13 h after infection, and subjected to S1 endonuclease analysis using M13-E1A, E2A or E4 single-stranded probe DNAs. S1-treated products were resolved by electrophoresis in a 5% polyacrylamide gel containing 8 M urea in a Tris-borate buffer. Bars immediately above the gel tracks mark groups of three RNAs (prepared at 5, 9 or 13 h after infection) analyzed by the indicated probe DNA. The M13-E2A probe (and to a lesser extent the E4 probe) consistently generates a large, major S1 endonuclease-protected fragment plus many smaller species. The largest fragment is that predicted to result from hybridization of the probe DNA with the major E2A mRNA.

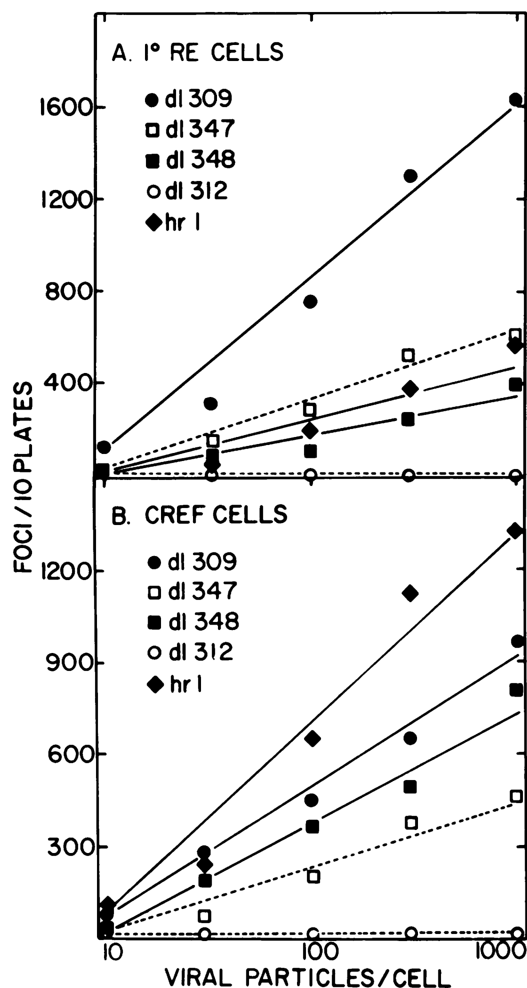

Fig. 6. Transformation of rat cells using parental or mutant viruses. Transformations were carried out using equilibrium density gradient-purified virus and either primary rat embryo cells (A) or the established CREF cell line (B). Foci were counted 6–7 weeks after infection. Symbols: ●, *dl*309; □, *dl*347; ■, *dl*348; ○, *dl*312; ◆, *hr*1.

cell lines (about one in 15 attempts) which were obtained displayed a morphology distinct from wild-type transformants (Figure 7). Whereas wild-type transformants were epitheloid, *dl*347 and *hr*1 transformants were fibroblastic.

We conclude that the products encoded by either the 12S or 13S mRNA are able to provide E1A transforming functions when assayed on an established rat cell line. In contrast, 13S but not 12S products function to transform primary rat cells. Optimal transformation efficiencies on primary cells require both E1A mRNAs.

Discussion

The two early E1A mRNAs have identical 5′ and 3′ ends, but differ in the way they are processed by RNA splicing. Both mRNAs utilize the same splice acceptor site at sequence position 1229, but the 12S and 13S species employ donor sites at 974 and 1112, respectively (Figure 1B). Splicing does not alter the reading frame. As a result, the polypeptides synthesized by these two mRNAs are identical in their N- and C-terminal domains but differ by 46 amino acids at an internal site. In addition to the difference in primary sequence, it is possible that the polypeptides encoded by the two mRNAs are differentially modified after translation. It is clear that they are modified since both mRNAs give rise to multiple polypeptides which migrate more slowly than predicted by their amino acid sequence.

During the lytic cycle either mRNA species was able to provide a function which enhanced the expression of early viral transcription units and virus yield as compared with *dl*312 which produces neither mRNA (Figures 2 and 5). *dl*348 expressed early mRNAs and grew as well as wild-type virus, indicating that the 13S mRNA encodes gene products sufficient for optimal growth of Ad5 in HeLa cells. This observation is in good agreement with the work of Montell *et al.* (1982) who mutagenized the 12S mRNA donor site to produce a virus (*pm*975) which produced no 12S mRNA but grew as well as the wild-type.

