mRNAs Containing the Unstructured 5' Leader Sequence of Alfalfa Mosaic Virus RNA 4 Translate Inefficiently in Lysates from Poliovirus-Infected HeLa Cells

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Poliovirus infection is accompanied by translational control that precludes translation of 5'-capped mRNAs and facilitates translation of the uncapped poliovirus RNA by an internal initiation mechanism. Previous reports have suggested that the capped alfalfa mosaic virus coat protein mRNA (AIMV CP RNA), which contains an unstructured 5' leader sequence, is unusual in being functionally active in extracts prepared from poliovirus-infected HeLa cells (PI-extracts). To identify the *cis*-acting nucleotide elements permitting selective AIMV CP expression, we tested capped mRNAs containing structured or unstructured 5' leader sequences in addition to an mRNA containing the poliovirus internal ribosome entry site (IRES). Translations were performed with PI-extracts and extracts prepared from mock-infected HeLa cells (MI-extracts). A number of control criteria demonstrated that the HeLa cells were infected by poliovirus and that the extracts were translationally active. The data strongly indicate that translation of RNAs lacking an internal ribosome entry site, including AIMV CP RNA, was severely compromised in PI-extracts, and we find no evidence that the unstructured AIMV CP RNA 5' leader sequence acts in *cis* to bypass the poliovirus translational control. Nevertheless, cotranslation assays in the MI-extracts demonstrate that mRNAs containing the unstructured AIMV CP RNA 5' untranslated region have a competitive advantage over those containing the rabbit α -globin 5' leader. Previous reports of AIMV CP RNA translation in PI-extracts likely describe inefficient expression that can be explained by residual cap-dependent initiation events, where AIMV CP RNA translation is competitive because of a diminished quantitative requirement for initiation factors.

Gene expression is modulated posttranscriptionally through the intrinsic ability of mRNAs to compete for components of the translational apparatus. Differential translation of specific mRNAs regulates gene expression during virus infection (for reviews, see references 32, 61, and 62), development (13, 22, 55), cellular differentiation (4, 33), and responses to environmental conditions (see references 25 and 66 for recent reviews). It has been proposed that efficiently translating mRNAs possess the highest affinity or lowest requirement for a limiting translational component(s) and that unequal translation rates among an mRNA population are most apparent when ribosomes or protein factors are limiting (37).

The nucleotide sequences and structural features that determine mRNA translational efficiency and competitive activity are poorly characterized. Regulatory elements that influence translation have been identified in the 5' untranslated leader (16, 27, 30, 51), the protein-coding region (15, 68), and the 3' untranslated region/poly(A) tail (14, 33, 40, 41, 58, 67). Furthermore, cellular proteins such as the iron-responsive element-binding protein can block translation by binding to specific *cis*-acting mRNA elements (8, 23, 56). Recent data suggest that *trans*-acting factors that regulate translation may include not only proteins but also small RNAs, such as the *lin-4* gene product in *Caenorhabditis elegans* (36, 67). It has been hypothesized that these small RNAs inhibit translation by

binding to complementary sequences in the 3' untranslated region (UTR) of *lin-14* RNA.

To identify and characterize nucleotide sequences and structures that regulate translational efficiency or mRNA competitive activity, we have focused on translation of alfalfa mosaic virus (AIMV) coat protein (CP) RNA 4, which encodes the plant viral coat protein. AIMV CP RNA is one of the most efficiently translated RNAs known (31). In previous studies, it has been shown that the 5' UTR of AIMV CP RNA is principally unstructured in solution (19) and is a translational enhancer sequence in vitro (27) and in vivo (18). Furthermore, chimeric mRNAs containing the AIMV CP RNA 5' UTR require lower levels of translation initiation factors for optimal translation in vitro (7). Sonenberg et al. reported that AIMV CP RNA translates in cell lysates prepared from poliovirus-infected HeLa cells (PI-extracts), in which translation of most capped cellular mRNAs is impaired (63). It was proposed that the lack of secondary structure in the AIMV CP RNA 5' UTR enabled the mRNA to overcome the poliovirus-induced translational inhibition (10, 63). Our goal was, therefore, to test this hypothesis directly and to define a mechanism for the reported selective AIMV CP RNA translation in PI-extracts.

