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The Adenovirus Type 5 i-Leader Open Reading Frame Functions in *cis* To Reduce the Half-Life of L1 mRNAs

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The 440-nucleotide adenovirus type 5 i-leader sequence, encoding a 13.6-kilodalton protein, is located between the second and third components of the tripartite leader sequence. It appears primarily on the L1 family of mRNAs. To study its function, we constructed two point mutations within the i leader. *pm*382 lacks the wild-type i-leader splice acceptor and failed to splice the leader onto L1 mRNAs. *pm*383 lacks the ATG used for translation of the i-leader protein; it synthesized i-leader-containing mRNAs, but failed to produce detectable levels of the polypeptide. Both mutants exhibited modestly reduced yields in some but not all cell lines tested and accumulated slightly elevated levels of L1 mRNA and L1 52- and 55-kilodalton proteins in infected cells. Mutant phenotypes were consistently more pronounced in *pm*382- than in *pm*383-infected cells. In wild-type virus-infected cells, L1 mRNAs lacking the i leader displayed a half-life of about 26 h, whereas L1 mRNAs containing the leader were much less stable, with a half-life of less than 4 h. In *pm*383-infected cells (ATG mutant), L1 mRNAs containing the i leader exhibited a half-life of 26 h. The abnormally long half-life of *pm*383-encoded L1 mRNAs containing a mutant i leader was not reduced by coinfection with wild-type virus, suggesting that synthesis of the i-leader protein leads to destabilization of the i-leader-containing L1 mRNA undergoing translation.

All adenovirus mRNAs transcribed from the major late promoter have a 200-nucleotide (nt) tripartite leader spliced to their 5′ ends (4, 11, 21). It consists of three segments, termed leaders 1, 2, and 3 that are 41, 71, and 88 nt in length and are encoded at 16.5, 19.5, and 22.5 map units, respectively (2, 38). mRNAs that carry the tripartite leader sequence are more efficiently translated within adenovirus-infected cells than are equivalent mRNAs that lack the leader (5, 6, 20, 24, 34). Tripartite leader-bearing mRNAs can be translated within poliovirus-infected cells in which cap-binding complex has been inactivated (9, 13). This raises the possibility that the increased efficiency with which these mRNAs are translated results from their ability to function in the absence of cap-binding complex, which may become rate limiting within adenovirus-infected cells.

The i leader is 440 nt in length and encoded at 22 to 23 map units on the chromosome. When it is spliced onto late mRNAs with the tripartite leader, the order is 1-2-i-3 (see Fig. 2A). At 16 h postinfection in HeLa cells, when more than half of the adenovirus mRNAs are made from the major late promoter, one-third of them contain the i leader; of these, more than half are members of the L1 family of mRNAs (10). Therefore, although the i leader is found on many RNAs, it is associated primarily with L1 mRNAs, specifically with the largest of the three major L1 species. Unlike any of the other adenovirus mRNA leader sequences, the i leader contains an open reading frame (see Fig. 2C) initiating 27 nt from its 5′ end, terminating at nt 22 of the third leader and encoding a 135-amino-acid (13.6-kilodalton [kDa]) protein (1, 14, 23, 35). Antibody generated against a peptide corresponding to the N terminus of the i-leader protein was used to show that it is made in vivo as early as 8 h postinfection in KB cells, accumulates maximally at 25 h, and is localized at the nuclear membrane (32).

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To investigate the function of the i leader, we generated two mutations in plasmids by oligonucleotide mutagenesis and rebuilt them into adenovirus type 5 (Ad5). The mutations (see Fig. 2B) were designed to distinguish the role of the i-leader protein from the i-leader RNA. In *pm*383, the ATG used for initiation of translation of the i-leader protein was changed to GTG. In *pm*382, the wild-type sequence GTAGGT containing the i-leader 5′ splice acceptor was changed to ATATGT (see Fig. 2B). Neither of these mutations altered the amino acid sequence of the viral DNA polymerase that is encoded by E2B mRNAs transcribed from the opposite DNA strand. Analysis of L1 mRNA metabolism within mutant virus-infected cells demonstrated that mRNAs containing a functional i-leader protein coding region exhibited a substantially reduced half-life as compared with L1 mRNAs that lack the coding region. A functional open reading frame providing the potential for synthesis of the i-leader protein was found to destabilize L1 mRNAs.

MATERIALS AND METHODS

Plasmids and mutagenesis. p*Xba*29 contains *dl*308/310 (33) sequences extending from the left end of the Ad5 chromosome to the *Xba*I site at sequence position 10589 inserted into the *Eco*RI and *Xba*I sites of a pBR322 variant to which an *Xba*I linker was added at the *Pvu*II site. The 2,922-nt *Hin*dIII-*Kpn*I fragment containing the i leader was subcloned into the corresponding sites of mp19 to generate pi6mut, from which single-stranded DNA was prepared as a template for mutagenesis. The method of Zoller and Smith (39) was used to generate mutations. Briefly, 20 pmol of mutant oligonucleotide (*pm*382, 5′-GATGTAGTATACACT GTTTC-3′; and *pm*383, 5′-CGGCTCGGCACCCTCGCAC-3′) was annealed to 2 pmol of single-stranded template by heating the mixture in 20 μl of a buffer containing 50 mM NaCl, 10 mM Tris (pH 8.0), 7 mM $MgCl_2$, and 1 mM EDTA in a 55°C water bath for 10 min. The heat was turned off, and the incubation was continued for 6 h. The mixture was then

