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Transcription Control Region Within the Protein-Coding Portion of Adenovirus E1A Genes

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A single-base deletion within the protein-coding region of the adenovirus type 5 early region 1A (E1A) genes, 399 bases downstream from the transcription start site, depresses transcription to 2% of the wild-type rate. Complementation studies demonstrated that this was due to two effects of the mutation: first, inactivation of an E1A protein, causing a reduction by a factor of 5; second, a defect which acts in *cis* to depress E1A mRNA and nuclear RNA concentrations by a factor of 10. A larger deletion within the protein-coding region of E1A which overlaps the single-base deletion produces the same phenotype. In contrast, a linker insertion which results in a similar truncated E1A protein does not produce the *cis*-acting defect in E1A transcription. These results demonstrate that a critical *cis*-acting transcription control region occurs within the protein coding sequence in adenovirus type 5 E1A. The single-base deletion occurs in a sequence which shows extensive homology with a sequence from the enhancer regions of simian virus 40 and polyomavirus. This region is not required for E1A transcription during the late phase of infection.

In *Escherichia coli*, promoter mutations generally result from changes in bases which interact directly with RNA polymerase and occur within 40 bases of the transcription initiation site (62, 68). In contrast to *E. coli* promoters, the DNA sequences which regulate the transcription of eucaryotic protein-coding genes, which are transcribed by RNA polymerase II, are more complex. The most highly conserved sequence element of polymerase II promoters, the TATA box (11; M. Goldberg, Ph.D. thesis, Stanford University, Stanford, Calif., 1978), centered about 27 bases upstream from the transcription start site, probably interacts directly with the polymerase. Deletion of this sequence usually leads to a reduction in the rate of transcription initiation by a factor of 5 to 10, and the residual transcripts usually have heterogeneous cap sites (18, 25, 32, 34, 49, 56), which are sites of transcription initiation (15, 36). Transcription initiation in vitro in most instances is completely dependent on the TATA sequence (16, 40, 59, 72, 78). However, in eucaryotic cells, mutations at much greater distances from the start site than are the TATA box or the −35 region of *E. coli* promoters can have profound effects on transcription initiation (5, 18, 21, 33–35, 49, 50, 53, 71). Several strong viral promoter regions contain sequences called enhancers, which can increase transcription from a variety of promoters by an unknown mechanism which is largely independent of their position relative to the transcription start site (4, 17, 21, 22, 44, 45, 52, 75). Since the function of these more distant transcription control signals is largely independent of their distance from the transcription initiation site, it seems unlikely that they interact directly with RNA polymerase II (44, 48, 52).

In most genes transcribed by RNA polymerase II which have been analyzed, DNA sequences required for transcription initiation lie upstream from the transcription start site. Important exceptions are immunoglobulin genes which contain B-lymphocyte-specific enhancer sequences located within the second intron of the rearranged, activated genes (3, 26). These internal enhancers are required for high rates of transcription of immunoglobulin genes (26, 60). For the early region 1A (E1A) of adenovirus type 2 (Ad2), Osborne et al. (57) found that deletion of the sequence to within 38 base pairs of the major transcription start site did not prevent expression of the gene. In this earlier study, a genetic test was used to measure the activity of E1A deletion mutants. More recently, this observation has been confirmed by direct analysis of E1A mRNA transcribed from deletion mutants of this transcription unit present on replicating plasmid DNAs in COS cells (27) or after transfection of nonreplicating plasmids into HeLa cells (Osborne et al., unpublished observations). These studies demonstrated that sequences upstream from the TATA box region are not required for E1A transcription from transfected plasmid DNA. However, Hearing and Shenk (39) found that when virus mutants were analyzed, deletion of the viral sequence extending from −304 to −141 decreased transcription by a factor of 20 during the early phase of infection but not during the late phase. In the present work, we introduced point mutations randomly throughout E1A and screened for mutations which prevented the synthesis of E1A mRNA. One mutant, Ad5 15606, was identified in which the rate of E1A transcription was reduced by a factor of 50 compared with the wild type. This phenotype was found to result from a single-base deletion 399 bases downstream from the major cap site, within the protein-coding portion of the gene. Thus, for this viral gene, as for immunoglobulin genes, a crucial DNA sequence required for transcription lies well within the transcribed region. In E1A, this transcription control region is located within the protein-coding portion of the gene.

E1A maps between nucleotides 499 and 1,632 in the 35-kilobase Ad5 sequence (2, 58, 76). It encodes a viral protein required for the early induction of transcription from at least five early promoters (6, 42, 54). E1A mRNAs have one major cap site (2) and multiple minor 5′ ends mapping up to 375 nucleotides upstream from the major cap site (55, 56, 64). The primary transcript is spliced into one of two

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alternative mRNAs during the early phase of infection (8, 58). A third spliced mRNA is synthesized during the late phase of infection (14, 69, 77; see Fig. 4). The viral DNA sequence contains a typical TATA box centered 27 base pairs upstream from the major cap site (2, 76), and in vitro transcription studies indicate that the region is transcribed by RNA polymerase II (79).

MATERIALS AND METHODS

Viruses and transfections. Purification of adenovirus virions, preparation of adenovirus DNA-protein complex, exonuclease III digestion of adenovirus DNA-protein complex with 10 U of exonuclease III per μg of DNA, and DNA-protein complex transfections were as described (19). The titers of wild-type Ad5 and Ad5 host range mutant stocks were determined by plaque assay on 293 cells (28).

$NaHSO_3$ mutagenesis. $NaHSO_3$ mutagenesis was as described by Shortle and Nathans (67), except that the concentration of $NaHSO_3$, (pH 6.0) was 1 M, and the incubation at 37°C was performed in capped polypropylene microcentrifuge tubes filled to the top with mineral oil. Incubation lasted 30 min to generate the 15300 series of mutants and 60 min to generate the 15600 series. The $NaHSO_3$ incubation was terminated by removing the aqueous phase to dialysis tubing and dialyzing for 2 h at 0°C against 1,000 volumes of 5 mM $KHPO_4$, (pH 6.8)–2 mM hydroquinone prepared in boiled deionized water and then proceeding with the procedure of Shortle and Nathans (67). The mutagenized DNA-protein complex was finally dialyzed into 10 mM Tris (pH 8.0)–0.1 mM EDTA and was used directly in transfections without ethanol precipitation.

Rapid screen for host range mutants. HeLa monolayer cells (4×10^5 per well) were seeded into 24-well microtiter plates (Costar, Cambridge, Mass.; well diameter, 16 mm). The next day, medium was aspirated from the wells, and two drops (0.1 ml) of phosphate-buffered saline containing picked virus plaques was added. The microtiter plates were incubated at 37°C for 1 h, 1 ml of agar overlay media was then added, and incubation at 37°C continued. The microtiter wells were examined microscopically at 2, 3, and 4 days postinfection for the development and spread of adenovirus cytopathic effect. Isolates which failed to induce a cytopathic effect were titrated on HeLa and 293 cells.

Complementation analysis. Complementation between isolated host range mutants was tested by coinfecting 60-mm plates of HeLa cells (containing 2×10^6 cells) with 5 PFU of each of two mutants per cell. The yield of virus was assayed by plaque titration on 293 cells and was compared with the yield after infection of HeLa cells with the individual mutants at a multiplicity of infection (MOI) of 10. Complementation indices equal the yield of mixed infections divided by the sum of the yield of single infections. A complementation index of less than 10 was considered as failure to complement.