We report here the results of translating AIMV CP RNA and chimeric mRNAs containing 5' UTRs of AIMV CP RNA or α -globin in PI-extracts and extracts prepared from mock-infected cells (MI-extracts). α -Globin mRNA translated poorly in the PI-extracts compared with a chimeric mRNA containing the poliovirus internal ribosome entry site (IRES). Replacing the structured α -globin 5' UTR with that of AIMV CP RNA did not restore α -globin translation to levels observed in MI-extracts, suggesting that the unstructured AIMV CP RNA 5'

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UTR does not act independently in *cis* to overcome the poliovirus-induced translational inhibition. Although others have suggested that AIMV CP RNA has a unique ability to translate in PI-extracts (10, 63), our data, generated by translating α -globin, vesicular stomatitis virus (VSV), and AIMV CP RNAs in parallel, demonstrate that AIMV CP RNA translation is severely inhibited in PI-extracts. In fact, AIMV CP RNA translation is impaired to the same relative degree as that of other capped mRNAs. We conclude that AIMV CP RNA translation is cap dependent and therefore sensitive to the translational inhibition observed for other capped mRNAs. The previously observed apparent translation of AIMV CP RNA in PI-extracts may therefore reflect residual cap-dependent translation. Thus, while the AIMV CP RNA 5' UTR increases translational efficiency and competitive activity, it does not support efficient translation in PI-extracts. Efficient translation of mRNAs in PI-extracts required an IRES element, which cannot be substituted for by the unstructured AIMV CP RNA 5' UTR.

MATERIALS AND METHODS

Cells, viruses, and viral RNAs. Cell lysates were prepared from HeLa S3 cells, which were obtained from Phillip Sharp (Center for Cancer Research, Massachusetts Institute of Technology). The HeLa R19 cells used for poliovirus plaque assays were provided by Richard Colonna (Merck, Sharp, and Dohme Research Laboratories). Poliovirus stocks (Mahoney strain) were obtained from Raul Adino and David Baltimore (Whitehead Institute, Cambridge, Mass.) and Nahum Sonenberg (McGill University, Montreal, Canada). Native VSV RNA and AIMV CP RNA were provided by Donald Coen (Harvard Medical School) and L. Sue Loesch-Fries (Purdue University), respectively.

Cell culture conditions. Calf serum and defined fetal calf serum (FCS) (HyClone Laboratories) were heat inactivated prior to use. HeLa S3 cells were amplified for suspension cultures by growing monolayers in Dulbecco's modified Eagle's medium (DMEM; Gibco or BioWhittaker) with 10% FCS and gentamicin (25 μ g/ml; Gibco). Two days before transfer to suspension cultures, the cells were plated at a low density (3×10^6 cells per 75-ml culture flask) in S3 medium (Joklik's minimal essential medium containing 5% calf serum, 10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.5] and gentamicin [25 μ g/ml]; all from Gibco). To prepare suspension cultures, nonconfluent cells were released by trypsin treatment (0.25% trypsin, 1 mM EDTA [Gibco]), washed with phosphate-buffered saline without Ca^{2+} or Mg^{2+} (PBS), resuspended in conditioned medium, transferred to spinner flasks (Bellco) at a concentration of 2.5×10^5 cells per ml, and maintained in a warm room at 37°C. Cells were counted each day and diluted to 2.5×10^5 to 3.0×10^5 /ml with warmed S3 medium. Only those cultures containing cells doubling every 24 h were used for viral stock amplification or extract preparation.

Amplification of virus stocks. To amplify viral stocks, HeLa S3 cells were washed twice with PBS and resuspended in infection medium (Joklik's minimal essential medium, 10 mM HEPES [pH 7.5], 1% FCS, equilibrated to room temperature) at 4×10^7 cells per ml. Virus was added at 5 PFU/cell, and the cells were incubated, with rocking, at room temperature for 45 min. The cells were then diluted 10-fold in S3 medium and incubated at 37°C for an additional 7 h. Cells were sedimented at $400 \times g$, washed three times with ice-cold PBS, and resuspended in RSB (10 mM Tris, 10 mM NaCl, 15 mM magnesium acetate) with 0.005 times the original suspension culture volume. The cells were subjected to three freeze (-20°C)-thaw cycles and centrifuged at $400 \times g$, and the resulting supernatant was removed and stored at -80°C .

Virus titers were determined by plaque assay (9) on R19 HeLa cells. Cells were plated at 10^6 cells per 60-mm culture plate and allowed to adhere. Monolayers were rinsed with cold PBS, infected with ~ 200 μ l of diluted viral stock, and incubated at room temperature for 45 min with occasional swirling. The virus was removed by aspiration, and the cultures were overlaid with 1% agar (DIFCO) and 1% FCS in DMEM, cooled to 38°C, and incubated for 2 to 3 days at 37°C. The agar overlay was removed, and plaques were identified by crystal violet staining (1% crystal violet, 6% ethanol).