diluted to 80 μl at a final concentration of 50 mM Tris (pH 8.0), 7 mM $MgCl_2$, 1 mM EDTA, 1.25 mM ATP, and 0.16 mM dATP, dCTP, dGTP, and dTTP. Klenow fragment of DNA polymerase I and T4 DNA ligase (New England BioLabs, Inc., Beverly, Mass.) were added (20 U of each), and the incubation was continued at 14°C for 14 h. JM101 cells were transformed with mutated DNA, and plaques were bound to filters and screened by selective thermal elution of labeled mutant oligonucleotides from wild-type templates. The efficiency with which mutations were introduced was about 3%. Mutations were subcloned back into p*Xba*29, and then wild-type Ad5 sequences were ligated from the *Xba*I site to a *Sal*I site at 45 map units to generate p45mut382 and p45mut383. Viruses were rebuilt by cotransfecting 293 cells with *Eco*RI-linearized p45mut382 or p45mut383 and a *dl*308 fragment extending from the *Bcl*I site at 35 map units to the right end of the chromosome by the calcium phosphate precipitate method (29).

RNA probes for use in RNase protection analysis were derived from a variety of plasmids. Plasmid sp65i contains a 680-nt *Sac*I fragment of Ad5 DNA cloned into the *Sac*I site of sp65 (27). SP6 polymerase transcription of *Hin*dIII-linearized template generates a 729-nt RNA that covers part of the intron between the second leader and the i leader, as well as all of the i leader except 17 nt at its 3′ end, protecting 423 i-leader nucleotides. pGEM45′L1 contains the 981-nt *Xba*I-*Hin*dIII fragment of Ad5 DNA covering the 5′ end of the L1 region, cloned into GEM4 (Promega Biotec, Madison, Wis.). Transcription of *Ava*II-digested template with T7 RNA polymerase generates a 639-nt transcript that protects 520 nt of L1. pGEM2L3, prepared by J. Wilusz in our laboratory, contains the *Hin*dIII-*Kpn*I fragment carrying the 3′ end of the L3 coding region cloned into the corresponding sites of GEM2 (Promega). Transcription by T7 polymerase of *Eco*RI-digested plasmid generates a 311-nt transcript which protects 207 nt of L3 mRNAs.

Cells and viruses. H5*dl*308 is a phenotypically wild-type derivative of Ad5 (19). 293 cells, which contain and express the E1A and E1B genes of Ad5 (15), were grown in medium containing 10% calf serum. Vero cells (obtained from G. Ketner, Johns Hopkins University, Baltimore, Md.) were propagated in medium containing 5% calf serum, and BJA-B cells (obtained from S. Chen-Kiang, Mount Sinai School of Medicine) were grown in medium containing 10% fetal calf serum.

Protein analysis. Infected cells were labeled with [^{35}S]methionine (200 μCi/ml; 1,200 Ci/mmol), and then either whole-cell extracts (17) or fractions enriched in the constituents of various cellular compartments (36) were prepared. For cell fractionation, the cells were first extracted with RSB (10 mM Tris hydrochloride [pH 7.6], 10 mM NaCl, 1.5 mM $MgCl_2$) plus 0.5% Nonidet P-40 to yield a cytoplasmic fraction and then extracted with RSB plus 1.5% Nonidet P-40 and 0.75% deoxycholate to produce a nuclear membrane fraction, and then the nuclear pellet was subjected to sonication in buffer containing 50 mM Tris hydrochloride (pH 8.0), 10 mM NaCl, and 0.5% Nonidet P-40 to liberate a nuclear fraction. Immunoprecipitations of the L1 52- and 55-kDa and i-leader proteins were performed with antipeptide sera generously provided by M. Green, St. Louis University Medical Center, as described by Lucher et al. (25) and Symington et al. (32). Electrophoresis was carried out in either 10 or 15% gels (acrylamide/bisacrylamide ratio, 100:1) as described by Laemmli (22). Gels were prepared for fluorography by being fixed in 40% methanol–10% acetic acid for 3 to 24 h and then rinsed twice for 60 min in dimethyl sulfoxide. The treated gels were agitated in a solution of 30% 2,5-diphenyloxazole in dimethyl sulfoxide, rinsed for 30 min in water, dried, and subjected to autoradiography with preflashed X-OMAT AR film. Bands of interest were quantified by scanning films with a densitometer.

RNA analysis. Cytoplasmic RNA was prepared by extraction of cells with isotonic buffer (140 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris [pH 7.5]) containing 0.1% Nonidet P-40 and 10 mM vanadyl ribonucleoside complexes (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Protein was removed by adding 1% sodium dodecyl sulfate (SDS), 200 mM Tris (pH 7.4), 50 mM EDTA, and 100 μg of proteinase K per ml, incubating the mixture at 37°C for 30 min, and extracting it with phenol-chloroform (1:1). Polyadenylated RNA was isolated as described by Maniatis et al. (26).

For RNase protection analysis, probe RNAs were prepared by the method of Melton et al. (27). Hybridizations were performed in 80% deionized formamide containing 0.4 M NaCl, 1 mM EDTA, and 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4). RNA plus probe mixtures were heated to 80°C for 10 min and then held at 55°C for 3 to 18 h. After they had cooled to room temperature, 0.5 ml of HSB (0.3 M NaCl, 10 mM Tris [pH 7.5], 5 mM EDTA) plus 1 μl of RNase A (5 mg/ml) and 150 U of RNase T_1 (grade V; Sigma Chemical Co., St. Louis, Mo.) were added, and the mixture was incubated at room temperature for 30 min. Proteinase K (100 μg/ml) and 25 μl of 10% SDS were added, and the incubation was continued for 30 min at 37°C. Then extraction with HSB-equilibrated phenol-chloroform (1:1) was carried out. Products were precipitated with ethanol and subjected to electrophoresis on a 5% sequencing gel.