Marker rescue mapping. Wild-type restriction fragments of Ad2 DNA were cloned in plasmid pBR322. The terminal *Pst*I fragment (nucleotides 1 through 1833) was obtained by *Pst*I digestion of BE5 (70). *Hae*III and *Hae*III-*Sma*I fragments (nucleotides 354 through 496, 497 through 605, 606 through 716, 717 through 1009, and 1010 through 1540) were cloned via *Bam*HI linkers (CCGGATCCGG; Collaborative Research, Inc., Waltham, Mass.) into the *Bam*HI site of pBR322. Ad2 sequences were released by *Hae*III digestion, or by digestion with *Hae*III plus *Bam*HI for the clones of nucleotides 717 through 1009 and 1010 through 1549. Fragments from nucleotides 729 through 923 and 923 through 1009 were obtained by *Sau*3A digestion of the clone from nucleotides 717 through 1009. Fragments from nucleotides 763 through 858 and 859 through 1011 were obtained by *Ava*I digestion of the same clone. These fragments were purified by gel electrophoresis through and elution from 5% polyacrylamide gels (47). The digested DNA was ethanol precipitated and dissolved in 10 mM Tris (pH 8.0)–1 mM EDTA at a concentration of 200 μg/ml. Twenty-five microliters of this solution was denatured by adding 3 μl of 1 N NaOH and incubating 10 min at room temperature. The solution was then chilled to 0°C on ice and neutralized by the addition of 32 μl of 1 M Tris, pH 7.4. This solution was rapidly added to 100 μl of a solution at 0°C containing 2 μg of Ad5 DNA-protein complex which had been digested for 7 min at 37°C with 10 U of exonuclease III per μg of DNA (19). The terminated exonuclease III digestion reaction contained 50 mM Tris (pH 8.0), 2 mM $MgCl_2$, and 10 mM EDTA. This solution now contained 2 μg of exonuclease III-digested 15606 DNA-protein complex and an approximately 10-fold molar excess of denatured strands of the wild-type restriction fragment from E1A. Fifteen microliters of 5 M NaCl was added, and the solution was incubated at 68°C for 10 min. The hybridization reaction was terminated by chilling on ice, and the solution was used the same day for preparation of a 1-ml calcium phosphate transfection precipitate (29). DNA-protein complex treated in this way yielded 10^3 to 10^4 PFU/μg of DNA on 293 cells, compared with 10^5 PFU/μg DNA for untreated DNA-protein complex.

Quantitative nuclease S1 analysis of cytoplasmic and nuclear RNAs. HeLa suspension cells were infected at an MOI of 10 (unless otherwise indicated in the figure legends) with Ad5 or Ad5 mutants as described (7). Cytoplasmic and nuclear RNAs were isolated as described (6). Fifty micrograms of RNAs was hybridized to an M13 clone of the Ad2 r-strand from nucleotides 360 through 2058 uniformly labeled with ^{32}P by growth in $^{32}PO_4$-containing media, all as described (56). Alternatively, 50 μg of RNA was hybridized to the 5′-end-labeled *Sau*3A fragment from nucleotides 1 through 631 (56) in 50 μl as described (7), except that hybridization was at 44°C for 12 to 16 h. After hybridization, the solution was diluted to 0.5 ml with 0.25 M NaCl–0.03 M sodium acetate (pH 4.5)–1 mM $ZnCl_2$–5% glycerol and was digested with 50 U of S1 (Bethesda Research Laboratories, Gaithersburg, Md.) at 37°C for 30 min for the uniformly labeled probe or with 200 U of S1 at 23°C for 60 min for the endlabeled probe. Digestion products were ethanol precipitated, denatured, and electrophoresed on 5% acrylamide–8 M urea-TBE gels (47). Gels were processed as described (56) and exposed to preflashed X-ray film with intensifying screens, and autoradiograms were analyzed with a Hoefer densitometer. Peak integration was performed by weighing.

In vitro nuclear run-off transcription, hybridization, and gel analysis. Log-phase HeLa suspension cells were infected with Ad5 or Ad5 15606 at an MOI of 10 or were mock infected. Cytosine arabinoside was added to 20 μg/ml, and an additional 20 μg/ml was added every 8 to 12 h. At 40 to 45 h postinfection (p.i.), nuclei were prepared as described (9), except that the homogenization buffer was Triton X-100 at 0.3 rather than at 0.14%. The nuclei were suspended at 2×10^7/0.1 ml of 25% glycerol–10 mM Tris (pH 7.9)–5 mM $MgCl_2$–1 mM EDTA–2 mM dithiothreitol. Fifty microliters of this suspension (10^7 nuclei) was added to 33 μl of 0.3 M Tris (pH 7.9)–3 mM $MnSO_4$–180 mM $(NH_4)_2SO_4$–1.2 mM EDTA–30% glycerol–2.4 mM dithiothreitol–1.8 mM ATP–1.8 mM GTP–1.8 mM CTP. Fifty microcuries of [α-^{32}P]UTP (450 Ci/mmol) was added, the total volume was brought to 100 μl with water, and the suspension was incubated at 25°C

for 30 min with frequent mixing. RNA was isolated as for nuclear RNA. Between 1×10^6 and 4×10^6 cpm was incorporated into the RNA isolated from a reaction with 10^7 nuclei.

RNA (10^5 cpm) was hybridized to 50 ng of an M13 clone of the r-strand from nucleotides 260 to 2068 in 0.375 M NaCl–10 mM Tris (pH 7.0)–1 mM EDTA–0.1% sodium dodecyl sulfate in 20 μl at 68°C for 60 min. Control experiments demonstrated that higher concentrations of the E1A M13 clone did not hybridize additional in vitro-labeled RNA from Ad5-infected cells. After the hybridization reaction was chilled on ice, 5 μl of 1-mg/ml ribonuclease A (Sigma Chemical Co., St. Louis, Mo.) and 1.0 μl of calf intestinal phosphatase (Boehringer Manheim, New York, N.Y.) at 2.5 U/μl were added and incubated at 37°C for 30 min. To inactivate these enzymes, 1 μl of pronase at 20 mg/ml (self-digested at 37°C for 30 min) was added, and the incubation continued for 15 min. Five microliters of 50% glycerol–0.1% bromophenol blue–0.1% xylene cyanol was added, and the material was layered on a vertical 1.0% agarose gel (thickness, 2 mm; length, 20 cm) cast in 0.3 M NaCl–0.09 M Tris–0.09 M boric acid–2.5 mM EDTA adjusted to pH 8.3 with HCl. The electrophoresis buffer was the same as that in which the gel was cast and was recirculated during electrophoresis at 30 V for 16 h. The gel was washed in 500 ml of 5% trichloroacetic acid at 4°C for 30 min. The trichloroacetic acid wash was repeated twice, and the gel was then washed in 95% ethanol at 4°C for 30 min. The gel was laid on six thicknesses of Whatman 3MM paper and dried. The dried gel was exposed to preflashed X-ray film with an intensifying screen at −70°C for 10 days.

Construction of *dl*1505 and *in*1512. Plasmid pHE3 (70) was digested to completion with *Sma*I and was partially digested with *Pvu*II. *Xho*I linkers were ligated to the blunt-ended molecules (5′-CCTCGAGG-3′, Collaborative Research), and the molecules were digested with *Xho*I and ligated, all as described (57). After transformation of C600 to tetracycline resistence, plasmids were isolated and screened for molecules with a deletion of the Ad2 sequence between the *Pvu*II site at 625 nucleotide and the *Sma*I site at nucleotide 1008 with the insertion of a single *Xho*I linker, pTF01505. *dl*1505 was constructed by digesting pTF01505 with *Xba*I, ligating a 10-fold molar excess to *Xba*I-cut *dl*309 (43), and transfecting 293 cells (70). Resulting plaques were screened for a host range phenotype and by restriction analysis of isolated viral DNA. Mutant 1512 was generated by isolating the Ad2 *Pvu*II-*Sma*I fragment from nucleotides 625 to 1008, ligating *Xho*I linkers to the ends, and cloning into the single *Xho*I site of PTF01505 to generate pTF01512. Mutant 1512 was generated by the method of Stow (70) as described for *dl*1505. Both *dl*1505 and *in*1512 were plaque purified twice, grown into stocks, and titrated by plaque formation, all on 293 cells (28).

RESULTS

Mutagenesis directed into E1A. To search for DNA or RNA sequences required for the transcription and processing of adenovirus E1A mRNA, this region of the Ad5 genome was mutagenized (Fig. 1). The Ad5 DNA-terminal protein complex was isolated and treated with exonuclease III to digest the 3′ 1,500 nucleotides of each strand. This rendered E1A, which maps at the left end of the genome, single stranded. These molecules were then treated with sodium bisulfite, a single-strand-specific mutagen (38, 67). DNA-terminal protein complex mutagenized in this way was then transfected into 293 cells. These cells are capable of

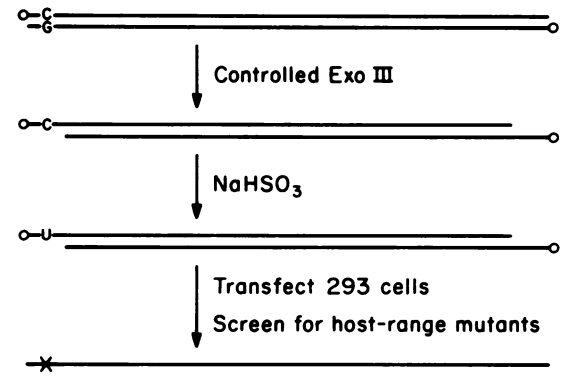

FIG. 1. Mutagenesis strategy. In the upper three lines, both strands of the viral DNA are represented, and the circle at their ends represents the terminal protein of the DNA-protein complex. The bottom line represents the genetic map of the resulting host range mutant, with X indicating the location of the mutation.

complementing lesions in essential E1A and E1B functions (28) because they contain an integrated copy of wild-type E1A and E1B (1) which is continuously expressed (1, 6). Plaques were picked from 293 cell monolayers and were tested for the ability to form plaques on HeLa cells, which cannot complement E1A or E1B mutants (37). Mutants which can form plaques on 293 cells but not on HeLa cells are termed host range mutants (37). A level of bisulfite mutagenesis was chosen to yield approximately 5% host range mutants from the picked plaques. Thus, the frequency of mutants with two lesions inactivating essential E1A or E1B functions was expected to be less than 1 in 2,000. This procedure should have produced mutations in E4, mapping at the right end of the genome, as well as in E1A. However, mutations which inactivated essential E4 functions would not have been complemented by 293 cells which do not express E4. Therefore, E4 mutants should not have been recovered.

