Translation Efficiency of Adenovirus Early Region 1A mRNAs Deleted in the 5' Untranslated Region

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Adenovirus deletion mutants were studied to examine the influence of the 5' untranslated sequence on the translation of early region 1A mRNAs. Alterations of the 5' untranslated sequence, including complete deletion of the wild-type 5' untranslated sequence, did not significantly affect the rate of translation.

The features necessary for the initiation of translation of eucaryotic mRNAs are not fully understood. The site of initiation of protein synthesis has been proposed to be determined by a scanning mechanism (reviewed in reference 20). In this model a 40S ribosomal subunit binds at the 5' end of the mRNA and migrates in a 3' direction until it encounters the first AUG codon (18, 19). Recent refinements of the model propose that sequences immediately surrounding the first AUG affect the efficiency with which the 40S subunit halts at the first AUG (22-24); there is experimental evidence for this proposition from several systems (21, 26; S. Baim and F. Sherman, personal communication). This modified scanning model allows for initiation at sites downstream from the first AUG in some mRNAs.

A prediction of this model is that specific sequences in mRNA 5' noncoding regions (except nucleotides immediately surrounding the AUG [see above]) are not required for efficient translation (20, 22). Evidence for this prediction is found in the observation that some closely related mRNAs exhibit considerable divergence in their 5' untranslated leaders (reviewed in reference 20). Also, viable virus mutants with deletions of, or insertions into, 5' noncoding sequences have been isolated (2, 3, 13, 29, 34, 36, 41, 42); however, in these cases the efficiency of translation of the altered mRNAs was not examined. In one polyomavirus mutant in which 5' leader sequences were deleted to within two nucleotides of the initiating AUG, translation efficiency was reduced approximately fivefold (4); however, protein and specific mRNA levels in the same infections were not compared. We describe here the use of adenovirus deletion mutants to investigate the efficiency of translation of early viral mRNAs.

Sequence complementarities between the 3' end of 18S rRNA and the 5' ends of eucaryotic mRNAs have been observed (14), although many mRNAs lack significant complementarity for interactions analogous to procaryotic Shine-Dalgarno sequence-ribosome binding (reviewed in reference 23; 32). The leader region of adenovirus early region 1A (E1A) mRNA has a complementarity with the 3' end of 18S rRNA (39); however, this sequence cannot be essential for E1A translation, since a mutant in which it is deleted is viable (29). Nevertheless, it is difficult to interpret the

significance of this earlier finding in terms of the efficiency of E1A mRNA translation, because as little as 1% of the wild-type level of E1A protein is sufficient to support viral replication (12). At the time of our earlier studies (28, 29), we could not directly determine the effects of this and other alterations of the E1A mRNA 5' untranslated sequence on the synthesis of E1A protein, because specific anti-E1A serological reagents were not available. Recently, we developed specific anti-E1A sera by immunizing animals with E1A fusion proteins expressed at high levels in *Escherichia coli* (35). We also found culture conditions which result in 10- to 30-fold increases in the concentration of E1A mRNAs after infection (8). Using these recent developments, we carried out a quantitative study of the effects of deletions in the 5' untranslated sequence on the efficiency of translation of adenovirus E1A mRNAs.

Early in adenovirus infection, the genes of E1A are transcribed into two mRNAs, 13S and 12S, which encode closely related proteins of 289 and 243 amino acids, respectively. These E1A proteins are of interest because of their transcriptional activation of viral and nonviral genes and their role in oncogenic transformation (5, 7a, 9-11, 15-17, 27, 30, 37, 38). Deletion mutants in the 5' portion of the E1A noncoding sequence have been analyzed for their ability to synthesize E1A mRNAs (28, 29). In this study, we used these deletion mutants to determine the effects of the 5' sequence on translational efficiency, examining both E1A mRNA levels and protein levels in the same infected cells.

