Translation by the Adenovirus Tripartite Leader: Elements Which Determine Independence from Cap-Binding Protein Complex

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Received 21 December 1989/Accepted 21 February 1990

The adenovirus tripartite leader is a 200-nucleotide-long 5' noncoding region which facilitates translation of viral mRNAs at late times after infection. The tripartite leader also confers the ability to initiate translation independent of the requirement for cap-binding protein complex or eIF-4F without any requirement for adenovirus gene products. To elucidate the manner by which the tripartite leader functions, the primary determinants of leader activity were investigated *in vivo* by testing a series of mutations expressed from transfected plasmids. The results of these experiments indicate that the tripartite leader does not promote internal ribosome binding, at least in a manner recently described for picornavirus mRNAs. In addition, despite an unusual arrangement of sequences complementary to the 3' end of 18S rRNA in the tripartite leader, we could find no evidence for involvement in its translation activity. Instead, our results are consistent with a model in which much of the first leader is maintained in an unstructured conformation which determines the ability of the tripartite leader to facilitate translation and bypass a normal requirement for eIF-4F activity. Several possible translation models are discussed, as well as the implications for translation of late viral mRNAs.

Adenovirus late mRNAs all contain an identical 200-nucleotide (nt)-long 5' noncoding region called the tripartite leader which enhances translation in infected (4, 31) and transfected (23) cells. Previous analyses demonstrated that large deletions abolish the preferential translation conferred by the tripartite leader (4, 23, 31). We have previously demonstrated that the tripartite leader eliminates or gradually reduces the requirement for a critical initiation factor called cap-binding protein (CBP) complex or eIF-4F (11). Factor eIF-4F is thought to facilitate ribosome binding by unwinding the 5' end of capped mRNAs (1, 28, 39; for a review, see references 12 and 45). Considerable interest has been directed toward investigating the relationship between 5' structure and the activity of eIF-4F in regulating translation of an mRNA. In general, translation efficiencies have been found to be inversely related to secondary structure in that enhanced structure decreases initiation and minimal structure increases it (12, 18, 24–26, 28, 37).

The mechanism by which the tripartite leader enhances translation and provides independence from CBP complex activity, as well as its biological significance, is not understood. We have previously shown that the tripartite leader very likely possesses a native conformation in which the first leader segment is predominantly unstructured (49), which could likely enhance translation and eliminate a requirement for eIF-4F activity. Nevertheless, both the secondary structure of the tripartite leader and elements of its nucleotide sequence can be implicated in its translation activity. As demonstrated in this report, each leader segment possesses a sequence complementary to a conserved hairpin structure found in the 3' end of 18S rRNA, which in leaders 2 and 3 is contained in identical 12-nt direct repeats. Although there is no evidence for a procaryotic Shine-Dalgarno-type interaction in animal cells, a flexible association between complementary 5' noncoding regions and 18S rRNA has often been suggested as a possible regulatory mechanism to promote

either internal initiation or to facilitate the binding of ribosomes (3, 14, 19, 36). Finally, it has been suggested that the tripartite leader might interact with adenovirus VAI RNA (23, 34), which maintains the function of initiation factor eIF-2 by inhibiting the activity of the interferon-induced DAI kinase (43, 46; for a review, see reference 44). An interaction between VAI RNA and the tripartite leader could, in principle, enhance translation by inhibiting the proposed local activation of this ribosome-associated kinase (9, 10, 40).

In this report, we have probed the mechanism by which the adenovirus tripartite leader facilitates translation and provides independence from a normal cap recognition process. We demonstrate that deletion of leader sequences complementary to 18S rRNA (or VAI RNA) have little if any specific effect on translation activity. These results therefore discount a specific interaction between 18S rRNA and the tripartite leader, at least in transfected cells, despite the unusual degree of complementarity with 18S rRNA. We also show that the tripartite leader does not promote translation through efficient internal initiation, as was found for picornavirus mRNAs that also translate in the absence of CBP complex activity (22, 38). Instead, our results support a model in which the 5' end of the tripartite leader is maintained in a relatively unstructured conformation, thereby permitting efficient translation in the absence of eIF-4F activity.