Seventeen host range mutants were isolated. The first 10 were tested for the ability to complement each other for replication in HeLa cells. Nine fell into one complementation group, and one (15303) complemented the other nine. The nine failed to complement Ad5 *hr*1 and therefore are defective in the E1A 289-amino-acid (aa) protein (37, 61). The other mutant, 15303, failed to complement Ad5 *hr*7, which has been mapped to E1B (23, 37). Therefore, it is probably an E1B mutant. The observation of one complementation group in E1A is consistent with earlier results which indicated that only the E1A 289-aa protein is required for replication in HeLa cells (51).

Ad5 15606 is defective in the synthesis of E1A mRNA. The early viral mRNAs synthesized in HeLa cells 6 h p.i. with each of the mutants were analyzed by S1 mapping with probes specific for five of the adenovirus early regions, E1A, E1B, E2, E3, and E4 (data not shown). Sixteen of the mutants had the phenotype previously defined for E1A mutants, greatly reduced levels of E1B, E2, E3, and E4 mRNAs compared with wild-type at this time p.i. (6, 42). The E1B mutant, 15303, produced wild-type levels of these mRNAs.

One of the E1A mutants, 15606, was unique in that it did not induce detectable E1A mRNAs (less than 5% of wild-type; Fig. 2). The concentration of 15606 E1A mRNA was also greatly depressed at 44 h p.i. in cytosine arabinoside-treated cells (Fig. 2B), conditions which yield 10- to 30-fold higher transcription rates from E1A than at 6 h p.i. (24).

Under these conditions, 15606 E1A mRNA could be detected at 2% of the level of wild-type E1A mRNA. Although the concentration of 15606 E1A mRNA was greatly reduced at 44 h p.i., the lengths of S1-protected fragments were the same as those produced by wild-type Ad5, indicating that the mutant mRNAs had essentially normal structure. (Longer exposures of the gel than that shown were required to detect the 12S-specific exon.) Similar results were observed in three successive experiments.

15606 is defective in E1A transcription. To determine the step of E1A mRNA synthesis which is defective in 15606, nuclear RNA was analyzed. The concentration of 15606 E1A nuclear RNA was much less than wild-type E1A nuclear RNA concentrations (Fig. 2). At 44 h p.i. in cytosine arabinoside. a wild-type nuclear RNA was readily detectable which extended from the cap site to at least 400 nucleotides beyond the E1A polyadenylic acid site (Fig. 2B, band 1550). This nuclear RNA was unspliced and not yet cleaved at the polyadenylic acid site. We interpret it to be the primary, unprocessed E1A transcript. The fact that the concentration of this primary transcript was much less in the mutant than in the wild type suggests that the mutant is defective in E1A transcription and not in a subsequent processing step. Similar results were observed in two successive experiments.

As another test for E1A transcription in the mutant, transcription of this region was analyzed in an in vitro nuclear run-off experiment. Nuclei were incubated with labeled ribonucleotide triphosphates under conditions which

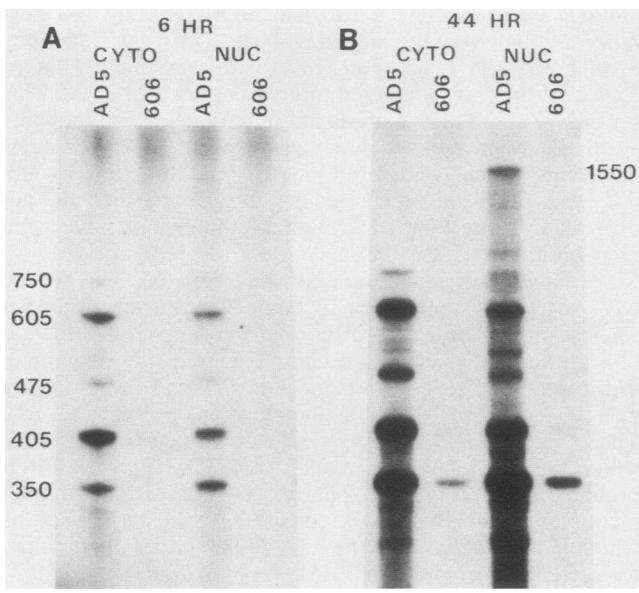

FIG. 2. Deficiency of mutant 15606 in the synthesis of early cytoplasmic E1A mRNA (CYTO) and early E1A nuclear RNA (NUC). E1A cytoplasmic RNAs and nuclear RNAs were analyzed by quantitative nuclease S1 analysis with a uniformly labeled M13 clone of E1A and the 5′ portion of E1B. (A) Band 605 was generated by the 5′ exon of the E1A 13S mRNA, band 475 by the 5′ exon of the E1A 12S mRNA, and band 405 by the 3′ exon common to both the E1A 13S and 12S mRNAs (see Fig. 4 and 6A). Band 350 was generated by the region of the E1B mRNAs complementary to the probe. Band 750 was generated by a fraction of 13S mRNAs transcribed from minor upstream start sites (55). Cytoplasmic and nuclear RNAs were isolated 6 h p.i. (B) Cytoplasmic and nuclear RNAs isolated 45 h p.i. from cytosine arabinoside-treated cells. Band 1550 was generated by the E1A unprocessed primary transcript extending from the major E1A cap site beyond the right end of the probe.

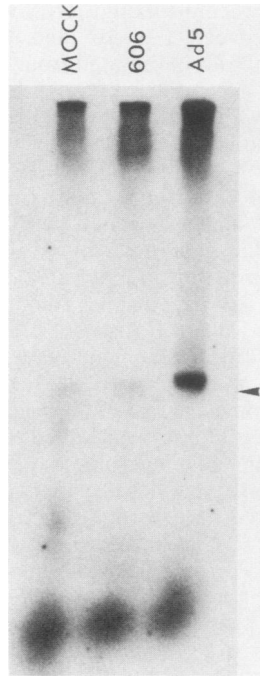

FIG. 3. Gel electrophoretic analysis of in vitro nuclear run-off transcription from Ad5-infected, 15606-infected, and mock-infected cells. Nuclei of AD5-, 15606-, or mock-infected cells were isolated and incubated in the presence of [α-^{32}P]UTP as described in the text. RNA was isolated and hybridized in solution to a large molar excess of the M13 E1A clone. After hybridization to completion, the products were digested extensively with RNase A in 0.3 M NaCl. The products were then subjected to electrophoresis through 1.0% agarose in TBE made 0.3 M NaCl. After being processed as described in the text, the gel was subjected to autoradiography. The arrow indicates the position of the M13 E1A clone as observed by ethidium bromide fluorescence.

allow RNA polymerases initiated in vivo to continue transcription but do not permit a significant fraction of transcription initiation (46). Therefore, incorporation of label into E1A sequences in this in vitro reaction is a measure of the in vivo transcription rate.

To measure in vitro E1A transcription, RNA was isolated at the end of the in vitro reaction and hybridized in solution to a molar excess of a single-stranded M13 clone of the coding strand of E1A. The hybridized material was digested with RNase, and the resulting hybrids of E1A RNA base paired to the 10-kilobase E1A M13 clone were subjected to electrophoresis through a neutral agarose gel in high salt (see above). The high-salt electrophoresis was performed to protect hybridized RNA from possible further degradation and to permit migration of the single-stranded M13 E1A clone to near the middle of the gel, well resolved from background label. Autoradiography of the gel showed E1A complementary labeled RNA migrating with the 10-kilobase M13 clone (Fig. 3). Small labeled oligonucleotides produced by RNAse digestion of the great bulk of label incorporated in vitro ran with the dye front and were largely washed from the gel before autoradiography. High-molecular-weight nuclear RNA which formed an RNA-RNA hybrid during the hybridization was resistant to RNase digestion but was present principally as slowly migrating species in the gel, well resolved from the M13 E1A DNA (Fig. 3). Far less E1A RNA was synthesized in nuclei prepared from 15606-infected cells than in Ad5-infected cells (Fig. 3). Densitometry of

the gel showed that nuclei from 15606-infected cells did not incorporate significantly more label into E1A-specific RNA than the background level observed with nuclei isolated from mock-infected cells. Nuclei from Ad5-infected cells incorporated 12 times this background value. Similar results were observed in three successive experiments. We conclude that the 15606 mutation inhibits E1A transcription.