The structures of the deletion mutants used in this study (29) are shown in Fig. 1. The mutants were constructed with adenovirus 5 (Ad5) *d*/309 (17), which is wild type in E1A sequence and therefore was used as a control in the experiments reported below. Mutant *d*/1501 has the smallest deletion, leaving the TATA homology intact. Because this deletion does not affect the concentration of E1A mRNAs significantly and results in mRNAs identical in sequence to those of *d*/309 (29), it serves as a control for the reproducibility of our mRNA and protein determinations. The deletion in *d*/1502 removes the TATA homology. This leads to a shift in the E1A mRNA 5' ends to a series of 5' ends located 160 to 230 bases upstream from the major wild-type cap site (29). The resulting extended 5' untranslated regions do not contain any AUG triplets. The TATA homology, the major wild-type mRNA cap site, and most of a region highly conserved in adenovirus groups, including 22 bases of the 61-base untranslated leader (Ad5, Ad7, and Ad12) (40) are deleted in *d*/1503. In *d*/1504 the deletion encompasses these same

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FIG. 1. Structures of E1A and deletion mutants. (A) DNA sequence of adenovirus E1A. The DNA sequence of adenovirus E1A is indicated by the double line, demarcated in kilobases (kb). The 12S and 13S mRNAs of E1A are indicated by arrows above the DNA sequence; carets represent the introns. The numbers above the exons indicate their sizes in nucleotides. The dotted lines symbolize mRNAs with upstream starts which were seen at low levels in the wild type, *dl1309*, and *dl1501*, but which were the predominant mRNA species of *dl1502*, *dl1503*, and *dl1504*. The sequences deleted in *dl1501*, *dl1502*, *dl1503*, and *dl1504* are indicated by the open boxes. The heavy line indicates the adenovirus sequences present in the single-stranded M13 DNA clone used as an S1 nuclease probe in Fig. 2. (B) Detailed map of deletion mutants. *dl1309*, the parent virus of *dl1501*, *dl1502*, *dl1503*, and *dl1504*, is wild type in E1A and is shown at the top of (B). Mutants *dl1501*, *dl1502*, *dl1503*, and *dl1504* have deletions which begin at -44 with respect to the major E1A cap site at +1 (nucleotide 498 in the adenovirus sequence [1]) and extend to the right to -39, -24, +21, and +63 nucleotides, respectively (29). The open boxes represent deleted sequences. The TATA box, the major cap site, and the first two in-frame ATG sequences are indicated.

sequences, extends completely through the wild-type 5' untranslated leader, and includes the AT corresponding to the initiator AUG of wild-type mRNA (33). The E1A proteins produced by *dl1504* are shorter than the wild-type proteins, corresponding to translation initiating at a second AUG 14 codons downstream in the wild-type sequence (7, 29, 31).

To examine whether these deletions had a significant effect on the translation of E1A mRNAs, we performed the experiments shown in Fig. 2. Suspension HeLa cells were infected with *dl1501*, *dl1502*, *dl1503*, *dl1504*, or *dl1309* (wild type in E1A sequence) at a multiplicity of infection of 200 in the presence of 20 μ g of cytosine arabinoside per ml (8); the cells were harvested at 45 h postinfection and analyzed for the presence of both E1A mRNAs and E1A proteins. E1A proteins were analyzed by a Western immunoblot technique (Fig. 2A). Cells (3×10^5) were washed three times with phosphate-buffered saline, and the cell pellets were lysed

and digested with micrococcal nuclease, RNase A, and DNase I as described previously (35). The extracts were then mixed with 0.25 volumes of 4 $\times$ Laemmli gel sample buffer and analyzed on 8 to 14% gradient polyacrylamide