For RNA blot analysis, RNA was subjected to electrophoresis in formaldehyde-containing agarose gels as described by Maniatis et al. (26). RNA was transferred onto a GeneScreen membrane (Du Pont, NEN Research Products, Boston, Mass.) by using 10× SSC (1.5 M NaCl, 0.15 M sodium citrate). Hybridizations were performed in 7% SDS–1% bovine serum albumin–1 mM EDTA–250 mM Na_2HPO_4, with the pH adjusted to 7.2 with H_3PO_4, by the method of Church and Gilbert (12). Filters were washed four times for 5 min each at room temperature with 2× SSC containing 0.1% SDS and then four more times for 15 min each at room temperature with 0.1× SSC containing 0.1% SDS.

For RNA half-life analysis, fresh medium containing 5 μg of dactinomycin (Sigma) per ml was added to infected cells at 24 h postinfection. At various times after addition of drug, polyadenylated RNA was prepared and assayed by Northern (RNA) or RNase protection analysis. After exposure, preflashed films were scanned with a densitometer.

RESULTS

Confirmation of i-leader sequences on the largest L1 mRNA. Polyadenylated cytoplasmic RNA was isolated at different times from 293 cells infected with phenotypically wild-type Ad5 (*dl*308) and subjected to RNA blot analysis probing for the late L1 mRNA family or the i leader (Fig. 1). A single i-leader-containing mRNA that comigrated with the largest L1 mRNA encoding the 52- and 55-kDa proteins was detected at 7 h postinfection. As the infection progressed, i-leader sequences were detected on a wide variety of different-sized RNAs, many of which did not belong to the L1 family. These results fit well with the original description of i-leader-containing L1 mRNAs by Chow et al. (10), who performed R-loop mapping of viral mRNA species.

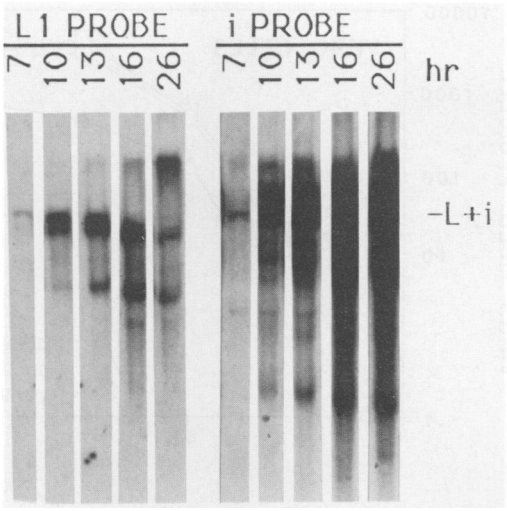

FIG. 1. RNA blot analysis of L1-specific mRNAs and RNAs that contain i-leader sequences. 293 cells were infected at a multiplicity of 20 PFU per cell with wild-type Ad5. Polyadenylated cytoplasmic RNA was isolated at the indicated times after infection and loaded in two sets on a 1.5% agarose–formaldehyde gel. After electrophoresis and transfer to nitrocellulose, the filter was cut to separate the two sets, hybridized to L1 or i-leader probe DNAs, washed, and autoradiographed. The position of L1 mRNA carrying the i-leader sequences (L+i) is indicated.

Mutations that block normal i-leader splicing or i-leader protein synthesis. i-leader function could depend on an activity of the i-leader protein, a structural feature of the i-leader RNA sequence, the presence of the i-leader reading frame on the mRNA, or any combination of the above. To discriminate among these possibilities, we constructed two mutant viruses (Fig. 2B). *pm*382 carries two single-base-pair changes in the i leader in the 5′ splice acceptor consensus. *pm*383 has a single-base-pair change in the ATG required for initiation of translation of the i-leader protein. Neither mutation altered the amino acid sequence of the viral DNA polymerase encoded on the DNA strand opposite the i-leader sequence.

The effect of the *pm*382 mutation on i-leader splicing was examined by RNase protection analysis (Fig. 3). Total cytoplasmic RNA was isolated at different times from Vero cells infected with *dl*308 (parental, wild-type virus), *pm*382, or *pm*383 and assayed by using an RNA complementary to the wild-type i-leader sequence as a probe. The 423-nt i-leader-specific band in *dl*308 and *pm*383 (ATG mutant) RNAs indicated the use of the wild-type splice site in cells infected with these two viruses. The faint 680-nt band represented an RNA including the i leader as well as the intron between leader 2 and the i leader. The 423- and 257-nt bands generated by *pm*382 (splice acceptor mutant)-infected cell RNA together represent the same 680-nt species. The two bands were produced since the RNA duplex formed between the wild-type probe and mutant RNA contained an RNase-sensitive 2-base-pair discontinuity at the i-leader splice site. This interpretation was confirmed by RNA blot analysis of cytoplasmic RNAs produced in *dl*382-infected cells (see Fig. 6). No species corresponding to the i-leader-containing L1 mRNA was evident.