The physiologically significant mutation in 15606 lies within the transcribed region. The mutation in 15606 responsible for the host range phenotype was mapped by a marker rescue procedure. The 15606 DNA-terminal protein complex was isolated and digested with exonuclease III as diagrammed in Fig. 1. Restriction fragments isolated from the E1A of wild-type virus were hybridized to the single-stranded left terminus of the mutant genome, and the resulting heteroduplexes were transfected into HeLa cells. After a time sufficient for two cycles of infection of wild-type adenovirus, progeny virus was harvested and assayed by plaque formation on HeLa cells. When cloned restriction fragments of a few hundred bases were used, only one fragment mapping from nucleotides 717 to 1009 in the Ad5 sequence (76) yielded rescued virus which could form plaques on HeLa cells (Fig. 4). In a parallel experiment, the host-range mutant Ad5 *hr*1 (37), which has a deletion of base pair 1055 (61), was rescued only by a fragment mapping from nucleotides 1010 to 1540 in the Ad5 sequence (data not shown). Marker rescue with smaller restriction fragments mapped the 15606 mutation between nucleotides 859 and 923 (Fig. 4).

The 15606 DNA sequence was determined between nucleotides 818 and 995. Only one mutation was found in the region responsible for the host range phenotype as determined by the marker rescue experiments. This was a single-base-pair deletion at nucleotide 897 in the Ad5 sequence (Fig. 5). This is a cytosine in the wild-type l-strand, the mutagenized strand. It is difficult to tell whether this mutation was generated by the bisulfite mutagenesis. Bisulfite treatment results in the deamination of cytosine residues to uracil residues (38). When $NaHSO_3$-mutagenized DNA is repaired in vitro before transfection, CG → TA transitions are usually observed (67). In this work, the mutagenized DNA was not repaired in vitro but rather was transfected directly. In this case, an in vivo repair process may have resulted in a single-base deletion of a deaminated cytidine residue. Alteratively, the single-base deletion may have occurred independently of the bisulfite treatment. However this mutation was generated, it is not surprising that it produces a host range phenotype since it should lead to out-of-phase translation of the E1A 289-aa protein previously determined to be required for plaque formation on HeLa cells (13, 37, 61). A second mutation was detected in the region of 15606 which was sequenced, a CG → GC transversion at nucleotide 855 in the Ad5 sequence (Fig. 5). However, this mutation lies outside the region which rescued plaque-forming activity on HeLa cells (Fig. 4). Although this second mutation produces a nonconservative amino acid change in the essential E1A protein (replacement of proline by arginine), this mutation is silent (see below).

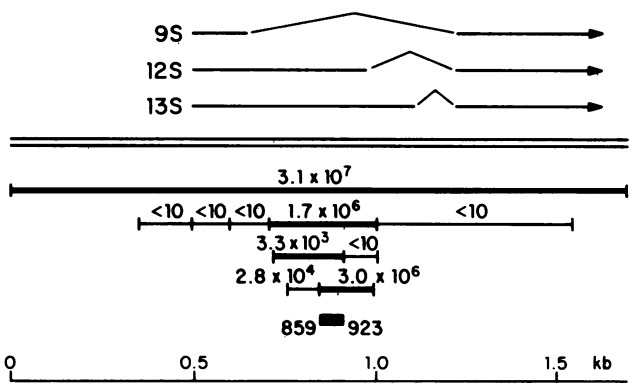

FIG. 4. Mapping the 15606 mutation by marker rescue. 15606 terminal protein-DNA complex was digested with exonuclease III, hybridized to cloned Ad2 restriction fragments, and transfected into HeLa cells. Five days after transfection, virus was harvested and assayed by plaque formation on HeLa cells. Tested fragments are indicated. Fragments yielding virus which could form plaques on HeLa cells are shown in bold lines. Actual titers were as follows: unhybridized, <10 PFU/ml; nucleotides 1 through 1833, 3.1×10^7 PFU/ml; 354 through 496, <10 PFU/ml; 497 through 605, <10 PFU/ml; 606 through 716, <10 PFU/ml; 717 through 1009, 1.7×10^6 PFU/ml; 1010 through 1540, <10 PFU/ml; 729 through 923, 3.3×10^3 PFU/ml; 923 through 1009, <10 PFU/ml; 763 through 858, 2.8×10^4 PFU/ml; 859 through 1011, 3.0×10^6 PFU/ml. The fragments from nucleotides 763 through 858 and 859 through 1011 were not purified by cloning but were purified by gel electrophoresis after *Ava*I cleavage of the clone from nucleotides 717 through 1009. The fragment from nucleotides 763 through 858 gave 1% of the number of plaques given by the fragment from nucleotides 859 through 1011. We interpret this to be due to contamination of the fragment from nucleotides 763 through 858 with that from 859 through 1011. The map locations of the early-phase 13S and 12S mRNA exons and of the late-phase-specific 9S mRNA exons are indicated.

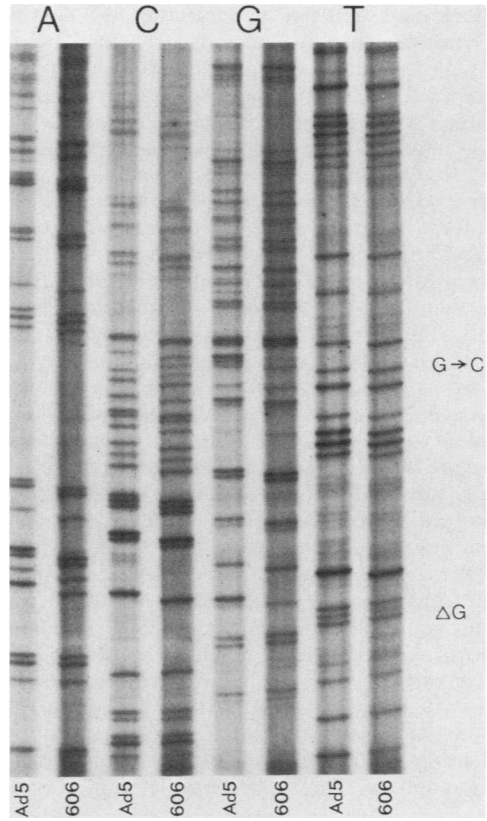

FIG. 5. Sequence of mutant 15606. This gel shows the sequence of Ad5 and 15606 between positions 800 and 917 determined by the chain termination method (65). The A, C, G, and T lanes are indicated with the Ad5 sequence to the left and the 15606 sequence to the right. Sequence is of the viral r-strand proceeding from left (top of gel) to right (bottom of gel). The regions of the G deletion (in r-strand) at 897 and the G→C transversion (in the r-strand) at 855 are indicated.

The single-base deletion at nucleotide 897 depresses E1A mRNA synthesis. For two reasons it seemed possible that 15606 might contain a second mutation responsible for depressed E1A mRNA synthesis separate from the mutation responsible for the host range phenotype which was identified in the marker rescue experiments. First, the mutation identified by marker rescue lies nearly 400 nucleotides downstream from the transcription start site, a surprising location for a mutation which affects transcription. Second, greatly reduced levels of E1A protein are still sufficient to permit replication in HeLa cells (31). Therefore, it seemed possible that the virus rescued by the internal restriction fragments (Fig. 4) might induce depressed levels of E1A mRNA (as the result of a second mutation in the virus nearer to the transcription start site) and yet be able to form plaques on HeLa cells. To determine whether this was the case, two independent viruses rescued by the fragment mapping from nucleotides 859 to 1009 (Fig. 4) were analyzed. Both of these rescued viruses maintained the silent mutation at nucleotide 855 as judged by the elimination of an *Ava*I restriction site present at this position in wild-type Ad5 (Fig. 6A). However, both of these rescued viruses induced wild-type levels of E1A mRNA at 6 h p.i. (Fig. 6B). These results prove that the single-base-pair deletion of nucleotide 897 is responsible for the depressed E1A mRNA levels induced by 15606. Any other base-pair changes which may occur in 15606 (including the CG → GC transversion at position 855) do not result in a host range phenotype or affect E1A transcription.