MATERIALS AND METHODS

Viruses and cells. COS 1 cells are derived from a CV-1 monkey cell line transformed with a replication origin-defective simian virus 40 (SV40) (16). Cells were grown in monolayers in Dulbecco modified Eagle medium containing 10% calf serum. DNA transfection of COS cells was performed by the DEAE-dextran technique (32). Poliovirus infections were performed 36 to 48 h after transfection with the wild-type P2 Lansing strain as previously described (11).

RNA and protein analysis. Poly (A)⁺ RNAs were prepared as previously described (11), subjected to Northern (RNA

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FIG. 1. Diagram of tripartite leaders (L1, L2, and L3) used in construction of variants. Restriction endonuclease sites used in the various mutagenesis schemes. Nucleotide positions are relative to the transcription start site of the tripartite leader. Splice junctions between leader exons are indicated at 40 and 112 nts which correspond to the cDNA used in this study (49). Symbols: $\square$, sequences complementary to 18S rRNA; ▨ , sequences complementary to adenovirus VAI RNA.

blot) analysis with equal amounts of RNAs (1.0 μ g) in formaldehyde-agarose gels, and transferred to nitrocellulose paper (48). DNA probes for the hepatitis B virus surface antigen (HBsAg) gene were prepared by the method of Feinberg and Vogelstein (13).

For polypeptide analysis, cells were labeled with [35 S] methionine with 50 μ Ci of tran[35 S]methionine per ml (ICN Pharmaceuticals, Inc.) in methionine-free media for 3 h at 48 h after transfection and when infected with poliovirus from 4 to 7 h after infection. When provided, VAI RNA was derived by cotransfection of plasmid pVAI, which contains the adenovirus type 2 (Ad2) VAI gene. Preparation of cell extracts, immunoprecipitation with HBsAg monoclonal antibodies, and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis were performed as previously described (11). Immunoprecipitations were quantitatively performed by employing equal amounts of protein from cell lysates and by recycling the supernatants through a second round of immunoprecipitations to ensure complete clearing. Fluorography of gels was performed with En 3 Hance (Dupont, NEN Research Products). Autoradiograms were quantitated by densitometry with an LKB Ultrospec 2. Chloramphenicol acetyltransferase (CAT) assays were performed by the method of Gorman et al. (17). Immunoprecipitation and CAT assays were independently repeated a minimum of three times.

Plasmid constructions. Plasmid pSV-HBV was described previously (11). It contains the Ad5 E1a enhancer fused to the Ad2 major late promoter, a full-length wild-type Ad2 cDNA of the tripartite leader, the HBsAg coding region, and Ad5 E1a polyadenylation signal. About 18 nts separate the third leader and HBsAg translational initiation codon. Mutagenesis of the tripartite leader involved a variety of techniques, including deletions, insertions, duplications, and inversions. BAL 31 exonuclease digestions, Klenow repair reactions, and restriction enzyme digestions were performed by the method of Maniatis et al. (33). Site-directed oligonucleotide mutagenesis was performed by the method of Kunkel (27). All mutations were verified by DNA sequence analysis (41). The positions of nucleotide sequences retained after mutagenesis are indicated in the figures and text.

Figure 1 shows the locations of restriction enzyme sites used in the various mutagenesis schemes. Deletion mutants *dl6* and *dl2* were made by unidirectional BAL 31 digestion from the *ScaI* site at nt position 79. The following variants were created by deleting fragments between the designated sites, and, where appropriate, repair of those sites with Klenow fragment. Thus, the nucleotides retained in variants do not always correspond precisely to the site of restriction enzyme cleavage. Mutant *dl30* was created by deleting between the *PvuII* (nt 33) and *SalI* (nt 211) sites, *dl8* was

created by deleting between *HinfI* (nt 142) and *XhoI* (nt 169), *dl9* was created by deleting between *XhoI* (nt 169) and *SalI* (nt 211), and *dl29* was created by deleting between *ScaI* (nt 79) and *XhoI* (nt 169). Deletion mutants *dl14* and *dl24* were constructed by site-directed oligonucleotide mutagenesis, which was used to delete sequences between nts 3 and 14 and 99 and 120, respectively. Oligonucleotides used for mutagenesis contained 10 bases complementary to both sides of the region to be deleted. Several "flip" variants were made by inverting the orientation of the indicated DNA fragments. In flip variant *fp1*, the intervening sequence between *ScaI* (nt 79) and *XhoI* (nt 169) was inverted so that this section of RNA is now the antisense orientation. In flip variant *fp31*, the sequence between *PvuII* (nt 33) and *SalI* (nt 211) was inverted. To construct insertion mutants *in32* and *in33*, a chemically synthesized double-stranded DNA fragment was synthesized corresponding to the first 33 nts of leader 1. This DNA fragment was inserted into the *PvuII* site (nt 33) to create either the sense (*in32*) or antisense (*in33*) orientations. To create insertion variants *in36* and *in37*, a DNA fragment corresponding to nts 3 to 17 was inserted in the *SalI* site (nt 211) in either the sense or antisense orientations, respectively.