To confirm that the mutations eliminated synthesis of the i-leader protein, we prepared [^{35}S]methionine-labeled extracts from infected Vero cells and immunoprecipitated them by using an antibody prepared against a synthetic peptide

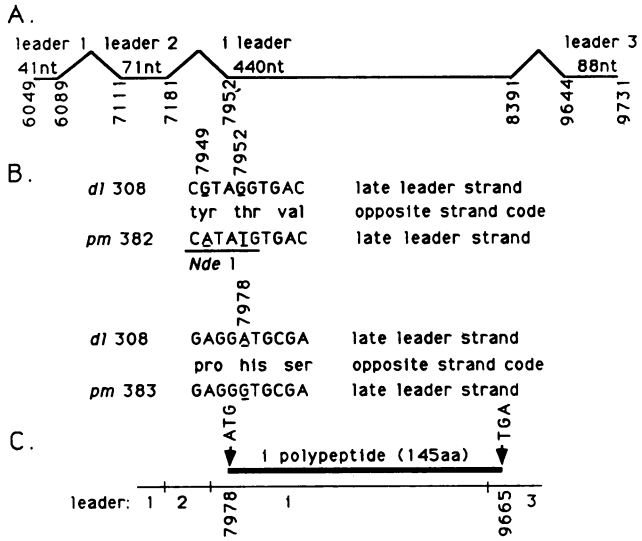

FIG. 2. Diagram of the i leader within the tripartite leader sequence, the i-leader coding region, and the mutations which alter the i leader. (A) Segments of the tripartite leader and i leader are represented by lines, and introns are indicated by inverted carat symbols. The sizes of leaders are indicated above the lines in nucleotides, and the sequence positions of the first and last nucleotides making up leader elements are indicated below the lines. (B) Nucleotide sequence alterations in *pm*382 and *pm*383 compared with their phenotypically wild-type parent, *dl*308. Mutated nucleotides are underlined. (C) i-leader polypeptide-coding region. The sequence positions of the first and last nucleotides encoding the polypeptide are indicated below the line. Abbreviation: aa, amino acids.

corresponding to the N terminus of the i-leader protein. Neither mutant encoded detectable levels of the protein (Fig. 4).

***pm*382 and *pm*383 exhibit modest growth defects and elevated L1 gene expression.** The yields of the two mutant

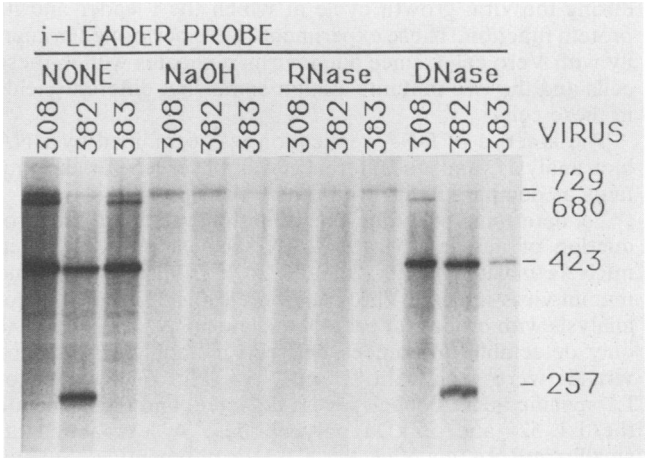

FIG. 3. RNase protection analysis of i-leader-containing RNAs produced in mutant- and wild-type-infected cells. At 24 h after infection of Vero cells at a multiplicity of 20 PFU per cell, cytoplasmic RNA was isolated and subjected to RNase protection analysis with in vitro-synthesized i-leader-specific RNA from *Hin*dIII-cut sp65i as probe. Cellular RNA was pretreated with NaOH, RNase A, or DNase I or was untreated prior to the assay. The sizes of protected bands are indicated in nucleotides.

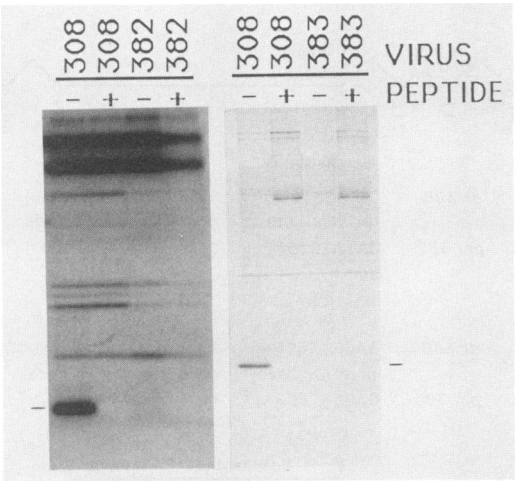

FIG. 4. Immunoprecipitation of i-leader protein from extracts of mutant- and wild-type-infected cells. At 16 h after infection of Vero cells at a multiplicity of 20 PFU per cell, proteins were labeled for 1 h with [^{35}S]methionine, subjected to immunoprecipitation, and analyzed by electrophoresis in SDS–12.5% polyacrylamide gels followed by fluorography and autoradiography. Antibody was either blocked by prior addition of peptide antigen (+) or used without blocking (−). Dashes beside the autoradiograms indicate the positions of the i-leader protein.

viruses were compared with those of *dl*308 on several human and monkey cell lines. Only modest differences were noted on HeLa, 293, WI38, Jurkat, COS, and CVI cells (data not shown). The greatest difference between mutant and wild-type viruses was apparent when viruses were propagated in Vero or B-JAB cells (Fig. 5). In the Vero monkey kidney cell line, *pm*383 (ATG mutant) grew as well as *dl*308 (wild-type virus), whereas *pm*382 (splice acceptor mutant) generated a yield that was reduced by a factor of 4. In the B-JAB human B cell line, *pm*383 and *pm*382 produced yields reduced by factors of 3 and 5, respectively, compared with the parental virus.