The defect in 15606 E1A transcription is due principally to a *cis*-acting mutation. The single-base deletion at position 897 should lead to out-of-phase translation and subsequent premature termination of both the 15606 E1A early proteins. Therefore, we considered the possibility that the 15606 E1A transcription defect might result from the production of defective E1A proteins rather than from a defect in a *cis*-acting transcription control region. Although transcription of E1A is not dependent on E1A proteins, wild-type E1A proteins stimulate E1A transcription about fivefold (6, 54).

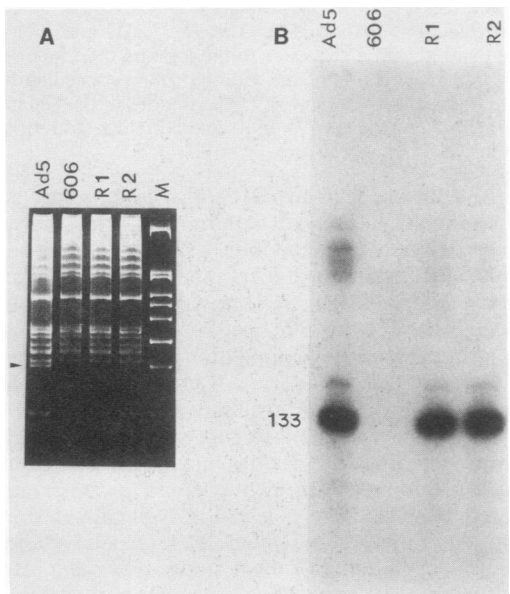

FIG. 6. E1A transcription defect caused by the deletion at position 897. (A) Ethidium bromide-stained polyacrylamide gel of *Ava*I-digested viral DNA. R1 and R2 are two independent viruses rescued for the ability to form plaques on HeLa cells by the fragment from nucleotides 859 through 1009. 606, R1, and R2 all have a mutation which eliminates the *Ava*I site at position 854. The resulting fused *Ava*I fragment is not well resolved on this polyacrylamide gel but could be observed on agarose gels (not shown). M, *Hin*fI digest of pBR322. (B) Quantitative S1 analysis of early E1A mRNA isolated 6 h p.i. from untreated cells. The probe was the 5'-end-labeled terminal *Sau*3A fragment, labeled 133 nucleotides from the major E1A cap site. The defect in E1A mRNA synthesis was completely rescued by the fragment from nucleotides 859 through 1009.

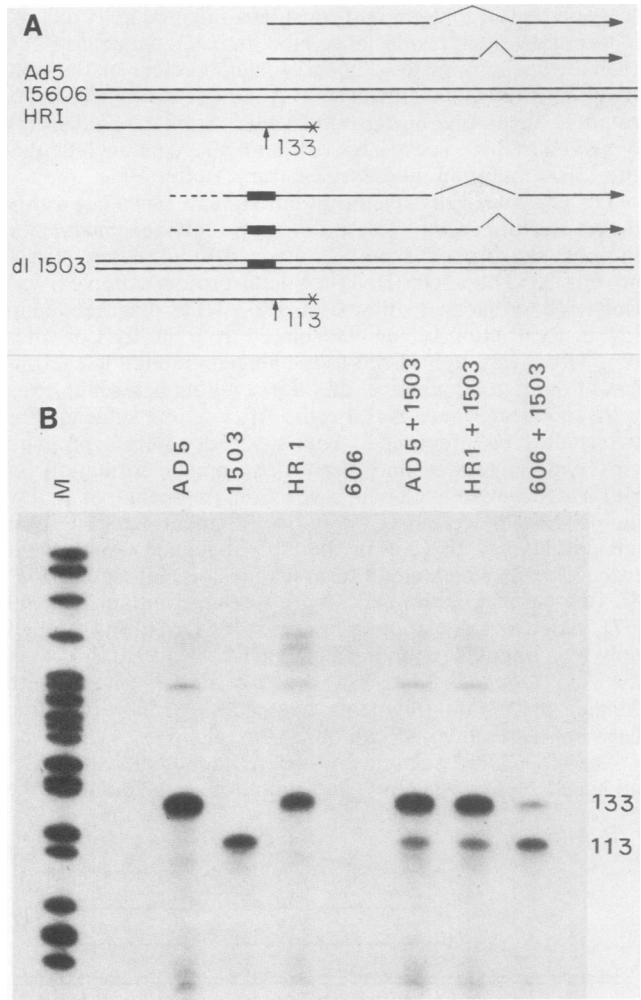

FIG. 7. E1A transcription defect in 15606 as a result primarily of a *cis*-acting mutation. (A) Diagram of the S1 analysis of E1A mRNA transcribed from the Ad5, 15606, or *hr*1 template and E1A mRNA transcribed from the *dl*1503 template by means of the wild-type 5'-end-labled *Sau*3A fragment. Ad5, 15606, and *hr*1 E1A mRNAs each generated a 133-nucleotide S1-protected fragment. *dl*1503 E1A mRNAs generated a 133-nucleotide S1-protected fragment. *dl*1503 has a deletion of the E1A TATA box region (solid rectangles) and generated E1A mRNAs with heterogeneous 5' termini (56) represented by the 5' ends of the *dl*1503 mRNAs (dashed lines). (B) Quantitative S1 analysis of cytoplasmic RNA isolated 6 h p.i. from HeLa cells singly infected with Ad5 or the indicated mutants (MOI, 20) or coinfected with the indicated mutants (each at MOI of 10). The 5'-end-labeled terminal *Sau*3A fragment was used as probe (shown in A). *hr*1 is an E1A host-range mutant (37) defective in the transcription-inducing activity of the E1A 289-aa protein (6, 61). Coinfection with *dl*1503 provided sufficient E1A protein to complement E1A mRNA synthesis from *hr*1 to wild-type levels, whereas 15606 E1A mRNA synthesis was depressed 10-fold compared with wild-type in coinfected cells. M, end-labeled, *Hpa*II-digested M13 RF.

Perhaps the defective E1A proteins synthesized by 15606 might actually repress E1A transcription.

To test whether the transcription-inhibiting defect in 15606 was due to mutant E1A protein or to a *cis*-acting mutation, we coinfected HeLa cells with 15606 and a helper virus to supply E1A proteins in *trans*. If the 15606 E1A proteins were repressors of E1A transcription, we would expect them to repress transcription of the helper E1A genes as well as those of 15606. Alternatively, if the 15606 transcription defect was due to a mutation in a transcription control region, we might expect some stimulation of 15606 E1A transcription by the wild-type E1A proteins provided by the helper virus, but the level of transcription would still remain significantly depressed compared with the wild type. This second alternative was observed.

The helper in these coinfection experiments was *dl*1503 (56), a mutant with a deletion from nucleotides 455 to 519 in the Ad5 sequence, so that E1A mRNAs transcribed from the helper virus could be distinguished from 15606 E1A mRNAs. The *dl*1503 deletion removes the E1A TATA box region, resulting in a reduction of *dl*1503 E1A mRNA levels to 10 to 20% of those in the wild type (56). However, the *dl*1503 deletion does not extend into the protein-coding portion of E1A, so wild-type E1A proteins are synthesized and *dl*1503 is able to replicate with normal kinetics in HeLa cells (56). To distinguish E1A mRNAs transcribed from 15606 and *dl*1503, E1A mRNAS were analyzed by S1 mapping with a wild-type 5'-end-labeled restriction fragment extending from nucleotides 1 to 631 in the Ad5 sequence (Fig. 7). This fragment produces a 113-nucleotide, S1-protected, labeled fragment when hybridized to *dl*1503 E1A mRNA and a 133-nucleotide fragment when hybridized to 15606 E1A mRNA (Fig. 7).

As a further control, HeLa cells were also infected with Ad5 *hr*1 and the same helper, *dl*1503. Ad5 *hr*1 is an E1A mutant defective in the transcription-inducing activity of the 289-aa protein as the result of a single-base-pair deletion at position 1055 (6, 37, 61). Infection with *hr*1 alone resulted in one-fifth the level of E1A mRNA found in infection with wild-type Ad5 (Fig. 7). Coinfection of *hr*1 plus *dl*1503 resulted in an increase in *hr*1 mRNA to the same level as that of the wild type in coinfected cells. This is because *dl*1503 supplied wild-type E1A proteins which stimulated transcription from the *hr*1 template. Coinfection with *dl*1503 also stimulated the level of 15606 E1A mRNA compared with that in cells singly infected with 15606 (Fig. 7; compare 606 with 606 + 1503). As with *hr*1, the stimulation was fivefold. However, the concentration of 15606 E1A mRNA after coinfection with *dl*1503 was depressed by a factor of 10 compared with wild-type or *hr*1 E1A mRNAs in cells coinfected with *dl*1503 (Fig. 7). This result demonstrates that the single-base deletion at nucleotide 897 causes a *cis*-acting defect in E1A transcription. Furthermore, this mutation has a more significant impact on E1A early mRNA levels than the TATA box region deletion of *dl*1503 (Fig. 7). Similar results were observed in three successive experiments.