FIG. 2. E1A proteins and mRNAs in cells infected with adenovirus E1A deletion mutants. (A) Protein analysis. HeLa cells infected with the indicated viruses were harvested 45 h postinfection and analyzed by Western immunoblot analysis with antisera to the E1A proteins and the E1B 21-kd protein (35). The E1A proteins and the E1B 21-kd protein are indicated. (B) S1 nuclease analysis of mRNAs. Cytoplasmic RNAs from the infections described in (A) were analyzed with the uniformly labeled M13 E1A-E1B probe shown in Fig. 1A (29). The numbers at the left indicate the sizes of the bands in *dl1309*. The solid symbols indicate 5' 13S-specific exons; the open symbols indicate 5' 12S-specific exons. The S1 nuclease-protected fragments generated by *dl1309* and *dl1501* major E1A mRNAs which initiate at nucleotide 498 are indicated by the triangles; minor mRNAs initiating upstream (see Fig. 1A) protect the bands indicated by the circles. In *dl1501*, *dl1502*, *dl1503*, and *dl1504* the bands indicated by the circles are fragments protected by the deleted mRNAs which initiate upstream of the wild-type start site (see Fig. 1A). The sizes of fragments protected by mRNAs with upstream starts (indicated by circles) are a result of S1 nuclease digestion at the point of nonhomology with the probe, i.e., at the left end of the adenovirus sequence in the M13 probe for *dl1309*, and at the deletions in *dl1501*, *dl1502*, *dl1503*, and *dl1504*. Common to all the viruses, the band at 405 represents the fragment protected by the 3' exon of the E1A mRNAs and the band at 350 represents the fragment protected by a portion of the E1B mRNAs.

gels (25). Proteins were transferred to nitrocellulose as described previously (35), and the filter was incubated with 9.6% nonfat dry milk to block nonspecific protein binding. The filter was incubated for 12 h with two antisera, one directed against a *trpE*-E1A fusion protein and the other directed against a *trpE*-early region 1B (E1B) 21-kilodalton (kd) fusion protein (35). The first-stage antibody was detected with the Immun-Blot system (a horseradish peroxidase-coupled antibody; Bio-Rad Laboratories). Each of the two full-length E1A proteins resolved into multiple bands in this gel system, presumably because of differences in posttranslational modifications (31, 43). The more rapidly migrating forms did not result from the cleavage of either amino- or carboxy-terminal sequences (7, 31). The *d1504* E1A proteins, which are shorter than the wild-type proteins (see above), reproducibly resolved into fewer species than did the full-length proteins in these gradient gels and comigrated with the rapidly migrating forms of the full-length proteins. (In other gel systems, we saw multiple species of E1A proteins in *d1504* which migrated faster than the corresponding wild-type proteins [data not shown]). The amount of E1A protein synthesized by each of the mutants was quantitated by densitometry of the immunoblots and summations of E1A protein bands. The densities of the bands measured here fell in the linear range of a dose-response curve obtained with various dilutions of the *d1309* extract (data not shown). The E1B 21-kd protein (6) was analyzed on the same immunoblots as an internal control for recovery of viral proteins. The Western blot analyses were performed in duplicate, and the values were averaged. Levels of mRNA were determined by the S1 nuclease technique with a single-stranded, uniformly labeled DNA probe (Fig. 2B), 100 µg of cytoplasmic RNA, and 100 U of S1 nuclease (Bethesda Research Laboratories) (29). Hybridizations were done with an excess of DNA and proceeded to near completion so that the quantity of the S1 nuclease-protected fragments was an accurate measure of E1A mRNA concentrations. The resulting autoradiograms were scanned with a densitometer, and the bands corresponding to the 13S 5' exon were used as a measure of E1A mRNA levels (Fig. 2B). The S1 nuclease analyses were performed in duplicate, and the values were averaged. The infections and analyses were repeated; the data from the two experiments are shown in Table 1.

None of the mutations analyzed decreased the levels of E1A proteins by a factor of three or more. Mutant mRNA concentrations varied from 25 to 100% of wild-type control mRNA concentrations (*d1309*), and the corresponding mutant E1A protein levels ranged from 40 to 100% of wild-type E1A protein levels. In general, when comparing mutants and the wild type, the concentrations of E1A proteins paralleled the concentrations of E1A mRNAs. Quantitation of mRNA and protein levels between the two experiments varied by a factor of less than two. Therefore, we cannot determine whether these mutations affect the efficiency of E1A mRNA translation by a factor of two or less. However, it is clear that these mutations do not have effects greater than twofold.