Construction of plasmid pCAT/S-Ag, which generates dicistronic mRNAs, was carried out as follows. The plasmid pSV2-CAT (called pCAT in this study) was digested with *HpaI* (position 3522) which is approximately 800 nts 3' to the CAT stop codon. A *KpnI-SacI* DNA fragment was derived from plasmid pYP55 (49), which contains the full-length tripartite leader fused to the HBsAg coding region, and was inserted into the *HpaI* site of pCAT. The resultant plasmid, pCAT/S-Ag, utilizes the SV40 early promoter and polyadenylation signal to generate a dicistronic mRNA in which cistron 1 encodes CAT and cistron 2 encodes the tripartite leader-HBsAg coding region.

RESULTS

The tripartite leader does not facilitate internal initiation of translation. There are two documented models by which the tripartite leader may facilitate translation and eliminate the requirement for CBP complex activity. First, the tripartite leader may be functionally analogous to an element in picornavirus mRNAs which promotes the internal binding of ribosomes (5, 22, 38). Second, analysis of the secondary structure of the tripartite leader indicates that the 5' end is rather unstructured (49), which could possibly eliminate the requirement for eIF-4F activity during translation initiation (1, 28). Either model accounts for initiation without CBP complex activity.

To determine whether the tripartite leader facilitates translation by promoting internal ribosome binding, a plasmid which transcribes a dicistronic mRNA directed by the SV40 early promoter was constructed (Fig. 2). The first cistron contains the bacterial CAT gene fused to a second cistron, the wild-type tripartite leader-HBsAg coding region. Approximately 800 nts of intercistronic spacer region separate the two genes. Since there are a number of initiation and termination codons in this spacer, it is extremely unlikely that translation could occur by reinitiation of scanning ribosomes at the second cistron (tripartite leader-HBsAg). Control plasmids consist of pCAT or pS-Ag, which encodes monocistronic mRNAs. Internal initiation at the tripartite leader in the dicistronic mRNA would be expected to generate CAT and HBsAg polypeptides at levels equal to that of either monocistronic mRNA.

FIG. 2. Schematic representation of dicistronic mRNA and translation expression of dicistronic and monocistronic mRNAs in transfected cells. Duplicate plates of COS cells were transfected with either the dicistronic mRNA plasmid pCAT/S-Ag or with either monocistronic mRNA plasmid pCAT (pSV2cat) or pS-Ag (pSV-HBV). After 48 h, one set was prepared for assay of CAT activity (17), and the other set was labeled for 3 h with [35 S]methionine. HBsAg polypeptides were analyzed by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. (top) Diagram of construct and transcript encoding dicistronic mRNA. Abbreviations: L, SV40 late promoter; E, SV40 early promoter; CML, CAT open reading frame (ORF), 3LDR, tripartite leader; pA+, SV40 polyadenylation signal. (A) Analysis of CAT activity from cells expressing dicistronic CAT and HBsAg or monocistronic CAT or HBsAg mRNAs. (B) Analysis of HBsAg polypeptides synthesized in cells expressing dicistronic or monocistronic mRNAs. Ac, Acetylated chloramphenicol; Cm, chloramphenicol.

Duplicate plates of COS cells were transfected with plasmids encoding either the dicistronic or monocistronic mRNAs. At 48 h after transfection, one set of plates was labeled with [35 S]methionine and HBsAg subjected to immunoprecipitation and SDS-polyacrylamide gel electrophoresis, and the other set was used for analysis of CAT activity. The level of CAT activity (Fig. 2A) was found to be similar in extracts containing mono- or dicistronic mRNAs. The slight decrease in CAT activity evident in the dicistronic mRNA-containing sample is not reproducible. HBsAg polypeptides, however, could be detected only in cells which express the monocistronic but not the dicistronic mRNA (Fig. 2B). The HBsAg gene produces roughly equal amounts of a 24-kilodalton (kDa) and glycosylated 27-kDa polypeptide. Northern analysis verified that mono- and dicistronic mRNAs accumulate to similar levels (data not shown). These results therefore demonstrate that the tripartite leader does not function to promote efficient internal ribosome binding, at least in a manner similar to that described for picornavirus mRNAs (5, 22, 38).