A further control experiment was performed to check that equal numbers of 15606 and *dl*1503 templates were present in the coinfected cells and to rule out any possible *cis*-acting repression by mutant 15606 proteins. E3 mRNA (mapping from approximately 77 to 86 map units [8]) isolated from the coinfected cells was analyzed by quantitative S1 mapping. Transcription of E3 is under the control of the E1A 289-aa protein (6, 42). 15606 is wild type in E3 and generates S1-protected fragments of 490 and 375 bases from the probe used in this analysis (Fig. 8A). *dl*1503 contains a deletion in E3 (56) and generates S1-protected fragments of 375, 300, and 190 nucleotides (Fig. 8A). Infection with 15606 or Ad5*hr*1 alone resulted in greatly reduced levels of E3 mRNA compared with Ad5-infected cells, as expected for E1A mutants (Fig. 8B). *dl*1503 induced somewhat lower concentrations of E3 mRNA than wild-type Ad5, as observed previously, because of the decreased E1A expression of *dl*1503 compared with the wild type (56). Most important, equal concentrations of E3 mRNAs were transcribed from both viral templates in the cells coinfected with *dl*1503 and 15606. Therefore, both templates were present in approximately equal numbers in the coinfected cells and were equally available for transcription of E3. Since the same RNA preparation showed reduced levels of 15606 E1A mRNA (Fig. 7), we conclude that the defect in E1A mRNA synthesis was due to a *cis*-acting mutation in 15606 which affected E1A transcription specifically.

The 15606 mutation does not affect E1A transcription

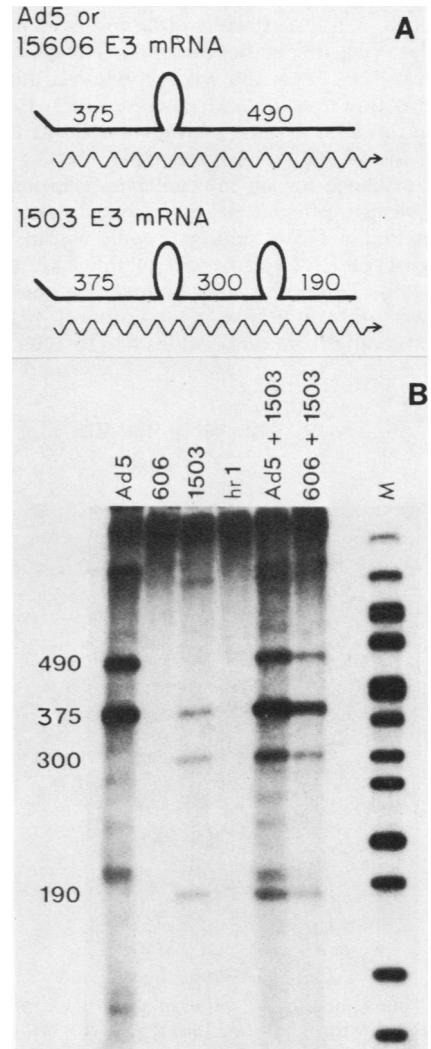

FIG. 8. Synthesis of equal amounts of E3 mRNAs from the *dl*1503 and 15606 templates in coinfected cells. (A) Diagram of the S1 analysis of 15606 and *dl*1503 E3 mRNAs. 15606 E3 mRNA generated S1-protected fragments of 490 and 375 nucleotides. *dl*1503, which contains a small deletion in the region corresponding to the probe, generated S1-protected fragments of 375, 300, and 190 nucleotides. (B) S1 analysis of E3 mRNAs in the same RNA preparations analyzed in Fig. 7.

during the late phase of infection. HeLa cells infected with 15606 alone do not enter the late phase of infection because of the defect in expression of early functions under E1A control. However, cells coinfected with 15606 and the helper *dl*1503 do progress into the late phase of infection. Cytoplasmic RNA was isolated from a culture of coinfected cells through the course of infection during the early (6 h p.i.) and late (12, 16, 20, and 28 h p.i.) phases. S1 analysis of E1A mRNA with the probe which distinguishes 15606 E1A mRNA from *dl*1503 E1A mRNA (Fig. 9) revealed that 15606 E1A mRNA reached wild-type concentrations during the late phase of infection. As observed previously, at 6 h p.i. there was considerably less 15606 E1A mRNA (133 band) than *dl*1503 E1A mRNA (113 band). However, during the late phase of infection, the 15606 E1A mRNA increased to levels equal to those of *dl*1503 E1A mRNA, and by 28 h p.i. it reached greater concentrations than *dl*1503 E1A mRNAs. These results indicate that the 897-base-pair deletion has a much less significant effect on E1A transcription during the late phase of infection than during the early phase. The activation of 15606 E1A transcription followed a time course typical of late-phase-specific functions. The observation that 15606 E1A mRNAs reached wild-type levels during the late phase of infection makes it extremely unlikely that the early-phase defect in E1A mRNA synthesis was due to decreased stability of the mutant E1A transcripts.

Further evidence for an internal transcription control region. The results presented above lead to the unexpected conclusion that a DNA sequence well within the protein-coding region of E1A forms part of the E1A transcription control region. To confirm this finding, we constructed and studied two other mutants. One mutant, *dl*1505, has a deletion extending from nucleotides 626 to 1009, completely within protein-coding sequence and overlapping the single-base deletion in 15606. An 8-base-pair synthetic *Xho*I restriction site linker was inserted at the position of the deletion to provide a convenient site for preparing an end-labeled probe for S1 analysis. A second mutant, *in*1512, has two insertions of this *Xho*I restriction site linker, one at position 625 and one at 1009. Thus, *in*1512 is identical to *dl*1505, except that the sequence from nucleotides 626 to 1009, containing the apparent internal transcription control region, has been reinserted into *in*1512 (Fig. 10A).

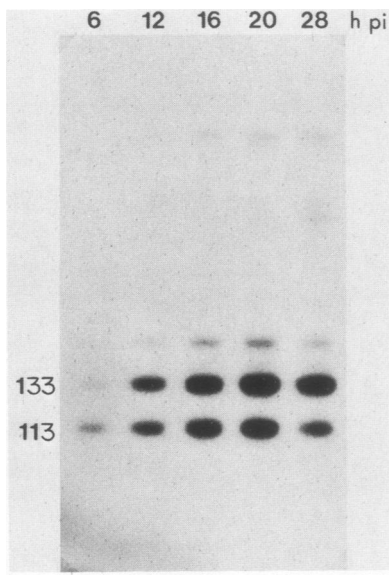

FIG. 9. Time course of induction of 15606 E1A mRNA in HeLa cells coinfected with 15606 and *dl*1503. Cytoplasmic RNA was isolated at the indicated times p.i. and assayed by S1 analysis with the 5′-end-labeled wild-type *Sau*3A fragment as probe. The 133-nucleotide band was generated by 15606 E1A mRNA, and the 113-nucleotide band was generated by *dl*1503 E1A mRNA. The band migrating more slowly than the 133-nucleotide band was due to S1 cutting at the A-T-rich TATA box region of hybrids between the probe and 15606 E1A mRNA initiated at sites upstream from the major cap site (55).

HeLa cells were coinfected with wild-type Ad5 to provide normal concentrations of E1A proteins in *trans* and with either *dl*1505 or *in*1512. Nuclear RNA was isolated at 2, 4, 6, (early phase), and 16 (late phase) h p.i. The concentrations of Ad5 and mutant nuclear RNAs isolated from the coinfected cells were determined separately by S1 analysis with specific end-labeled probes. To measure wild-type E1A RNA, a probe generated by *Sau*3A digestion was used extending from nucleotide 1 to a 5′-end label at nucleotide 631 in the wild-type Ad5 sequence. A hybrid between this probe and wild-type E1A RNA is fully duplex to the 5′-labeled end of the probe and results in a labeled fragment after S1 digestion (Fig. 10B). The major E1A mRNAs generate a fragment of 133 nucleotides. However, a hybrid between this probe and either *dl*1505 E1A RNA or *in*1512 E1A RNA is single stranded to the right of nucleotide 625 so that the 5′-end label is digested by S1 (Fig. 10B). Consequently, this probe detects Ad5 RNA specifically.