Similar results were obtained with cells infected with the deletion mutants 18 h postinfection in the absence of cytosine arabinoside (data not shown). The E1A proteins had a short half-life, ca. 30 min (unpublished observations); thus, the levels of accumulated proteins as analyzed in Table 1 represent the levels of newly synthesized proteins. (Greater than 90% of the accumulated E1A proteins were synthesized in the preceding 2 h.) Accordingly, analysis of E1A proteins of the deletion mutants by pulse-labeling with [³⁵S]methio-

TABLE 1. Comparison of E1A mRNA and protein levels in Ad5 E1A 5' deletion mutants

Expt	Mutant	Level of:	
		mRNA ^a	Protein ^b
1	<i>d1309</i> (Control)	1.00	1.00
	<i>d1501</i>	0.60	0.97
	<i>d1502</i>	0.35	0.52
	<i>d1503</i>	0.48	0.64
	<i>d1504</i>	0.39	0.52
2	<i>d1309</i> (Control)	1.00	1.00
	<i>d1501</i>	1.02	0.86
	<i>d1502</i>	0.35	0.49
	<i>d1503</i>	0.27	0.41
	<i>d1504</i>	0.35	0.40

^a mRNA levels were quantitated by densitometric scanning (with a Hoefer densitometer) of the autoradiograms from two independent S1 nuclease analyses, one of which is shown in Fig. 2B (experiment 1), or two independent S1 nuclease analyses from a duplicate experiment (data not shown, experiment 2). The E1A 13S 5' exon bands (solid symbols in Fig. 2B) were used as a measure of the E1A mRNA levels; values were corrected for size, as the probe was uniformly labeled, and then were normalized to 1.0 for *d1309* mRNAs and averaged for the two analyses.

^b Protein levels were quantitated by densitometric scanning of the E1A bands in the Western immunoblot nitrocellulose filter (Fig. 2A) and in a duplicate filter (not shown), normalized to a value of 1.0 for *d1309*, and averaged (experiment 1). Similarly, two filters were analyzed and values were averaged for experiment 2.

nine for 2 h gave results similar to those shown in Table 1 (data not shown).

These results indicate that specific sequences in the 5' noncoding regions of adenovirus E1A mRNAs are not necessary for efficient translation of E1A proteins. Protein and mRNA levels were directly compared in infections with a series of deletion mutants, and although E1A protein levels in several of the mutants were reduced relative to wild-type E1A protein levels, these levels correlated with reduced mRNA levels. The AUG sequence contexts of both the first (AAAAUGA) and the second (GAAAUGG) AUGs (used in *d1504*) of E1A fit the consensus sequence proposed to be necessary for efficient recognition by the migrating 40S ribosomal subunit, that is, ANNAAUGN or GNNAAUGR (N = any nucleotide; R = a purine) (23). Thus, in support of the scanning model, which requires only specific sequences immediately surrounding the initiator AUG, adenovirus E1A mRNAs can tolerate major 5' sequence alterations without significantly affecting the efficiency of translation. The deletion mutants which remove the TATA homology (*d1502*, *d1503*, and *d1504*) initiate transcription at a variety of upstream sites which are also used at low frequencies in wild-type virus during the early phase of infection (28, 29). The resulting extended untranslated sequences do not include any AUG triplets 5' to the initiation codon for the 289- and 243-amino-acid E1A proteins (29). These heterogeneous start sites thus delineate a diverse population of E1A mRNAs with various 5' untranslated leader sequences. The efficiencies of translation cannot be measured individually for each of these messages, but the results presented here indicate that translational efficiencies overall for these mutant E1A mRNAs are not dependent on specific 5' sequences. Mutants *d1503* and *d1504* have lost some or all of the 5' untranslated sequences of wild-type E1A mRNAs and yet are translated essentially as efficiently as wild-type messengers. We conclude that the 5' untranslated sequences of adenovirus E1A mRNAs do not have a significant function in protein synthesis.

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