Construction of tripartite leader variants and cytoplasmic accumulation of mRNAs. To study elements of the tripartite leader involved in translation activity, mutations were generated in DNA segments subcloned into M13mp10 with restriction enzyme sites (Fig. 1 and described in Materials and Methods). Mutations were confirmed by nucleotide

FIG. 3. Stability of wild-type (WT) and variant tripartite leader containing mRNAs produced after transfection of COS cells. Plasmids were transfected which express HBsAg-specific mRNAs with wild-type or variant tripartite leaders. Cytoplasmic poly(A) $^{+}$ RNA was prepared 48 h after transfection and was subjected to Northern-type analysis. Specific probes were derived by labeling HBsAg DNA fragments by the method of Feinberg and Vogelstein (13).

sequence analysis, and variants then were rebuilt into expression vector pSV-HBV, which has been described previously (11). The vector is a cassette consisting of the left end of Ad5 (1 to 357 base pairs), the Ad2 major late promoter, a complete cDNA copy of the Ad2 tripartite leader which terminates with a *Sa*I linker, the HBsAg coding region, and the Ad5 E1a polyadenylation signal. All variants therefore utilize the same transcription signals and encode the HBsAg polypeptide. The nucleotide boundaries for each mutation identify the last nucleotide retained on either side of the alteration. Plasmids contain the SV40 origin of replication to permit amplification after transfection of COS cells.

The levels of tripartite leader variant mRNAs were first investigated to determine whether any mutations altered their cytoplasmic accumulation. Poly (A) $^{+}$ RNAs were prepared from COS cells transfected with plasmids expressing each variant and were subjected to Northern analysis. Filters were probed with labeled DNA fragments specific for the HBsAg coding region to eliminate signal differences that were due to leader length. For the most part, variants were found to accumulate to levels similar to that of the wild-type tripartite leader mRNA (Fig. 3). Experimental variations in individual mRNA levels (about twofold) were found between different analyses. Deletion mutant *dl15* mRNAs, however, were found to consistently accumulate to three- and four-fold-lower levels. The reason for this decrease is currently under investigation. Although it was generally not feasible to construct deletion mutations in the first leader because it includes the 3' boundary of the adenovirus major late promoter (29), *dl14* is an exception and mRNAs accumulate to the same level as does the wild type. All mRNAs were analyzed by Northern analysis, of which 10 are shown (Fig. 2; see Fig. 7). The minor RNA bands are probably products of the SV40 early promoter, which is located 800 base pairs upstream of the adenovirus major late promoter. These mRNAs cannot encode HBsAg polypeptides. We can conclude that, except for mutant *dl15*, similar levels of mRNAs accumulate regardless of the nature of the tripartite leader mutation. In addition, infection by poliovirus was not found to alter the steady-state level of mRNA species (11; see Fig. 7A; data not shown).

Characterization of potential mRNA-rRNA interactions in tripartite leader activity. All three tripartite leader segments contain a sequence with significant complementarity to a

FIG. 4. Comparison of complementary sequences between tripartite leader and 18S rRNA. The wild-type Ad2 tripartite leader is shown in comparison to eucaryotic 18S rRNA (19). Potential base pairs are designated by vertical lines, and identical sequences are designated by dots. (A) The putative 3' hairpin structure in 18S rRNA is shown. (B) Potential regions of complementarity in all three leaders with 18S rRNA. Regions that potentially interact with VAI RNA correspond to the junction between leaders 2 and 3 (nts 100 through 119; 23) and to the terminal part of leader 3 (34). Nucleotides (in parentheses) are shown corresponding to the 3' end of 18S and the 5' end of 3 LDR RNAs.

highly conserved hairpin structure at the 3' end of 18S rRNA. Two of these elements, in leaders 2 and 3, comprise identical direct repeats (Fig. 4). Although an interaction between mRNAs and rRNAs is unprecedented in eucaryotic translation, the organization and conservation of these sequences are compelling and could conceivably be important for translation of the tripartite leader.