Several experiments were undertaken to identify the point during the viral growth cycle at which the i leader and its protein function. These experiments were performed primarily with Vero cells, since adenovirus replicates well in these cells and the two mutants exhibit somewhat different yields in these cells.

The kinetics of DNA replication was examined by DNA blot analysis, and no difference was observed for the mutants as compared with wild-type virus (data not shown).

To determine whether the mutations affected the production of any late viral mRNA species, polyadenylated mRNAs isolated at 16, 20, and 24 h from wild-type and mutant virus-infected Vero cells were subjected to RNA blot analysis with probes for each of the late mRNA families. The only detectable differences between mutant and wild-type viruses were seen with L1 mRNAs (Fig. 6). Two major L1-specific mRNA species were detected, and these encode the L1 52- and 55-kDa polypeptides. A less abundant, smaller mRNA encodes the L1 IIIa polypeptide. Several additional large RNAs were also evident. These probably represent incompletely spliced major late transcripts that contain L1 sequences.

The larger of the major mRNA species seen in wild-type virus-infected cells was missing in *pm*382-infected cells (Fig. 6). This L1 mRNA species contains the i leader (Fig. 1). Its absence in *pm*382-infected cells is consistent with the mutant's lack of a functional splice acceptor sequence for the i-leader exon. The amount of the larger i-leader-negative mRNA was modestly elevated (about twofold) at 20 and 24 h after the cells were infected with *pm*382 compared with the effect of infection with *dl*308. All of the normal L1 mRNA species were synthesized in *pm*383-infected cells. However, the level of the i-leader-containing species was elevated by a factor of about 4 compared with *dl*308-infected cells at 20 and 24 h after infection.

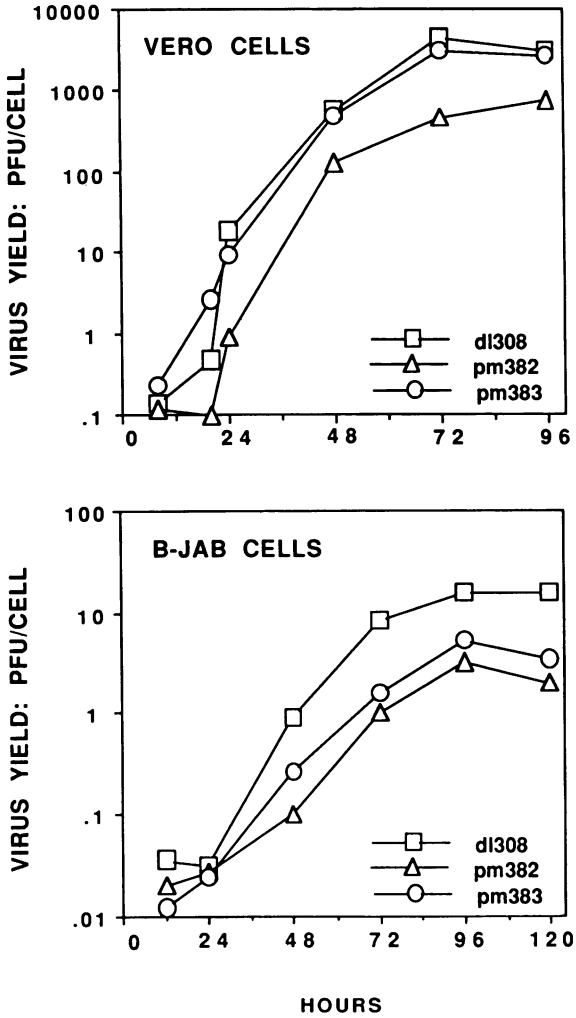

FIG. 5. Growth kinetics of mutant and wild-type viruses. At several times after infection of B-JAB or Vero cells at a multiplicity of 3 PFU per cell, cells were scraped into medium, adjusted to 50 mm Tris (pH 8.0), and subjected to three freeze-thaw cycles, and virus titers were determined on 293 cells. Yields are reported as PFU produced per infected cell.

Next, accumulation of virus-specific proteins was examined. Cytoplasmic, nuclear membrane, and nuclear fractions were prepared from [^{35}S]methionine-labeled infected cells by the method of Weinberg and Penman (36) at various times postinfection. The only visible difference in protein synthesized in mutant virus-infected cells was observed upon electrophoretic analysis of the nuclear membrane fraction (Fig. 7A). The 52- and 55-kDa polypeptides were overexpressed at 24 h after infection with either *pm*382 or *pm*383. This is the size of the proteins encoded by the two major L1 mRNAs. To confirm the identity of these polypeptides, fractions from *dl*308 and *pm*382-infected cells were immuno-