Preparation of HeLa cell lysates. HeLa cell lysates were prepared by previously published methods (54), with minor modifications. Cells were infected as described above except that virus was added at 50 PFU/cell. Cells were incubated, with rocking, at room temperature for 45 min, diluted 10-fold in warm S3 medium, and incubated at 37°C for 3.5 h (6). Cells were washed three times with PBS, resuspended in 1.5 volumes of hypotonic buffer (10 μ M HEPES-KOH [pH 7.6], 10 μ M potassium acetate, 1.5 mM magnesium acetate, 2.0 μ M dithiothreitol [DTT]), swelled on ice for 15 min, and then lysed by approximately 25 strokes with a Dounce homogenizer, type B pestle. Microscopic analysis of trypan blue-stained homogenate showed that more than 90% of the cells were lysed. The homogenate was transferred to Corex or Falcon polypropylene tubes and cen-

trifuged in an HB-4 rotor at $2,000 \times g$ for 5 min; next, the supernatant was transferred to a fresh tube and centrifuged for 20 min at $10,000 \times g$. The S10 supernatant was removed, transferred to dialysis tubing (Spectrapor; molecular cutoff, 6,000 to 8,000), and dialyzed against 100 volumes of dialysis buffer (10 mM HEPES-KOH [pH 7.6], 90 μ M potassium acetate, 1.5 mM magnesium acetate, 1.0 mM DTT) for 2 h at 4°C. Small aliquots of the lysate (30 to 50 μ l) were snap-frozen in liquid nitrogen and stored in liquid nitrogen.

cDNAs and plasmid constructs. All enzymes and linkers were purchased from New England Biolabs (Beverly, Mass.) unless otherwise noted. Constructs were prepared by standard molecular techniques (59). Plasmid pAMV-4, containing AIMV RNA-4 cDNA in pTZ18R (Pharmacia), was constructed with an AIMV CP RNA cDNA (38) by Karen Browning, University of Texas (12). Plasmid pHST405 is a derivative of pAMV4 in which 4 vector nucleotides immediately downstream of the T7 RNA polymerase transcriptional initiation site were deleted. Removal of the vector nucleotides permitted transcription of AIMV CP RNA that contained only 4 vector-derived nucleotides at the 5' end and 2 vector-derived nucleotides at the 3' end.

Rabbit α -globin cDNA was cloned with the Okayama-Berg vectors (43, 44) and subcloned into an in vitro transcription vector to generate pHST101. Plasmid pHST106 is a related construct in which the 5' UTR of rabbit α -globin was replaced by the 5' UTR of AIMV RNA-4. Transcripts from plasmids pHST101 and pHST106 contain no vector-derived nucleotides at the 5' UTR and a 48-nucleotide poly(A) tail following the 3' UTR. Constructs involving replacement of the AIMV CP RNA 5' UTR are based upon plasmid pMON9822 (65), in which a unique *NcoI* restriction enzyme site (CCATGG) was engineered at the initiation site for protein synthesis. Plasmid pHST416 contains the AIMV RNA-4 coding region and 3' UTR preceded by the α -globin 5' UTR.

The cDNA encoding the VSV NS protein (New Jersey strain; pGEM-NS) was a gift from Amiya K. Banerjee, Roche Institute of Molecular Biology, Roche Research Center (20). Plasmid pP2CAT, containing the poliovirus IRES followed by the chloramphenicol acetyltransferase (CAT) gene, was obtained from Jerry Pelletier, McGill University (48).

In vitro transcription and RNA preparation. RNA was transcribed for the in vitro translation reactions with commercial kits designed for high mRNA yield (Promega Ribomax System or Ambion's mMessage mMachine). All mRNAs used for translation experiments were purified by denaturing polyacrylamide gel electrophoresis (PAGE). Following phenol-chloroform extraction and prior to ethanol precipitation, RNA was passed through a G-50 Sephadex (Pharmacia) column. Thin-layer chromatography (28) showed that greater than 90% of the transcripts were capped at the 5' terminus. Quantitation of transcripts was routinely carried out by measuring the A_{260} (1 optical density [OD] unit = 40 μ g of RNA per ml). Control experiments demonstrated that the RNA concentration determined spectrophotometrically was not significantly different from that determined by calculating the specific activity of a radiolabeled ribonucleotide incorporated during in vitro transcription. Prior to translation, RNA aggregates were disrupted by heating the RNA to 65°C for 8 min, followed by slow cooling to 25°C over 20 min in a Perkin Elmer Cetus DNA Thermal Cycler.