Tripartite leader variants were therefore constructed in which each element was systematically deleted (Fig. 5), and variants were tested by assaying the levels of HBsAg after transfection of COS cells. Cells were labeled with [³⁵S]methionine 48 h after transfection, and immunoprecipitation was performed on cell extracts with a monoclonal antibody specific for HBsAg, followed by SDS-polyacrylamide gel electrophoresis and autoradiography. Autoradiograms were analyzed by densitometry to quantitate the effect of mutations on translational efficiency. Variants were tested in at least three independent experiments and were quantitated (see Fig. 8). Translational efficiencies represent the percent deviation from that observed for the wild-type tripartite leader mRNA.

Characterization of mutants presented in Fig. 5 demonstrates that deletion of the putative binding site in the first leader for 18S rRNA (*dl14*) had no discernable effect on mRNA translation. Levels of both the 24-kDa and glycosylated 27-kDa HBsAg polypeptides were indistinguishable from that of the wild-type tripartite leader-containing mRNAs. Deletion of the direct repeat sequence in the second (*dl2*) and third leaders (*dl18*), both of which are complementary to 18S rRNA, also had no detectable effect on translation. A double mutant (*dl29*) was constructed which deletes sequences complementary to 18S rRNA in leaders 2 and 3. Translation of this mutant was found to be slightly impaired (Fig. 5), with HBsAg levels accumulating to within 60% of that of the wild type (see Fig. 8). To investigate a possible function of these sequences in eIF-4F-independent translation, duplicate plates of transfected cells were infected with wild-type poliovirus before being labeled with [³⁵S]methionine and HBsAg polypeptide levels

FIG. 5. Diagram of tripartite leader variants and translation expression in transfected cells. (A) Wild-type (top) and tripartite leader variants are shown. Nucleotide positions are located relative to the start site of transcription. Open boxes designate sequences complementary to 18S rRNA. Deletion mutations are represented by heavy lines, and boundaries are represented by the last nucleotide retained in the sequence. (B) Duplicate plates of COS cells were transfected with plasmids encoding the indicated mRNAs. One set was labeled with [³⁵S]methionine, and cell extracts were prepared as previously described (11). The other set was infected with poliovirus for 3 h before labeling. Immunoprecipitation of HBsAg and analysis of polypeptides have been described previously (11). The two forms of HBsAg correspond to the 24-kDa (p24) and glycosylated 27-kDa (gp27) polypeptides.

were examined (Fig. 5; see Fig. 8). None of the presumptive 18S rRNA binding sites were found to be required for translation independent of eIF-4F activity. We must conclude that there is little evidence to support an obligatory Shine-Dalgarno-type interaction in the translation of tripartite leader mRNAs, despite a compelling arrangement of

mRNA sequences complementary to 18S rRNA. It should be noted, however, that these observations may be limited to the transfection assay employed since variants have not yet been constructed into adenoviruses and have not been analyzed in infected cells.

Leaders 2 and 3 contain two segments for which a possible interaction with adenovirus VAI RNA has been suggested (23, 34). This is an attractive proposal, since it potentially accounts for the selective translation of mRNAs expressed at late times during adenovirus infection or from plasmids in transfected cells (23, 34). Variants deleted of the potential binding sites were therefore cotransfected with a plasmid expressing VAI RNA. Deletion of the potential binding site in leader 2 (*dl24*) was found to decrease translation by only 30%, and in leader 3 (*dl19*), it had no effect (Fig. 5; see Fig. 8). A duplicate experiment performed without VAI RNA did not detect any change in the phenotypes of *dl24* and *dl19* (data not shown). In addition, these sequences apparently played no role in eIF-4F-independent translation (Fig. 5, lower panel).