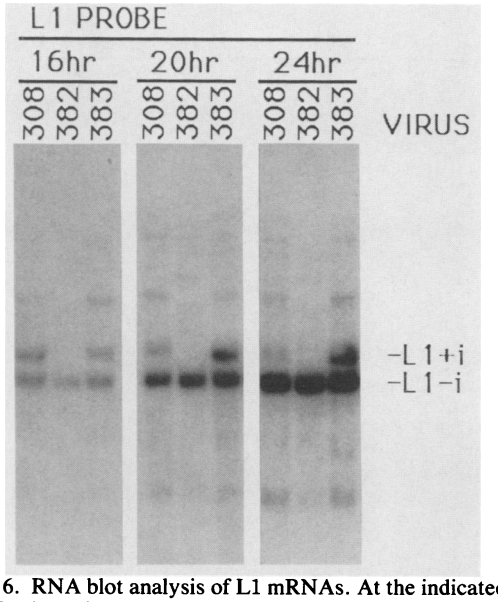

FIG. 6. RNA blot analysis of L1 mRNAs. At the indicated times after infection of Vero cells at a multiplicity of 20 PFU per cell, cytoplasmic RNA was prepared and analyzed by RNA blot analysis with an L1-specific probe DNA. Bands representing the large L1 mRNAs that contain (L1+i) and lack (L1−i) the i-leader sequence are labeled.

precipitated by using a polyclonal antibody prepared against an L1 52- and 55-kDa-specific peptide (Fig. 7B). The L1-encoded proteins were overrepresented in the nuclear membrane fraction of *pm*382-infected cells. The overrepresentation in this fraction did not result from a failure of the polypeptides to be transported to their normal location. Maximal accumulation of the L1 proteins in the three cell fractions was attained within 10 min of chase after a 5-min pulse with [^{35}S]methionine (data not shown). Although the final levels of L1 52- and 55-kDa proteins in the fractions differed for mutant and wild-type viruses, the time needed to reach steady state did not.

The levels of L1-encoded mRNAs and polypeptides produced in mutant virus-infected cells appeared different from the levels produced in wild-type virus-infected cells, but the differences were fairly modest. To be more confident of the magnitude of the effects, we quantified mRNA and protein levels by densitometry (Table 1). The major L1 mRNAs and the 52- and 55-kDa polypeptides were elevated by factors of about 2 and 2 to 4, respectively.

Functional i-leader sequences destabilize L1 mRNA. The half-lives of L1 mRNAs that carry or lack the i leader were measured in a dactinomycin chase experiment (Fig. 8). The drug was added to cultures at 24 h after infection with *dl*308, *pm*382, or *pm*383; cells were harvested after various time intervals; and cytoplasmic polyadenylated RNA was prepared. RNA blot analysis with an L1-specific probe was then performed to distinguish between RNAs that contain or lack i-leader sequences. The resulting autoradiograms (Fig. 8A) were scanned by densitometry and normalized to L3 RNA signals (L3 mRNA levels were equal for wild-type and i-leader mutant viruses), and the results were plotted (Fig. 8B). L1 mRNAs lacking the i leader produced by any of the viruses exhibited similar half-lives (20 to 27 h). The half-life of the i-leader-containing L1 mRNA produced by *dl*308 (wild-type virus) was much shorter (3.4 h), whereas the