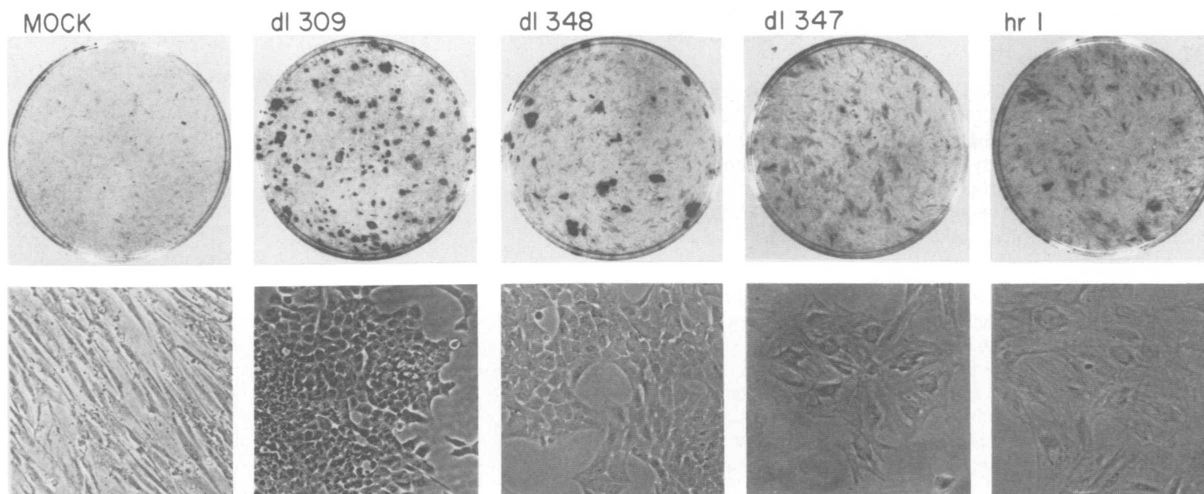

Fig. 7. Photographs of colonies and cell lines resulting from transformation of primary rat embryo cells with parental and mutant viruses. Petri dishes contain primary rat cell cultures which were infected at a multiplicity of 1000 particles/cell and stained with Giemsa 6 – 7 weeks after infection. Below each dish is a photomicrograph of a cell line derived from a transformed focus which appeared in a culture carried in parallel to the stained samples.

Cells infected with *dl*347 contained ~4-fold less E1A 12S mRNA than wild-type virus-infected cells (Figure 3), and expressed mRNAs from other early transcription units after a delay (Figure 5). The delayed expression of mRNAs likely results from relatively inefficient function of the 12S as compared to the 13S gene product. The delay could also be due to the reduced level of E1A 12S expression observed in *dl*347-infected cells. The reason for the reduced expression is not clear. Perhaps the mRNA is poorly expressed because its normal processing pathway is altered (it no longer undergoes splicing). Alternatively, the 347 deletion could alter a *cis*-acting transcriptional control element or the 13S mRNA product could regulate expression of the E1A unit in a positive fashion. Experiments are underway to distinguish these possibilities. *dl*347 behaves similarly in its growth to *hr*1 (Figure 2) whose single base-pair deletion alters the 13S but not 12S coding region (Harrison *et al.*, 1977; Ricciardi *et al.*, 1981; Figure 1B). The similarity suggests that residual E1A function displayed by *hr*1 is due to its 12S mRNA product and not to a truncated product of the 13S mRNA.

Both *dl*347 and 348 were better able to transform an established rat cell line (CREF cells) than primary rat embryo cells (Figure 6). The E1A gene is known to express a function which is able to confer on primary cells the ability to grow indefinitely in culture (Van der Eb *et al.*, 1977; Houweling *et al.*, 1980). Further, Ruley (1983) has recently shown that the adenovirus E1A gene can facilitate transformation of primary cells by transforming genes (polyoma middle T antigen and T24 Harvey *ras*1) which by themselves can only transform established cells. So, it makes sense that alterations in the E1A gene impact more severely on transformation of primary than established cells. Since neither the 12S nor 13S products alone function at wild-type efficiency, it seems clear that the products of both mRNAs play a role in the transformation of primary cells.

Although it functioned at reduced efficiency, the product of the 13S mRNA could provide the immortalizing function during transformation of primary cells more effectively than the 12S product. *dl*348-induced foci could readily be established as transformed cell lines with epitheloid morphology. In contrast, *dl*347 (E1A-12S)-infected foci appeared abnormal, they generally failed to give rise to transformed cell lines, and on the rare occasion when a line was obtained it displayed a fibroblastic morphology (Figure 7).