In vitro translations and protein quantitation. All cell extracts used for in vitro translation were thawed immediately prior to the experiment and never refrozen. Endogenous mRNA was removed by micrococcal nuclease digestion (Boehringer Mannheim; 75 U/ml of lysate with 1 mM CaCl_2 at 20°C for 15 min) (47). The nuclease digestion was stopped by adding the calcium chelator EGTA (ethylene glycol tetraacetic acid) to a final concentration of 2.8 mM. A 100- μ l lysate mixture contained 66.5 μ l of nuclease-treated lysate, 20 mM HEPES-KOH (pH 7.5), 1 mM ATP, 55 μ M GTP, 0.2 mM spermidine, 10 μ M amino acids minus methionine, 2.5 mM DTT, 9 mM creatine phosphate, creatine phosphokinase (23 μ g/ml), 3 mM magnesium acetate, and 140 mM potassium acetate. All components were added from frozen stocks except for the creatine phosphate, which was prepared immediately prior to the experiment. Stocks of 1 M DTT were used once and discarded. A 25- μ l translation reaction mix contained 13 μ l of lysate mixture, 5 μ Ci of L-[^{35}S]methionine (Dupont-New England Nuclear), and mRNA as indicated in the figure legends. All time course analyses of protein synthesis in the HeLa cell extracts were carried out at saturating concentrations of added RNA unless otherwise noted. Translation was initiated by transfer from ice to the incubation temperature (30°C). In titration experiments, aliquots of the reaction mixture were removed during linear incorporation of [^{35}S]methionine. Protein synthesis was monitored by analyzing an aliquot (2 μ l) of the translation reaction mixes by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (34). Quantitation of radioactive methionine in the specific protein bands was performed on a Molecular Dynamics PhosphorImager. Values were corrected to reflect the number of methionines in the proteins, assuming that the initiator methionine is removed (26, 64): 3 methionines per mole of coat protein, 1 methionine per mole of α -globin, 8 methionines per mole of pP2CAT, or 7 methionines per mole of VSV NS protein (New Jersey strain).

Analysis of RNA stability by Northern (RNA) blotting. To assess mRNA integrity, 2- μ l aliquots were removed from the translation reaction mixes as described above and processed for Northern blot analysis. RNAs were separated by electrophoresis into a 1% denaturing agarose gel (1% agarose in 10 mM MOPS [morpholinepropanesulfonic acid, pH 7.0], 4 mM sodium acetate, 0.5 mM EDTA, 20% formaldehyde) at 100 V in 10 mM MOPS (pH 7.0)–4 mM sodium acetate–0.5 mM EDTA (59) and then transferred overnight to Duralose membranes (Stratagene) in 20 $\times$ SSC (3 M NaCl, 0.3 M sodium citrate [pH 7.0]) prior

FIG. 1. Western blot analysis of p220 integrity in MI- and PI-extracts. Experimental details are described in Materials and Methods. (A) Immunoblot of MI- and PI-extracts, showing poliovirus-specific cleavage of p220. Comparable amounts of protein were added each lane. Lane 1, MI-extract; lane 2, PI-extract; lane 3, purified eIF-4F; lane 4, purified eIF-4A. (B) Ponceau Red staining of the same blot, indicating protein loading. Sizes are marked in kilodaltons.

to cross-linking to the membrane with a Bio-Rad GS Gene Linker UV chamber. Northern blot hybridization was carried out with QuikHyb (Stratagene) according to the manufacturer's instructions. Hybridized blots were washed twice in $2\times$ SSC-0.1% SDS at room temperature for 15 min and then once at 60°C in $0.2\times$ SSC-0.1% SDS. To generate probes for hybridization, the appropriate cDNA was first digested with restriction endonucleases, and the specific fragment was isolated by nondenaturing agarose gel electrophoresis and electroelution. DNA fragments were then radiolabeled with a random primer kit (Boehringer Mannheim or Prime-It II [Stratagene]) according to the manufacturer's instructions. Probes generated by this method routinely had a specific activity of 10^9 cpm/ μg and were added to the hybridization solution at a concentration of 1.5×10^6 to 2.0×10^6 cpm/ml with 500 μg of yeast tRNA.

Western (immunoblot) analysis of the p220 component of eIF-4F. The integrity of the p220 component of eIF-4F in the HeLa cell extracts was analyzed by Western blotting. Aliquots of lysates containing equivalent amounts of protein were separated by electrophoresis into an SDS-12.5% polyacrylamide gel. Proteins were transferred overnight at 40 V onto nitrocellulose (Schleicher and Schuell) at 4°C in 20 mM Tris-150 mM glycine-20% methanol with a Western transfer apparatus (Bio-Rad Laboratories). Transferred proteins were visualized by staining in 0.1% Ponceau red and 5% acetic acid. The membranes were subsequently destained in water. Nonspecific antibody-binding sites were blocked by soaking the membrane in 3% bovine serum albumin (BSA) for 30 min, followed by washes in PBS. The membrane was incubated for 2 h at room temperature in PBS containing a 1:1,000 dilution of mouse anti-human p220 antibody (provided by Diane Etchison, University of Kansas Medical Center). The blot was then washed three times for 15 min each in PBS prior to adding conjugated second antibody (alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G [IgG]; Promega; 1:7,500 dilution) and incubating for an additional 2 h. The blot was washed twice in PBS and once in 100 mM Tris (pH 9.5)-100 mM NaCl-5 mM MgCl_2 and then developed with the Nitro Blue Tetrazolium-BCIP system (Promega).