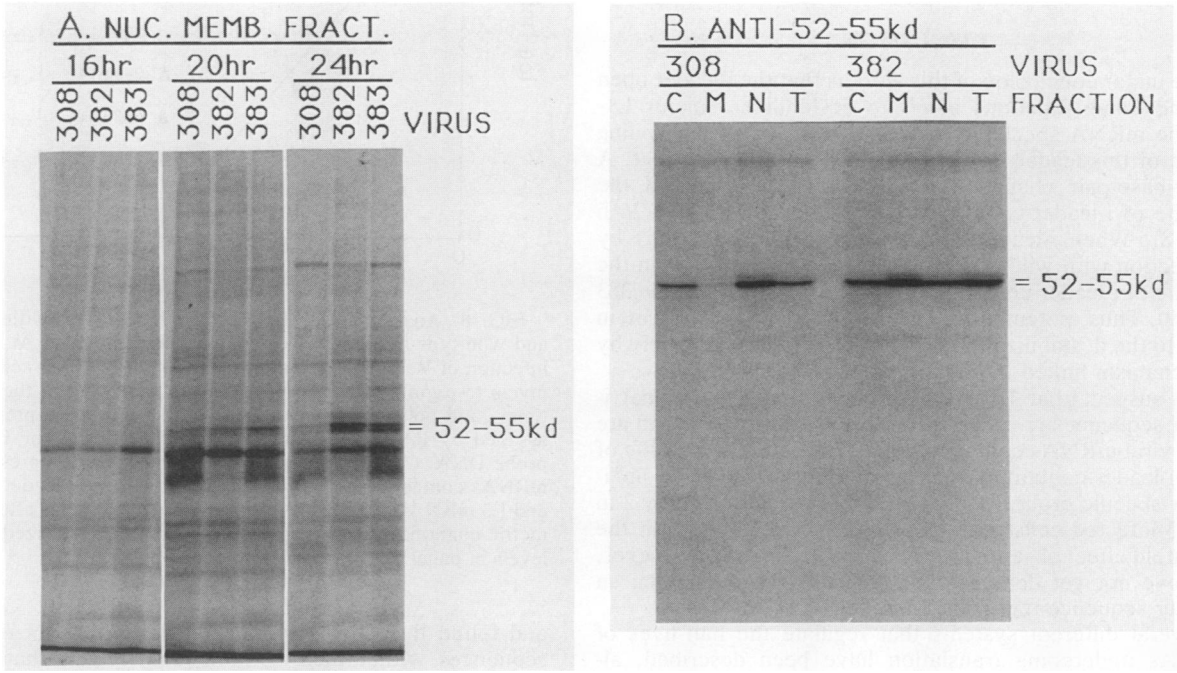

FIG. 7. Electrophoretic analysis of proteins synthesized in mutant- and wild-type-infected cells. At indicated times after infection of Vero cells at a multiplicity of 20 PFU per cell, cells were labeled with [^{35}S]methionine for 1 h and then fractionated. (A) Portions (10 μg) of protein from the nuclear membrane fraction were analyzed by electrophoresis in an SDS–12.5% polyacrylamide gel followed by fluorography and autoradiography. (B) Portions of total cell extracts were subjected to immunoprecipitation by using antibody to the L1 52- and 55-kDa polypeptides and then analyzed as for panel A. The cell fractions assayed were cytoplasmic (lanes C), nuclear membrane (lanes M), nuclear (lanes N), and total cell (lanes T). Bands corresponding to the L1 52- and 55-kDa polypeptides are labeled.

TABLE 1. Relative levels of L1 mRNAs and 52- and 55-kDa polypeptides in mutant- and wild-type-infected Vero cells[a]

Time after infection (h)	Relative mRNA levels[b]			Relative protein levels[b]		
	*dl*308	*pm*382	*pm*383	*dl*308	*pm*382	*pm*383
16	1.0	0.8	1.7	1.0	2.2	1.4
20	1.0	2.2	1.8	1.0	3.9	2.7
24	1.0	2.2	1.2	1.0	3.8	3.0

[a] Appropriate exposures of the autoradiograms shown in Fig. 6 and 7A were produced by using preflashed film, and the relative intensities of the bands of interest were quantified with a densitometer.

[b] The intensities of mutant virus-generated bands were calculated relative to those of the parental, wild-type virus (*dl*308), which were set to 1.0.

equivalent i-leader-containing mRNAs produced by *pm*383 (ATG mutant) exhibited a half-life equivalent to that of mRNAs lacking the leader (26 h). The sevenfold difference in half-life can account for the increase observed in steady-state levels of i-leader-containing L1 mRNA within *pm*383-infected cells (Fig. 6).

The i-leader open reading frame acts in *cis* to destabilize L1 mRNAs. It was possible that the i-leader protein acted in *trans* to reduce the half-lives of L1 mRNAs that carry the i-leader sequence. Alternatively, the synthesis of the i-leader protein or the presence of a functional i-leader open reading frame might lead to destabilization of L1 mRNAs which are translated. To distinguish between these possibilities, a dactinomycin chase was performed on cells infected with both *pm*383 and *dl*308 (Fig. 9). The i-leader-containing L1 mRNA in coinfected cells displayed the long half-life characteristic of *pm*383-infected cells. Thus, the i-leader protein does not act in *trans* to reduce the half-life of *pm*383-encoded L1 mRNAs, indicating that translation of the i-leader sequence destabilizes the L1 mRNA undergoing translation.

DISCUSSION

The major conclusion of this work is that the i-leader open reading frame functions in *cis* to destabilize a major L1-specific mRNA species. Ongoing translation of the coding region of this leader is probably required for this effect. A single-base-pair change in *pm*383 (Fig. 2) increased the half-life of i-leader-containing L1 mRNA from 3.4 to 26 h (Fig. 8). When i-leader protein was supplied in *trans* by coinfection with wild-type virus, there was no effect on the half life of i-leader-containing L1 mRNA encoded by *pm*383 (Fig. 9). Thus, potential for the synthesis of i-leader protein leads to the destabilization of L1 mRNAs, most probably by a mechanism linked to ongoing translation.

We suspect that L1 mRNAs containing a functional i-leader sequence are destabilized to a greater extent than are other viral mRNAs containing the sequence. The half-life of total i-leader-containing RNAs, monitored by using an i-leader-specific probe, was increased by a factor of only 2 in *pm*383-infected cells (data not shown), compared with the sevenfold effect observed for L1 mRNAs (Fig. 8). However, we have not yet demonstrated that all RNAs carrying an i-leader sequence represent bona fide viral mRNAs.

Several different systems that regulate the half-lives of mRNAs undergoing translation have been described, although none are understood at the mechanistic level. A variety of mRNAs with short half-lives contain A+U-rich sequences within their 3′ noncoding regions. Shaw and Kamen (31) deleted a segment containing multiple AUUUA sequences from the 3′ noncoding region of the short-lived granulocyte-macrophage colony-stimulating factor mRNA