As was the case for lytic growth properties, *dl*347 appears similar to *hr*1 in its transforming phenotype. Both *hr*1 and *dl*347 give rise to incomplete or abortive transformants of primary cells (Figure 7 and Graham *et al.*, 1978; Ruben *et al.*, 1979). Like *dl*347, *hr*1 can transform the established CREF cell line (Figure 6B and Babiss *et al.*, 1983). The *hr*1 transformation phenotype is cold-sensitive (Ho *et al.*, 1982; Babiss *et al.*, 1983). Experiments are in progress to evaluate the temperature-dependence of *dl*347 transformation.

Materials and methods

Plasmids, viruses and cells

Mutations were originally constructed in a recombinant plasmid (pE1A-WT) which contains the left-end 1339 bp of the Ad5 genome inserted between the unique *Eco*RI and *Pvu*II sites of pBR322. The DNA segment containing 12 and 13S mRNA introns was removed from pE1A-WT by cleavage with the *Cla*I and *Xba*I endonucleases and corresponding DNA copies (cDNAs) of the 12 and 13S mRNAs were substituted. The source of 12 and 13S cDNAs were p133 and p131, respectively (Perricaudet *et al.*, 1979). Mutations constructed in pE1A-WT were rebuilt into intact viruses using the procedure of Stow (1981).

H5*dl*309 was selected as an Ad5 variant that contained only one *Xba*I cleavage site (1339 bp from the left end of the chromosome) (Jones and Shenk, 1979b), and served as the parent for the mutants described in this report (*dl*312, 347 and 348). H5*hr*1 was isolated as a host range mutant subsequent to mutagenesis with nitrous acid (Harrison *et al.*, 1977).

The 293 cell line (a human embryonic kidney cell line transformed with a DNA fragment carrying the left 11% of the Ad5 genome) was obtained from H.Young and has been described by Graham *et al.* (1977). Cells were maintained in medium containing 10% calf serum. HeLa cells were from the American Type Culture Collection and were maintained in medium containing 7% calf serum. CREF cells were from P.Fisher and have been described (Fisher *et al.*, 1982). They were maintained in medium containing 10% fetal calf serum. Primary rat embryo cells were prepared by trypsinization of 15- to 17-day-old embryos from inbred Fisher rats. Cells were grown in medium supplemented with 10% calf serum.

RNA preparation and analysis

To prepare RNAs, HeLa spinner cells were infected at a multiplicity of 20 p.f.u./cell and harvested either 5, 9 or 13 h later. RNA was prepared using the procedure of McGrogan *et al.* (1979), and S1 endonuclease analysis using M13 single-stranded probes was essentially as described by Gaynor *et al.* (1982). M13 probe DNAs carried the following Ad5 DNA segments: E1A, 0 – 4.5 map units; E2A, 59.5 – 70 map units, E4, 89 – 100 map units. S1-treated DNA fragments were analyzed by electrophoresis in a 5% polyacrylamide gel

containing 8 M urea prepared in a Tris-borate buffer. After electrophoresis, gels were dried onto filter paper and exposed to X-ray film.

Analysis of polypeptides

At 12 h after infection at a multiplicity of 20 p.f.u./cell, HeLa cells were labeled for 1 h using [^{35}S]methionine (1100 Ci/mmol, 50 μCi/ml). Immunoprecipitations were performed as described by Sarnow *et al.* (1982) using an Ad5 E1A-specific monoclonal antibody provided by A.Zantema and A.van der Eb. SDS-polyacrylamide gel electrophoresis was carried out according to Sarnow *et al.* (1982). Bands corresponding to radioactively labeled polypeptides were identified by fluorography (Laskey and Mills, 1975).

Transformation experiments

Transformation assays utilized either the established CREF cell line or primary rat embryo cells. Cells were infected at ~80% confluence with virions which had been purified by equilibrium density centrifugation and suspended in Tris-saline plus 2% calf serum. Adsorption was for 1 h at 37°C. Medium containing 10% calf serum was added and incubation continued for 1 day at 37°C. The cultures were then passaged (1:5) and after three additional days incubation in standard medium were placed in medium containing 0.1 mM $CaCl_2$ (Freeman *et al.*, 1967). Cells were re-fed with this low-calcium medium twice a week, and final counts of foci were made at 6–7 weeks.

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