To measure *dl*1505 or *in*1512 RNA, a probe was used extending from nucleotide 1 to a 5′-end label at the end generated by *Xho*I digestion of these mutant DNAs, i.e., at nucleotide 631 in both the *dl*1505 and the *in*1512 sequences. This probe forms a hybrid with either *dl*1505 or *in*1512 RNA in which the end label is resistant to S1 digestion. The major mutant E1A RNAs generate an S1-protected fragment of 133 nucleotides. However, a hybrid between wild-type E1A RNA and the mutant-specific probe is single stranded to the right of nucleotide 625 so that the end label is sensitive to S1 (Fig. 10B). Consequently, this probe is specific for *dl*1505 and *in*1512 RNA.

S1 analyses performed with these specific probes upon nuclear RNA isolated from the coinfected cells were fully consistent with the conclusions of the experiments performed with 15606. In cells coinfected with equal multiplicities of Ad5 and *dl*1505 (10 PFU per cell), Ad5 nuclear RNA was just detectable at 2 h p.i., whereas *dl*1505 nuclear RNA could not be detected (Fig. 10C). At 4 and 6 h p.i., the level of wild-type nuclear RNA was 10 times that of *dl*1505 nuclear RNA in the same cells and transcribed from a equal number of templates per cell. However, during the late phase of infection (16 h p.i.), the level of Ad5 and *dl*1505 nuclear RNAs were approximately equal. Analyses of E3 RNAs showed equal concentrations of Ad5 and *dl*1505 RNAs in the 4- and 6-h samples (data not shown). E3 RNA was not detectable at 2 h p.i. Similar results were observed in three successive experiments. The relative concentrations of Ad5 and *dl*1505 nuclear RNAs at 4 h p.i. in these three experiments are shown in Table 1. The mutant *dl*1505 cannot synthesize an equivalent of the E1A 12S mRNA because the 12S mRNA 5′ splice site has been deleted. However, this cannot be the cause of the decreased levels of E1A nuclear RNAs. Another mutant, *pm*975, is also defective in the synthesis of the 12S mRNA (51). Nonetheless, infection of HeLa cells with mutant *pm*975 leads to wild-type levels of the 13S mRNA during the early phase of infection and of the 13S and 9S E1A mRNAs during the late phase (51). Mutant

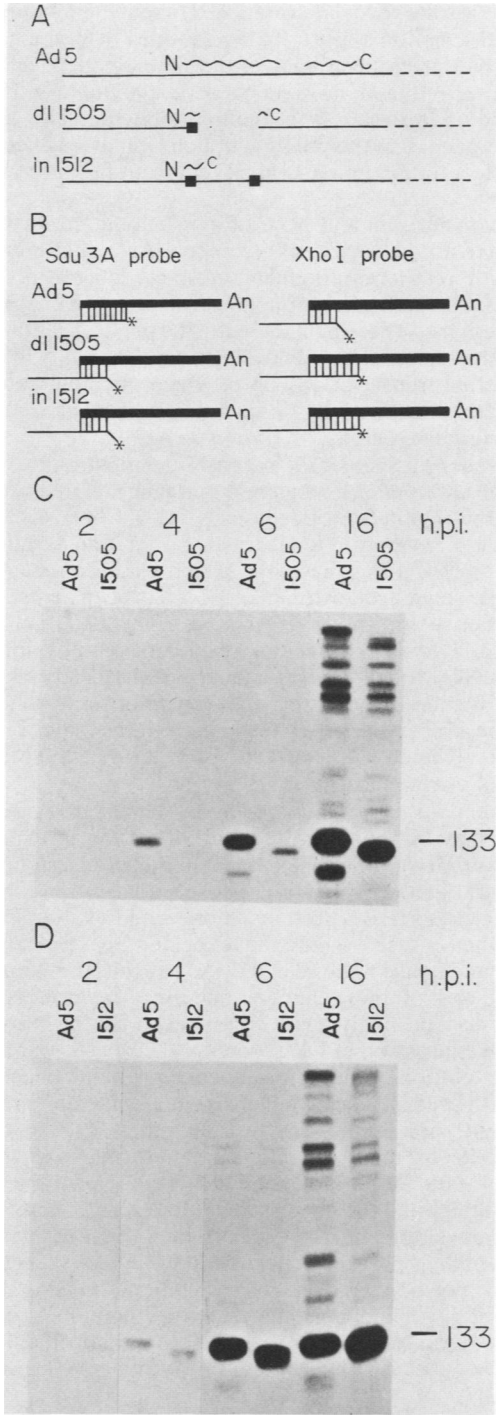

FIG. 10. *cis*-acting defect in E1A transcription in cells coinfected with wild-type Ad5 and *dl*1505. (A) Diagram of the left end of the *dl*1505 and *in*1512 genomes. The left 1,600 base pairs of the Ad5 genome are represented by the line at the top. The region deleted in *dl*1505 is represented by the missing portion of the *dl*1505 line. Inserted *Xho*I linkers are represented by the dark squares in *dl*1505 and *in*1512. The sequence translated into the 289-aa E1A protein by Ad5, and the truncated E1A proteins translated from the mutant sequences, are represented by the wavy lines between N and C. (B) Diagram of hybrids formed between the wild-type or mutant RNAs and the wild-type *Sau*3A end-labeled probe (left) or the mutant *Xho*I end-labeled probe (right). (C) S1 analyses of nuclear RNAs isolated from cells coinfected with wild-type Ad5 and *dl*1505 at 2, 4, 6, and 16 h p.i. Ad5 E1A RNA was detected specifically with the wild-type probe end labeled at the *Sau*3A site. *dl*1505 E1A RNA was detected specifically with the *dl*1505 probe end labeled at the *Xho*I site. Both major S1-protected fragments are 133 nucleotides long, but they migrated to slightly different positions in the gel owing to differences in sequence. The 16-h samples show the late-phase-specific upstream starts (55). The high levels of S1 used to digest completely the label in nonhomologous hybrids led to additional cleavage in a fraction of the Ad5 RNA-*Sau*3A probe hybrids, generating the band below band 133 in the Ad5 tracks. (D) S1 analysis of nuclear RNA isolated from cells coinfected with wild-type Ad5 and *in*1512. Ad5 E1A RNA was specifically detected with the *Sau*3A probe, and *in*1512 E1A RNA was detected with the *Xho*I probe.

TABLE 1. Quantitative comparison of E1A nuclear RNA levels[a]

Expt	Mutant RNA/wild-type RNA[b]	
	*dl*1505	*in*1512
1	0.07	
2	0.10	1.0
3	0.15	0.95

[a] Coinfections were performed as described in the text with wild-type Ad5 and the mutants indicated. In each experiment, analysis of E3 transcription showed equal levels of Ad5 and mutant-specific E3 RNA (see the text).
[b] Ratio of mutant-specific RNA to wild-type Ad5-specific RNA at 4 h p.i.

*pm*975 also induces wild-type levels of E1A nuclear RNAs (C. Montell and A. J. Berk, unpublished observations).

As a control for the experiments with *dl*1505, we performed similar experiments with *in*1512, which contains the internal control region. HeLa cells coinfected with Ad5 and *in*1512 expressed similar levels of wild-type and *in*1512 nuclear RNAs during both the early (2, 4, and 6 h) and late (16 h) phases of infection (Fig. 10D, Table 1). These control experiments were performed with the same probes used in the experiments with *dl*1505 and confirm that the method of analysis was sound. Since *dl*1505 and *in*1512 should give rise to very similar truncated E1A proteins (Fig. 10A), these control experiments also rule out the possibility that the defect in *dl*1505 and 15606 was due to a *cis*-acting effect of the mutant E1A proteins. We conclude that a sequence mapping entirely within the protein-coding region of E1A is required in *cis* to achieve maximum rates of E1A transcription. When this internal transcription control region is defective, as in 15606 and *dl*1505, and E1A protein is provided in *trans*, the rate of transcription during the early phase is approximately 1/10 the rate of transcription of the wild-type template.

High concentrations of E1A protein do not relieve the early-phase-specific requirement for the internal transcription control region. Recent studies have demonstrated that the E1A protein encoded by the 13S mRNA will stimulate transcription from nonviral genes as well as adenovirus genes which are newly introduced into mammalian cells (24a, 30, 74a). Treisman et al. (74a) showed that E1A protein activation of the early simian virus 40 (SV40) promoter eliminates the requirement for the SV40 72-base-pair enhancer sequence. This finding suggested to us that the decreased requirement of E1A transcription for the internal control region during the late phase of infection might be due to the high concentrations of E1A protein synthesized during the late phase. That is, it seemed possible that once sufficient E1A protein accumulated, the stimulation of E1A transcription it produced might not require the internal sequence. To test this

possibility, we assayed E1A nuclear and cytoplasmic RNA synthesized after the infection of 293 cells with *dl*1505 and *in*1512. 293 cells constitutively express concentrations of E1A mRNAs similar to that observed in HeLa cells at the end of the early phase of infection (6). Using the mutant-specific S1 probe, we observed that at 4 h p.i., *in*1512 RNA was present at 10-fold higher concentrations than *dl*1505 RNA in both nuclear and cytoplasmic fractions (Fig. 11). We conclude that some late-phase-specific factor(s), and not simply E1A protein itself, is required to suppress the effect of the *dl*1505 deletion.