Translational expression of large deletion and inversion mutations in the tripartite leader. Several mutations were constructed to investigate the effect of tripartite leader length and primary sequence on the ability of the leader to facilitate translation and provide independence from factor eIF-4F. It was generally not possible to analyze leader 1 by this approach since the 3' boundary of the viral major late promoter extends into it. As shown below, the first leader was analyzed instead by insertion mutagenesis. Two large deletion mutants were constructed, one of which lacks all of the second and half of the third leader (*dl15*) and another which lacks both leader segments (*dl30*). Both large deletion mutants translate quite poorly (Fig. 6). Taking into account the lower accumulation of *dl15* mRNAs, densitometric quantitation indicates an impairment of 10- to 20-fold in translation activity (see Fig. 8). Because both *dl15* and *dl30* mRNAs translate so poorly, it was not possible to unambiguously determine whether they are also more dependent upon eIF-4F activity, although the results from *dl30* tend to support this notion (Fig. 6, compare lanes 12 and 15).

In order to determine whether the length or primary sequence of tripartite leader segments 2 and 3 are important for translation activity, several additional variants were constructed in which particular segments were inverted or "flipped" into the antisense orientation (Fig. 6). Remarkably, whereas deletion of the 33- to 211-nt segment in *dl30* severely inhibits translation, its inversion in *fp31* only slightly alters translational efficiency (20% reduction) and has no effect on eIF-4F independence (see Fig. 8). Previous structure mapping studies indicate that leaders 2 and 3 may form an intrastrand duplex (49), and a computer-generated structure of the *fp31* antisense tripartite leader predicts a conformation with a similar free energy of formation to that of the wild type, although the predicted secondary structure is of course different (unpublished observations). From these results, it is tempting to speculate that tripartite leader translation activity may depend on the degree of structure in leaders 2 and 3 rather than on any particular conformation. In comparison to deletion mutant *dl15*, inversion of the 79- to 169-nt fragment in *fp1*, or deletion of most of the remaining segment in *dl6*, also does not alter the translational activity of the tripartite leader or increase its requirement for eIF-4F. However, the inversion which creates variant *fp1* does not symmetrically flip the second and third leader segments as does *fp31*, and, accordingly, it is difficult to predict whether the stability of the antisense structure will be less or greater

FIG. 6. Diagram and expression of tripartite leader variants containing large deletion and inversion mutations in transfected cells. Nomenclature and experimental procedures are the same as those described in the legend to Fig. 5. The boundaries of inversion mutations are designated by vertical lines. The polypeptide migrating above gp27 is a poliovirus (Polio) protein which is coimmunoprecipitated with HBsAg.

than that of the native conformation. Thus, the results reported in this section clearly indicate that translation is inhibited by deletion of leaders 2 and 3 but that the actual sequences deleted are apparently not important. Nevertheless, the precise role of this segment is not clear and its contribution to translation activity may involve either leader length, a required degree of secondary structure, or both.

The conformation of leader 1 determines translational efficiency and eIF-4F independence. The previous results indicate that the primary sequence of the second and third leader segments is not involved in facilitating translation or eIF-4F independence. Since structural analyses indicate that much of the first leader may be predominantly unstructured, we next tested the translational properties of this segment by constructing two sets of mutations. Variants were produced by inserting a DNA fragment corresponding to nts 1 through

FIG. 7. Diagram and expression of tripartite leader insertion variants. Nomenclature and experimental procedures are the same as those described in the legends to Fig. 2 and 5. (A) Northern-type analysis of wild-type (WT) and variant tripartite leaders containing cytoplasmic mRNAs (arrow) in uninfected (–) and poliovirus (Polio)-infected (+) cells. (B) Translation expression of HBsAg polypeptides from tripartite leader containing mRNAs. The site of insertions are designated by triangles at 33 or 212 nts, and the orientation of the inserted sequences is designated by the 5' nucleotide position.

33 of the tripartite leader into the *PvuII* site at position 33 in either the sense (*in32*) or antisense (*in33*) orientation. Variant *in32* therefore duplicates the potentially unstructured 5' end of the tripartite leader, whereas variant *in33* should generate a relatively stable duplex. An additional set of variants was also constructed in which a fragment corresponding to nts 3 through 17 of leader 1 was inserted at the 3' end of the tripartite leader at position 212 in either the sense (*in36*) or antisense (*in37*) orientation. Variant *in37* should therefore generate a 5' noncoding region in which the mRNA cap is sequestered within the body of the mRNA and is therefore inaccessible to ribosomes. Northern analysis (Fig. 7A) demonstrates that wild-type and variant mRNAs accumulate to similar levels (within twofold) in uninfected or poliovirus-infected cells, as shown for variants *in33* and *in37*.