RESULTS

Characterization of HeLa extracts. Several criteria were evaluated to ensure that the cells used to prepare the PI-extracts were infected with poliovirus and that the resulting PI- and MI-extracts would actively translate exogenous mRNAs. Proteolytic cleavage of p220, a subunit of the cap-binding protein complex (eIF-4F), is characteristic of poliovirus infection, and it is thought that inhibition of host capped mRNA translation is a consequence of p220 proteolysis (11, 35). Thus, the integrity of p220 in the poliovirus-infected cells was determined by immunoblot analysis with p220 antiserum (Fig. 1A). In the MI-lysate, p220 is intact, migrating with an apparent molecular mass of 220 kDa (Fig. 1A, lane 1). In contrast, no intact p220 is detectable in the PI-lysate (Fig. 1A, lane 2); the p220 antiserum reacts only with lower-molecular-weight cleavage products. The controls presented in Fig. 1A (lanes 3 and 4) show that the antibody reacts with purified cap-binding protein complex (eIF-4F), but there is no reactivity against purified eIF-4A alone. The equivalent intensity of Ponceau red staining (Fig. 1B) indicates that approximately equal amounts of protein were loaded in each lane of the gel. The absence of detectable intact p220 is evidence that the cells used to prepare the PI-extracts were indeed infected with poliovirus.

A second characteristic of extracts prepared from poliovirus-infected HeLa cells is that translation of capped mRNAs such

FIG. 2. Translation of VSV mRNAs is depressed in PI-extracts. (A) Autoradiogram of an SDS-polyacrylamide gel showing the translational profile of native VSV (Indiana strain) mRNA in MI- and PI-extracts. Sizes are shown in kilodaltons. (B) Autoradiogram of an SDS-polyacrylamide gel showing translation of VSV NS transcripts (pGEM-NS) synthesized in vitro with SP6 RNA polymerase. Aliquots (2 μl) were removed from translation reaction mixes at 10 min and analyzed as described in Materials and Methods. (C) Data generated from the autoradiogram depicted in panel B; ■, MI-extracts; ♦, PI-extracts. (D) Stability of the SP6-generated NS transcripts in MI- and PI-extracts. Northern blot analysis was performed as described in Materials and Methods.

as VSV RNA is compromised (54), while translation of certain viral and cellular mRNAs containing an IRES is unimpaired (39, 50). Native VSV mRNA (i.e., isolated from viral particles) and an in vitro transcript of VSV NS mRNA were translated in the PI- and MI-extracts; the electrophoretic separation of radiolabeled translation products is shown in Fig. 2A and 2B. Quantitation of the radiolabeled protein bands from the VSV NS transcript is shown in Fig. 2C. Both the native mRNA and the VSV NS transcript translate in the MI-extracts, whereas translation is severely depressed in the PI-extracts. Northern blot hybridization results demonstrate that the NS RNA extracted from the translation reaction mixes shown in Fig. 2B

A. **B.**

FIG. 3. Translation of pP2CAT mRNA in MI- and PI-extracts. (A) Autoradiogram of an SDS-polyacrylamide gel showing P2CAT translation in MI- and PI-extracts. P2CAT transcripts were added to extracts at a concentration of 50 pmol of RNA per ml and analyzed as described in Materials and Methods. Time is in minutes. (B) Quantification of data shown in panel A. Levels of CAT protein in PI-extracts (♦) and MI-extracts (■) were determined as described in Materials and Methods.

and 2C is stable in both lysates (Fig. 2D). The results presented in Fig. 2 show that capped NS mRNA translation is compromised in the PI-extracts.

To demonstrate that the poor translation of NS mRNA observed in the PI-extract was not due to use of an inactive lysate, we translated P2CAT mRNA, which contains 730 nucleotides of the poliovirus 5' UTR, including the IRES, linked to the CAT coding region (49). Although poliovirus infection depresses translation of capped mRNAs, mRNAs containing an IRES initiate translation by a cap-independent mechanism and are therefore unaffected (39, 52). The results presented in Fig. 3 show that P2CAT mRNA translates efficiently in both MI- and PI-extracts; in fact, P2CAT often translated with greater efficiency in the PI-extracts. As an additional control to ensure that the HeLa cells used to prepare PI-extracts were indeed infected with poliovirus, protein synthesis was analyzed

in a PI-extract that was not treated with micrococcal nuclease. Analysis of the labeled polypeptides by SDS-PAGE clearly demonstrated the presence of labeled viral proteins (data not shown). These results, coupled with the data presented in Fig. 1 to 3, demonstrate that the MI-extracts support both cap-dependent and cap-independent translation, while translation of capped mRNAs is compromised in the PI-extracts. Thus, the extracts exhibit the characteristics of PI-extracts described previously (11, 52, 54), and with the criteria described above, we proceeded to use the extracts for translational analyses with chimeric mRNAs.