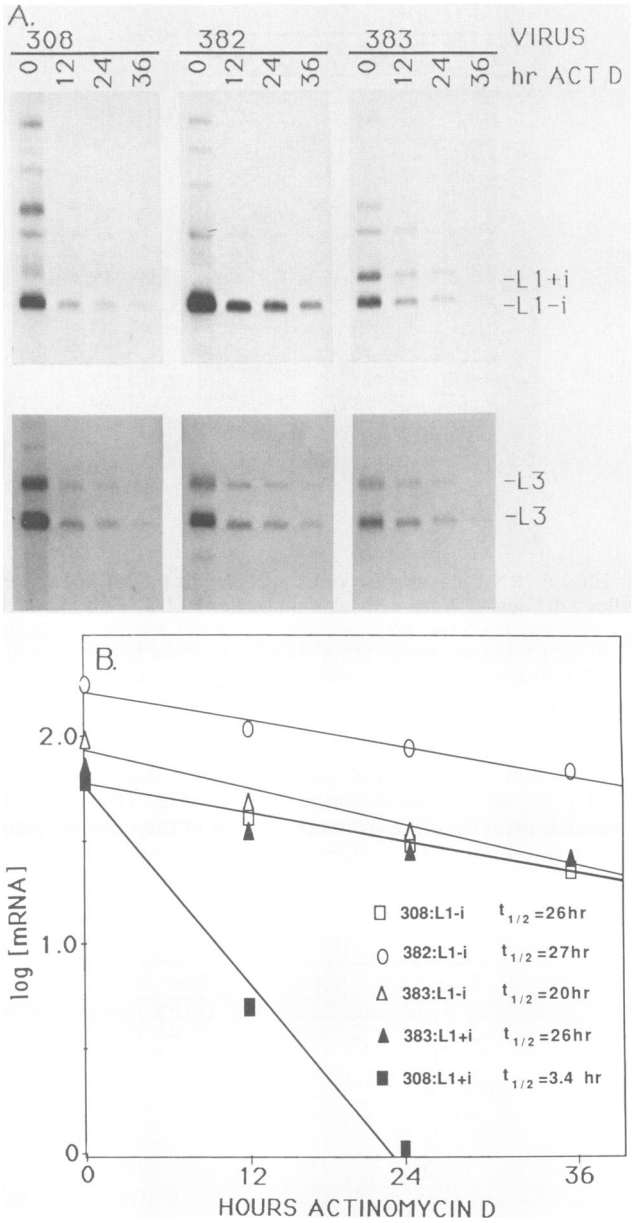

FIG. 8. Analysis of L1 and L3 mRNA half-lives within mutant- and wild-type-infected cells by dactinomycin chase. At 24 h after infection of Vero cells at a multiplicity of 20 PFU per cell, dactinomycin (5 μg/ml) was added. Cells were harvested at the indicated times, and polyadenylated cytoplasmic RNAs were prepared and assayed by RNA blot analysis with either an L1- or L3-specific probe DNA. (A) Autoradiograms on which bands representing L1 mRNAs containing (L1+i) or lacking (L1−i) the i-leader sequence and L3 mRNAs are labeled. (B) Plot of mRNA levels after densitometric quantification of the L1-specific bands normalized to the L3 levels in panel A.

and found that it was stabilized. Histone mRNAs contain sequences within their 3′ noncoding region that regulate their stability, but only in a cell cycle-dependent fashion (7, 16). The half-lives of α- and β-tubulin mRNAs are also regulated. Tubulin synthesis is reduced in response to increases in the intracellular pool of tubulin subunits (3), and the rapid reduction is caused by degradation of tubulin mRNA (8, 30). Degradation requires the presence of the

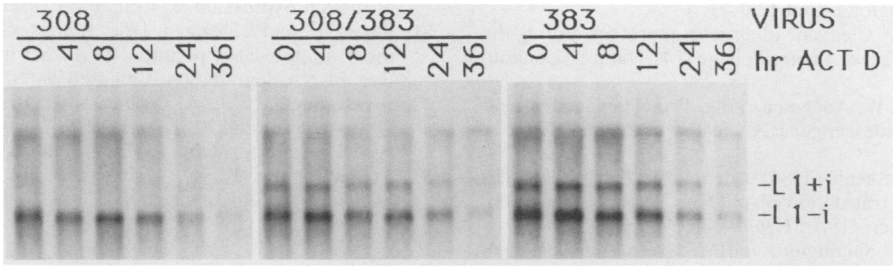

FIG. 9. Analysis of L1 mRNA half-lives by dactinomycin chase in single and mixed infections. Vero cells were infected with one virus at a multiplicity of 20 PFU per cell or two viruses at a multiplicity of 10 PFU per cell each, and the dactinomycin chase was performed as described in the legend to Fig. 8.

mRNA on polysomes (28). Fusion of the sequence encoding the four N-terminal amino acid residues (Met-Arg-Glu-Ile) of the tubulin protein can confer regulation on a heterologous mRNA if the new amino acids are positioned at the N terminus of the resulting chimeric polypeptide (37).

Our limited understanding of the influence of i-leader translation on L1 mRNA stability tempts us to speculate that it may regulate mRNA half-life through a mechanism similar to the tubulin system. However, its first four amino acids (Met-Arg-Ala-Asp) are different from those of the tubulins. Additional experiments are in progress to better define the mechanism underlying the translation-dependent destabilization of L1 mRNAs carrying the i leader.

Why does adenovirus contain a system to reduce the half-life of only half of the L1 mRNAs that encode the 52- and 55-kDa polypeptides (50% of these mRNAs carry the i leader [Fig. 6] [10])? The shortened half-life of i-leader-containing L1 mRNAs reduced the overall levels of the largest L1 mRNAs and the 52- and 55-kDa polypeptides by a factor of only 2 to 4 (Fig. 6 and 7; Table 1). It is possible that the i-leader-induced destabilization is used as a fine-tuning mechanism to more precisely control levels of the 52- and 55-kDa polypeptides which have been shown to play a role in the assembly of virions (18). Alternatively, it could very effectively regulate the level of i-leader protein or i-leader-encoding RNA sequences within infected cells. Modest variations in 52- and 55-kDa polypeptide levels in the nuclear membrane and the complete lack of i-leader protein (*pm*383) or both protein and mRNA sequences (*pm*382) had small but significant effects on Ad5 yields in a variety of cultured cells (Fig. 5; data not shown). Conceivably, these alterations exert more profound effects on adenovirus growth within an infected host animal or human.

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