Characterization of insertion mutations in leader 1 demonstrates that the 5' end of the tripartite leader is responsible

FIG. 8. Quantitation of HBsAg levels produced from tripartite leader variants. Values are expressed as percentage of wild-type (wt) control and represent averages obtained from densitometric scans of at least three independent experiments. Experimental error between independent analyses averaged $\pm 10\%$ of the value shown. Symbols: ■, uninfected cells; ▨, poliovirus-infected cells.

for facilitating translation independent of eIF-4F activity. Whereas control mRNAs from variant *in32* translate identically to that of the wild type, those from antisense variant *in33* are now fully dependent on eIF-4F activity (Fig. 7B, compare lanes 3 and 8). In fact, the dependence on eIF-4F activity for translation of *in33* is almost absolute, as demonstrated by a greater-than-20-fold drop in protein synthesis in poliovirus-infected cells (Fig. 8). Although translation of *in33* is reduced by only half in the presence of eIF-4F activity, for unexplained reasons, secondary structure has been found to be less inhibitory to translation in COS cells (8). Thus, the tripartite leader can acquire eIF-4F-dependent translation, a property associated with most mRNAs, simply by eliminating the proposed structureless 5' end. Because *in33* mRNA can be made dependent on eIF-4F by providing an adjacent antisense sequence, this suggests that the 5' end is very likely unstructured in its native conformation; otherwise, this mutation would have had little effect on eIF-4F dependence. Interestingly, translation of mutant *in37* mRNAs was almost abolished in the presence or absence of eIF-4F activity whereas control *in36* mRNAs were found to translate normally (Fig. 7B and 8). Mutant *in37* presumably sequesters the 5' noncoding region within the body of the mRNA. These results therefore indicate that permitting long-range base-pair interactions between the first leader and the body of the mRNA severely inhibits translation, presumably because the 5' cap is now inaccessible to translation factors and ribosomes.

DISCUSSION

Only a few mRNAs have been shown to translate in the absence of CBP complex or eIF-4F. Examples include several picornavirus mRNAs (5, 22, 38), the *Drosophila* hsp 70 mRNA (35), alfalfa mosaic virus 4 mRNA (15), and an immunoglobulin heavy-chain binding protein mRNA (42). Thus, the previous finding that eIF-4F-independent translation can be conferred on a variety of mRNAs by a 5' noncoding region, the adenovirus tripartite leader (11), is significant considering the small number of mRNAs in this class. In this report, we have therefore investigated the molecular mechanism by which the tripartite leader directs protein synthesis without CBP complex activity.

Picornavirus mRNAs represent the best-studied examples of eIF-4F-independent translation, which is believed to occur by the internal binding of ribosomes (5, 22, 38). To determine whether the tripartite leader also promotes internal initiation, it was located as the second cistron in a dicistronic mRNA (Fig. 2). The results of these experiments demonstrate that the tripartite leader does not guide the efficient internal entry of ribosomes, at least in a manner shown to occur for picornavirus mRNAs. However, we cannot currently exclude theoretical variations of internal initiation, such as "jumping" ribosomes that require binding to a 5' cap (7, 21). We also failed to find any evidence to support a Shine-Dalgarno-type interaction between the tripartite leader and 18S rRNA in the translation process. Although the tripartite leader possesses three regions with surprising complementarity to 18S rRNA (Fig. 4), deletion of these elements individually (*dl14*, *dl2*, and *dl8*) had little or no effect on translation efficiency or on eIF-4F independence (Fig. 5 and 8). Furthermore, the 40% impairment of the double deletion variant (*dl29*) is probably attributable to the size of the deletion rather than to any specific effect, as underscored by the results from large deletion and inversion variants. Because large deletions of the tripartite leader inhibit translation, we did not construct a mutant deleted of all three regions complementary to 18S rRNA. However, considering the results from insertion mutations in leader 1, we think it extremely unlikely that the complementarity to rRNA in the first leader is responsible for the translation activity of the tripartite leader. Nevertheless, given the unusual conservation of these elements in different adenovirus serotypes (unpublished observations), we are currently determining whether they serve any translation function at late times in adenovirus-infected cells.