Analysis of AIMV CP RNA translation in MI- and PI-extracts. To identify *cis*-acting nucleotide elements that might permit AIMV CP RNA to translate selectively in the PI-extracts, we analyzed translation of chimeric mRNAs containing combinations of untranslated and coding regions from AIMV CP RNA and α -globin mRNA (Fig. 4). AIMV CP RNA is a naturally nonadenylated mRNA, and thus we used a cell-free translation system instead of cellular transfection to assess the translational contribution of individual AIMV CP RNA regions. Since mRNAs transcribed from cellular transfection vectors become polyadenylated, direct translation of chimeric mRNAs in HeLa cell extracts greatly simplified interpretation of the translation data. The mRNAs tested included AIMV CP RNA (Fig. 4A), a chimeric AIMV CP RNA containing the rabbit α -globin 5' UTR leader sequence ($\alpha/A/A$, Fig. 4B), rabbit α -globin (Fig. 4C), and a chimeric α -globin mRNA containing the unstructured AIMV CP 5' UTR leader sequence ($A/\alpha/\alpha$, Fig. 4D).

The unstructured 5' UTR of AIMV CP RNA enhances translation of chimeric mRNAs in rabbit reticulocyte and wheat germ lysates (27), while stable secondary structure in the 5' untranslated leader sequence diminishes translational efficiency (51). We reasoned therefore that substituting the structured rabbit α -globin leader (46) for the unstructured AIMV CP RNA leader (see Fig. 4) might decrease translational efficiency and diminish coat protein synthesis significantly in the HeLa extracts. The data presented in Fig. 5A show that during the initial synthetic period, when incorporation of [35 S]methionine is linear with respect to time, the translational efficiency of the chimeric $\alpha/A/A$ in the MI-extracts was less than that of the wild-type AIMV CP RNA; however, after 2 h of translation, the levels of accumulated protein were similar. These results provide further evidence for a correlation between ef-

FIG. 4. Schematic drawings of chimeric RNAs used in this study. The cDNAs representing these RNAs were prepared as described in Materials and Methods. The thin black and gray lines represent the untranslated regions of AIMV CP RNA and α -globin mRNA, respectively; the thick black and gray lines represent the AIMV CP and α -globin coding regions, respectively. (A) AIMV CP RNA; (B) chimeric AIMV CP RNA with the rabbit α -globin 5' UTR; (C) rabbit α -globin mRNA; (D) chimeric α -globin mRNA with the AIMV CP RNA 5' UTR.

FIG. 5. Translational profile of AIMV CP RNA and chimeric $\alpha/A/A$ transcripts in MI- and PI-extracts. (A) Translational time course of AIMV CP RNA (■) and chimeric $\alpha/A/A$ (▼) mRNAs in MI-extracts. Below the graph is an autoradiogram of the SDS-polyacrylamide gel used to generate the quantified data. (B) Translation of the same RNAs in PI-extracts. Below the graph is an autoradiogram of the SDS-polyacrylamide gel used to generate the data. (C) Northern blot hybridization of AIMV CP RNA transcripts extracted from MI- and PI-extracts. To control for equal RNA loading, the blots were stripped and rehybridized with a probe recognizing 28S rRNA. Time is in minutes.

efficient translation of AIMV CP RNA and the presence of the unstructured 5' UTR.

The results of translating the mRNAs in the PI-extracts are plotted in Fig. 5B with the same relative scale used in Fig. 5A. Translation of both AIMV CP RNA and the chimeric $\alpha/A/A$ transcripts was severely depressed in the PI-extracts; in fact, AIMV CP RNA translation was diminished by up to 85% in the PI-extracts (compare the 60-min time point of AIMV CP RNA translation [squares] in Fig. 5A and B). Control experiments (Fig. 3) demonstrated that the PI-extracts support efficient translation of P2CAT mRNA; therefore, the poor translation of AIMV CP RNA did not result from the use of inactive extracts. Furthermore, the inefficient AIMV CP RNA transla-

tion cannot be explained in terms of differential mRNA stability (Fig. 5C). Because incorporation of radioactive methionine into AIMV CP with the PI-extracts reached a plateau at approximately 20 to 25 min of incubation, Northern blot analysis was routinely carried out over a 30-min period. However, other experiments showed that the stability of AIMV CP RNA was similar in both extracts after 60 min of translation (data not shown).