DISCUSSION

A single-base deletion 399 base pairs downstream from the major start site of Ad5 E1A transcription, within a protein-coding region, was found to decrease the rate of E1A transcription to 2% of the wild-type level. This decrease in the rate of transcription by a factor of 50 was due to two effects of the mutation. The first was a fivefold effect resulting from inactivation of the 289-aa E1A protein, an effect which has been noted previously (6, 54). The second effect of the mutation has not been observed in studies with other E1A mutants, neither the 15 generated here nor 6 others reported in the literature (13, 37). This second effect of the single-base deletion at position 897 in the Ad5 sequence is a *cis*-acting defect which depresses E1A mRNA and nuclear RNA concentrations by a factor of 10 when E1A protein is provided in *trans*. A second mutation, a deletion from base pair 626 to 1009 in the Ad5 sequence, completely within the coding region of E1A, has a similar phenotype.

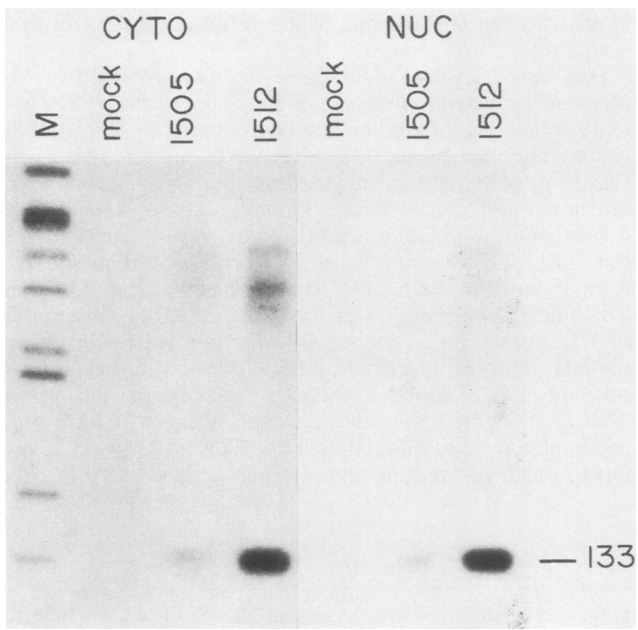

FIG. 11. S1 analyses of *dl*1505 and *in*1512 E1A RNA isolated 4 h p.i. from 293 cells. RNA isolated from mock-infected cells or mutant-infected cells was analyzed with the *Xho*I end-labeled probe specific for mutant RNA. The concentration of Ad5 E1A RNA transcribed from the integrated Ad5 DNA in 293 cells was several-fold higher than the *in*1512 RNA (data not shown). The low signal in the lanes from mock-infected cells demonstrates the specificity of the probe. The nuclear RNA analysis (NUC) was exposed four times longer than the cytoplasmic RNA analysis (CYTO). M, Marker.

These results reveal a critical DNA sequence required for E1A transcription almost 400 nucleotides from the cap site, within the transcribed region of the gene. Sequences regulating transcription of most eucaryotic polymerase II genes analyzed lie upstream from the initiation site. The adenovirus E1A gene, like the immunoglobulin genes, is exceptional in having a transcription control region so far interior to the gene.

O. M. Andrisani and N. C. Jones (submitted for publication) have used a variety of techniques to assay for enhancer activity in restriction fragments from the left end of the Ad5 genome. Several nonoverlapping fragments were found to have activity. The fragment with the highest activity contained the region of the 15606 deletion. Thus, it is likely that the internal transcription control region demonstrated here has the enhancer activity described for the elements of other viral promoter regions.

Hearing and Shenk (39) recently demonstrated the existence of an enhancer sequence mapping upstream of E1A within the region from positions −141 to −305. Deletion of this region resulted in a decrease in E1A transcription by a factor of 20 during the early phase of infection. Another enhancer sequence upstream of E1A has been mapped to the region between positions −302 and −349 (39a). Yet another enhancer activity has been mapped between positions −188 and −45 (41). Finally, an enhancer activity has been demonstrated for the inverted terminal repeat of the Ad2 genome, mapping at positions −498 to −396 from the major E1A start site (R. Kumar, S. Hartzell, and K. N. Subramanian, personal communication).

Why should E1A contain so many enhancer regions? The answer may be found in the functions of the E1A proteins. The larger E1A protein (289 aa) facilitates transcription of all other viral genes (6, 42, 54). Those viruses in a population which express E1A proteins more rapidly after infection than other viruses would be expected to replicate more rapidly and hence to be selected. As a result, the sequence of the left end of the genome, including the protein-coding region, has probably evolved to result in the most rapid possible expression of E1A proteins. This has apparently led to the evolution of multiple sequences with enhancer activity. Furthermore, since enhancers may operate more efficiently in some cell types than in others (3, 26, 60), the occurrence of multiple enhancers may increase the host range of Ad5. Apparently, the structural constraints of the E1A proteins and the degeneracy of the genetic code permitted the development of a transcription enhancer within the protein-coding portion of E1A in the vicinity of nucleotide 897, the position of the 15606 mutation. Indeed, the Ad5 sequence in this region shows strong homology with the proposed core sequence of the SV40 enhancer (80) and a similar sequence within the polyomavirus enhancer (17, 75) (Fig. 12).

Although the deletion of nucleotide 897 has such a significant effect early in infection, late in infection the mutant E1A mRNAs are present at the same concentration as those of the wild type. This is also true of mutants which delete the upstream enhancer identified by Hearing and Shenk (39). In terms of initiation rates per template, E1A transcription rates are much lower during the late phase than during the early phase; the template copy number is amplified greatly (thousands of times), whereas the E1A transcription rate increases only about 10 times compared with the early phase (66, 69). The difference between the effect of the 15606 mutation on E1A mRNA synthesis at early and late times indicates that the DNA sequences required for transcription

SV40 TTTCCACACC 241-250
Py TTTCCACcCA 5236-5245
Ad5 TTTCTATGC*C 888-897

FIG. 12. Homology between the Ad5 sequence in the vicinity of the 15606 deletion and the SV40 sequence near the proposed core of the SV40 enhancer (80). A homologous sequence from the region of the polyomavirus enhancer region (Py) is also shown. Nucleotide numbers shown at the right are from the sequences in Tooze (74). The SV40 sequence in the sense of the late mRNAs is shown. The polyomavirus and Ad5 sequences are shown in the sense of the early mRNAs. The asterisk marks the position of the single C deletion in 15606.

during the late phase are different from those required during the early phase. This change in sequences required for E1A transcription is not due entirely to the activities of E1A proteins since *dl*1505 was defective for E1A transcription in 293 cells, cells which express E1A proteins from integrated Ad5 DNA. There is ample evidence that the specificity of transcription initiation is altered during the late phase of infection. The IX gene, IVa2 gene, and E2 late gene are not transcribed early but are transcribed during the late phase of infection (14). The activity of the major late promoter and the VA polymerase III promoters turn up greatly during the late phase (42, 66). Thomas and Mathews (73) have shown that the early-to-late switch in mRNA synthesis from the major late transcription unit requires that the template undergo DNA replication. DNA replication was also found to be necessary for the synthesis of a minor class of late-phase-specific E1A mRNAs which have 5′ ends mapping far upstream from the major cap site (55). DNA replication may also be necessary for the change in sequence requirements for E1A transcription observed in the studies presented here.

DNA viruses go to extraordinary lengths to encode genetic information in the limited amount of DNA which can be packaged into their capsids. The internal transcription control region of adenovirus E1A may be an example of this remarkable economy. A similar situation may occur in the early region of bovine papillomavirus, in which a restriction fragment from within the coding sequence was found to have enhancer activity (12). Of course, the precedent of an internal transcription control region already exists for genes transcribed by RNA polymerase III (10, 63). Future studies are required to determine whether other protein-coding genes besides adenovirus E1A and immunoglobulin genes contain internal control regions. But in the future, it cannot be assumed that all elements of polymerase II promoters lie upstream from the transcription start site.

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