It is surprising that most mutations in the tripartite leader do not alter its translational efficiency or eIF-4F independence. In fact, most of leaders 2 and 3 can be inverted into the antisense orientation, as was shown with variants *fp1* and *fp31* (Fig. 6), and still not disrupt the normal translation activity of the tripartite leader. However, deletion of leaders 2 and 3, as shown with mutants *dl5* and *dl30* (Fig. 6), severely impairs translation. In previous studies, large deletions were also found to inhibit translation of tripartite leader-containing mRNAs (4, 23, 31). Our results therefore demonstrate that the primary sequence of leader segments 2 and 3 is not involved in facilitating enhanced translation or eIF-4F independence.

Previous structure mapping experiments performed on the tripartite leader indicate that much of the first leader is rather unstructured, whereas the second and third leaders are probably paired in a moderately stable structure (49). We therefore inserted within the first leader a complementary sequence (*in33*), which now renders the mRNA fully dependent on CBP complex for its translation (Fig. 7). These results suggest that the 5' end of the first leader is indeed unstructured *in vivo* and that this conformation is responsible for the translation properties of the tripartite leader. Deletion variant *dl14* very likely preserves the translation activity of the tripartite leader because only 10 of the unstructured nucleotides have been deleted. In addition, the severe translation defect observed with large deletion mutants *dl15* and *dl30* may possibly be explained by mutant *in37*, in which the 5' end of leader 1 is forced to pair with a complementary sequence inserted within the body of the mRNA. Mutants *dl15* and *dl30* do not alter the essential 5' end of the tripartite leader but eliminate much of its secondary structure. In all three mutants, the 5' end of the message

is very likely sequestered within the body of the mRNA and the cap is probably inaccessible to translation factors and ribosomes. In fact, similar results have been reported for the zein mRNA from *Zea mays*, in which stable long-range base-pair interactions with the 5' end of the message also severely impair translation efficiency (47).

How does the tripartite leader facilitate translation and bypass the normal requirement for CBP complex activity? The proposed conformation of the tripartite leader offers one attractive explanation for its activity and accounts for some of the results obtained in this study. Our earlier results (49) strongly suggest that the 5' end of the tripartite leader is in fact unstructured under native conditions, which could reduce the dependence on translation factor eIF-4F, which presumably unwinds the 5' end of capped mRNAs. Experimentally, minimal 5' structure also correlates with more efficient ribosome binding and translation initiation, particularly under competitive conditions (for a review, see reference 12). The precise role of leader segments 2 and 3 in translation of the tripartite leader has not been established by this study. Structure mapping studies predict that they may form a moderately stable duplex structure independent of the first leader (49), although this remains to be demonstrated *in vivo*. In one model, the pairing between the second and third leaders may be responsible for preventing the unstructured first leader from base pairing with the remainder of the RNA molecule, possibly by disrupting all but the most stable long-range interactions with the first leader. This model seems plausible, since it has been shown that moderately stable structures placed some distance from the 5' cap do not necessarily interfere with translation (6, 25, 30). Alternatively, the effect of 5' noncoding region length on translation activity is clearly complicated and poorly understood, involving considerations of inherent secondary structure and ribosome-binding efficiencies. For example, it has been suggested that long 5' noncoding regions may enhance translation of an mRNA, particularly during competitive or stressful conditions (25), although the reasons for this are not clear.

Finally, unrecognized features of the tripartite leader may also be involved in the selective recruitment or utilization of individual components of the CBP complex. In particular, initiation factors eIF-4A and eIF-4B have been shown to bind to short, unstructured regions of RNA in a cap-independent fashion and display a limited RNA unwinding activity (1, 2). It has been proposed that such an interaction could potentially serve as a signal for the binding of other initiation factors and ribosomes in a cap-independent manner (20).

The results presented in this report have investigated several possible mechanisms by which the adenovirus tripartite leader directs translation in the absence of CBP complex activity. The fundamental purpose of this alternate translation mechanism, however, is not known. Although a reduced requirement for eIF-4F has been shown to correlate with enhanced translation of an mRNA (1, 12), particularly under competitive conditions (28), the role of eIF-4F independence in the translation of late mRNAs during adenovirus infection remains to be determined.

ACKNOWLEDGMENTS

We thank Yan Zhang for technical assistance during parts of this work and V. Racaniello and P. Palese for valuable discussions.

This work was supported by Public Health Service grant CA-42357 from the National Institutes of Health. R.J.S. is a recipient of an Irma T. Hirsch Career Scientist Award.

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