To determine if the suppressed translation of AIMV CP RNA in the PI-extracts was similar to that observed with another mRNA, α -globin, we translated AIMV CP RNA and α -globin mRNA in MI- and PI-extracts. In addition, we assessed the ability of the unstructured AIMV CP RNA 5' UTR to rescue α -globin translation in the PI-extracts. The results shown in Fig. 6A indicate that both α -globin and the chimeric A/ α /A transcripts translate similarly, though the initial translational rate of the chimeric mRNA is slightly greater. Interestingly, we consistently observed that both α -globin and the chimeric A/ α /A translated with higher efficiency than AIMV CP RNA in the MI-extracts. This is not due to the preferential degradation of AIMV CP RNA over the α -globin and the chimeric A/ α /A transcripts (Fig. 7C). At the same time, all three mRNAs translate poorly in the PI-extracts, though the mRNAs are stable over the time sampled (Fig. 7C). These data suggest that the unstructured AIMV CP RNA 5' UTR does

FIG. 6. Translational time course of α -globin, chimeric A/ α /A, and AIMV CP RNA transcripts in MI- and PI-extracts. (A) Translation of α -globin (●), A/ α /A (○), and AIMV CP (■) transcripts in MI-extracts. (B) Translation of the same transcripts in PI-extracts; symbols are the same as in panel A.

FIG. 7. Titration of α -globin, chimeric A/ α / α , and AIMV CP RNA transcripts in MI- and PI-extracts. (A) Titration of α -globin (●), A/ α / α (○), and AIMV CP (■) transcripts in MI-extracts. Aliquots (2 μ l) were removed at 10 min following the initiation of translation and analyzed as described in Materials and Methods. (B) Titration of the same transcripts in PI-extracts; symbols are the same as in panel A. (C) Northern blot hybridization of transcripts extracted from translation reaction mixes in the same experiment depicted in panels A and B. Aliquots (2 μ l) were removed at the times indicated and processed as described in Materials and Methods. Time is in minutes.

not function in *cis* to stimulate translation of a chimeric mRNA in the PI-extracts; moreover, the results provide additional evidence that AIMV CP RNA is not selectively translated with high efficiency in the PI-extracts.

We considered the possibility that the poor translation of AIMV CP RNA in the PI-extracts might be an mRNA transcript concentration artifact. The data shown in Fig. 7 argue against this possibility, because the translation of AIMV CP RNA, α -globin mRNA, and the α /A/ α mRNA is diminished over a wide concentration range in the PI-extracts. Furthermore, we carried out additional control experiments to determine if the reduced AIMV CP RNA translation in the PI-extracts resulted from suboptimal ion concentrations (data not shown). Regardless of the ionic conditions, translation of AIMV CP RNA in the PI-extracts was severely compromised relative to that in the MI-extracts. Our interpretation of the results shown in Fig. 5 to 7 is that the translation of all capped mRNAs tested, including that of AIMV CP RNA, is markedly depressed in the PI-extracts without significant loss of mRNA

FIG. 8. Cotranslation of AIMV CP, α -globin, and chimeric A/ α / α mRNAs in MI- and PI-extracts; autoradiogram of translation reaction mixes analyzed on an SDS-12.5% polyacrylamide gel. AIMV CP, α -globin, and chimeric A/ α / α transcripts were either translated independently or cotranslated (AIMV CP and α -globin RNAs or AIMV CP and chimeric A/ α / α RNAs) in MI- and PI-extracts. For cotranslation experiments, equal molar amounts of RNA (final RNA concentration equals 100 pmol/ml) were translated. For translation in PI-extracts, analysis of competitive translation was precluded by the low levels of protein synthesis. Experiments were performed as described in Materials and Methods.

stability. There is no evidence from these experiments that AIMV CP RNA circumvents poliovirus-induced translational inhibition.

Role of AIMV CP RNA 5'-unstructured leader in translation under competitive conditions. Although the translation experiments shown in Fig. 5 to 7 indicate that the AIMV CP RNA 5' UTR does not permit selective translation in PI-extracts, it has been demonstrated that the unstructured leader is a translational enhancer sequence (16, 17, 27). For other experiments presented in this report, mRNAs were translated under non-competitive conditions at subsaturating RNA concentrations. However, in intact cells, it is more likely that mRNAs must compete for components of the translational apparatus. To test the possibility that the AIMV CP RNA 5' UTR contributes to mRNA competitive activity, we cotranslated AIMV CP RNA with α -globin or the chimeric A/ α / α mRNA in MI-extracts. Figure 8 (lanes 1 to 3) shows that all three transcripts, when analyzed individually, translate efficiently in the MI-extracts. However, when CP RNA was cotranslated with α -globin mRNA, there was no apparent globin protein synthesized (Fig. 8, lane 4), indicating that the AIMV CP RNA outcompeted the globin mRNA for translational components. At the same time, if AIMV CP RNA was cotranslated with the chimeric α -globin mRNA containing the AIMV CP 5' UTR (A/ α / α), both globin and coat protein translation products were observed (Fig. 8, lane 5), suggesting that the chimeric mRNA had greater competitive activity than the wild-type globin transcript. These data strongly suggest that the 5' UTR of AIMV CP plays a significant role in determining mRNA competitive activity. Consistent with previous data (Fig. 5 and 7), translation of these mRNAs was inefficient in the PI-extracts (Fig. 8, lanes 6 to 8).

DISCUSSION

The initial aim of this study was to probe mechanisms of selective mRNA translation by identifying the *cis*-acting nucleotide elements that permitted AIMV CP RNA translation in PI-extracts, in which translation of other capped mRNAs is compromised. The rationale for the experiments was based on reports stating that AIMV CP RNA, a naturally capped mRNA, translates at much higher efficiency than other capped mRNAs in PI-extracts and that translation of AIMV CP RNA is not strongly dependent upon the cap structure (10, 19, 63). One interpretation of these reports is that AIMV CP RNA translates by a cap-independent mechanism (1, 19), and in fact, examples of cellular (capped) mRNAs that translate by cap-

independent mechanisms have been described (39, 42, 60). AIMV CP RNA is structurally distinct from other mRNAs that translate by cap-independent mechanisms because it is both 5' capped and nonpolyadenylated (5, 29, 53).

The data presented in this paper show, first, that all extracts used for protein synthesis were synthetically active; moreover, several criteria were satisfied to verify the characteristic translational control that accompanies poliovirus infection (Fig. 1 to 3). Control experiments provided evidence that the template activity of mRNAs prepared by *in vitro* transcription was comparable to that of native mRNAs (data not shown). To minimize the possibility of *in vitro* translation artifacts, protein synthesis was carried out over a wide range of mRNA and salt concentrations, and the experiments were repeated at least three times each with several different extract preparations. Furthermore, two different sources of poliovirus were used to make PI-extracts, without significant differences in the translation results. The amounts of protein expressed were quantified by phosphorimaging analysis of individual radioactive protein bands on SDS-polyacrylamide gels, and the data were normalized to reflect the molar concentration of methionine residues in AIMV CP and rabbit α -globin. The stability of the mRNAs was also confirmed by Northern blot analysis.

The data presented in this study show that AIMV CP RNA translation is severely compromised in PI-extracts and suggest that limited translation of capped mRNAs in these extracts is due to residual cap-dependent mechanisms. Furthermore, transfer of the unstructured 5' UTR of AIMV CP RNA to α -globin mRNA did not restore α -globin relative translational efficiency in the PI-extracts to the level observed in the MI-extracts (Fig. 6 and 7), while P2CAT mRNA translated similarly in both extracts (Fig. 3). These data lead us to conclude that initiation of AIMV CP RNA translation is cap dependent; moreover, we found no evidence of *cis*-acting nucleotide sequences that permit selective translation in the PI-extracts.

Translation of AIMV CP RNA is apparently less sensitive to the inhibitory effects of the cap analogs m^7 GTP and m^7 GDP than some other capped mRNAs, and this has been interpreted to indicate that efficient AIMV CP RNA translation has a diminished dependence on an intact cap (10, 19, 63). Others, however, have reported that AIMV CP RNA translation is indeed inhibited by cap analogs (2, 12, 21, 57). One explanation for the disparity in results may relate to experimental details involving mRNA and analog concentrations (12, 21), coupled with the observation that AIMV CP RNA requires lower concentrations of eIF-4F for efficient translation than other mRNAs (7, 12). AIMV CP RNA is a highly competitive mRNA, and its saturation concentration in *in vitro* translation systems is significantly higher than that of many other mRNAs (3, 24). Technical details may explain in part why others have reported that AIMV CP RNA translates in PI-extracts (10, 63). In this study, we have presented data, both as gel electrophoresis autoradiograms and as graphs, in which quantified data have been normalized to reflect the molar concentration of methionine residues in each protein. A second possibility relates to the inherent synthetic activity of the HeLa cell extracts. If the baseline protein-synthetic activity of an extract were insufficient to translate α -globin mRNA but adequate to support AIMV CP RNA translation, then differences between translation of the two templates would be artifactually exaggerated.

The possibility of selective translation of capped AIMV CP RNA in PI-extracts is not consistent with current models of capped mRNA translation, and we find no evidence for a unique mechanism that overcomes the poliovirus translational control. In a similar manner, tobacco mosaic virus RNA, which

also contains an unstructured 5' translational enhancer sequence (16, 17), fails to translate in PI-extracts (10). Pause et al. recently reported that eIF-4F was required for the translation of all mRNAs tested, including AIMV CP RNA (45). These data strongly suggest that the poliovirus translational control mechanism effectively inhibits translation of mRNAs lacking an IRES element. We find no evidence to support the hypothesis that AIMV CP RNA translates selectively or by a cap-independent mechanism in the PI-extracts. The results remain consistent, however, with the observation that efficient translation of AIMV CP RNA requires a lower concentration of initiation factors than that of some other mRNAs and that the 5' UTR is important in determining mRNA competitive activity.

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