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Identification of a Competitive Translation Determinant in the 3′ Untranslated Region of Alfalfa Mosaic Virus Coat Protein mRNA

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We report that the competitive translational activity of alfalfa mosaic virus coat protein mRNA (CP RNA), a nonadenylated mRNA, is determined in part by the 3′ untranslated region (UTR). Competitive translation was characterized both in vitro, with cotranslation assays, and in vivo, with microinjected *Xenopus laevis* oocytes. In wheat germ extracts, coat protein synthesis was constant when a fixed amount of full-length CP RNA was cotranslated with increasing concentrations of competitor globin mRNA. However, translation of CP RNA lacking the 3′ UTR decreased significantly under competitive conditions. RNA stabilities were equivalent. In *X. laevis* oocytes, which are translationally saturated and are an inherently competitive translational environment, full-length CP RNA assembled into large polysomes and coat protein synthesis was readily detectable. Alternatively, CP RNA lacking the 3′ UTR sedimented as small polysomes, and little coat protein was detected. Again, RNA stabilities were equivalent. Site-directed mutagenesis was used to localize RNA sequences or structures required for competitive translation. Since the CP RNA 3′ UTR has an unusually large number of AUG nucleotide triplets, two AUG-containing sites were altered in full-length RNA prior to oocyte injections. Nucleotide substitutions at the sequence GAUG, 20 nucleotides downstream of the coat protein termination codon, specifically reduced full-length CP RNA translation, while similar substitutions at the next AUG triplet had little effect on translation. The competitive influence of the 3′ UTR could be explained by RNA-protein interactions that affect translation initiation or by ribosome reinitiation at downstream AUG codons, which would increase the number of ribosomes committed to coat protein synthesis.

Among mechanisms of regulated gene expression, mRNA competition for limiting amounts of translational components is evident during development (56, 61, 62) and viral infection (43, 77). Competitive translation often reflects changing environmental conditions, providing a selective advantage to some mRNAs. Lodish (49) proposed that protein synthesis is regulated by the availability of translational components, so mRNAs with the highest rate constants for initiation have the highest translational efficiencies. Competitive differences among mRNAs are most apparent when translational components are limiting; moreover, the translational efficiency of a given mRNA is not necessarily constant, especially if it is a poor competitor. The features of mRNAs that define competitive activity are not understood.

mRNA translational efficiency, defined as the number of completed polypeptide chains synthesized per polysomal mRNA molecule per unit of time (9), encompasses mRNA competitive activity; however, competition is rarely discussed. Experimental evidence indicates that in vitro, the translational efficiency of competitive mRNAs is relatively constant during cotranslation with increasing concentrations of a second (competitor) mRNA (3, 30), while noncompetitive mRNAs perform poorly under the same conditions. One interpretation of this phenomenon is that competitive mRNAs have a greater affinity for limiting translational components (49, 59). Alternatively, competitive mRNAs may not require high factor concentrations, thereby permitting efficient expression even when levels of translational components are low (5). In theory, a competitive advantage at any point during the course of initiation, elongation, or termination in protein synthesis could enhance expression.

Although translational control studies have long focused on the 5′ mRNA terminus as the key regulatory locus, more recent evidence suggests important roles for 3′ sequences and structures (35). Both the 3′ untranslated region (UTR) (7, 8, 11, 29, 44, 69, 70, 72, 79) and the poly(A) tail (15, 20, 40, 54, 62, 65, 68) contain important translational efficiency determinants, but the mechanisms of their effects are poorly understood.

Viral systems have retained significance as experimental models for elucidating translational control mechanisms. This paper focuses on the translational activity of the coat protein mRNA (CP RNA) of alfalfa mosaic virus (AMV) a highly efficient and competitive mRNA molecule (4, 23, 30, 41). AMV RNAs are capped but not polyadenylated, offering the potential to study the role of 3′ sequences exclusive of a poly(A) tail. In addition, these RNAs lack the 3′-terminal tRNA-like structure that has been implicated in translation of other plant viral RNAs (16). Although other positive-strand RNA viruses, such as poliovirus, actively disable host mRNA translation (67), AMV encodes only four known gene products, none of which is known to adversely affect host mRNA translation. We reasoned, therefore, that efficient and competitive AMV coat protein expression must be inherent to the

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sequence and/or structure of CP RNA, and the goal of this work was to begin to define the relevant elements.

While the 5′ leader sequences of plant viral RNAs contain translational enhancer sequences (18, 38), other regions apparently contribute to translational efficiency as well. For example, a pseudoknot domain in the tobacco mosaic virus RNA 3′ UTR may substitute for a poly(A) tail (14, 17). In the case of AMV, Loesch-Fries et al. reported that only diminished levels of coat protein were detected in *Xenopus laevis* oocytes injected with AMV CP RNA lacking the 3′ UTR (50); moreover, in vitro translation experiments with fractionated wheat germ extracts suggested that the AMV CP RNA 3′ UTR contributes to translational efficiency (64). These results suggested that the AMV CP RNA 3′ UTR plays an important role in regulating CP RNA translation. In this paper, we present both in vitro and in vivo evidence that the 3′ UTR of AMV CP RNA is a determinant of competitive translational activity.

MATERIALS AND METHODS

Preparation of HeLa cell lysates. HeLa cell lysates were prepared as described previously (27).

cDNA and constructs. All enzymes and linkers were obtained from New England Biolabs (Beverly, Mass.) unless otherwise noted. Constructs were prepared by standard molecular techniques (66). The plasmid pAMV-4, containing AMV RNA 4 cDNA in pTZ18R (Pharmacia), was a gift from Karen Browning, University of Texas (12). Plasmid pHST405, containing AMV coat protein cDNA, has been described previously (27). Transcription of pHST405 yielded AMV CP RNA containing only 4 vector-derived nucleotides at the 5′ end and 2 vector-derived nucleotides at the 3′ end. AMV RNA 4 transcripts lacking the 3′ UTR (AMV CP RNAΔ3′) were transcribed from plasmid pHST448. This plasmid was generated by inserting a *Sma*I site 10 bp 3′ to the translational termination codon (positions 712 to 713; the translational termination site is at nucleotides 700 to 702). Plasmid pHST448 AMV CP RNAΔ3′ contained 4 vector-derived nucleotides at the 5′ end and the 10 remaining nucleotides of the AMV CP RNA 3′ UTR in addition to 2 cytosines derived from the *Sma*I site. Plasmid pHST101, which contains the rabbit α-globin cDNA, has also been described previously (27). Transcripts from this plasmid lack vector-derived nucleotides at the 5′ end and contain a 48-nucleotide poly(A) tail following the 3′ UTR. The plasmids containing the full-length tobacco streak virus (TSV) coat protein cDNA or the cDNA representing the 3′-UTR-truncated transcript were prepared by reverse transcriptase PCR techniques from TSV RNA 3 (1). Transcripts derived from these plasmids contain 23 nucleotides 5′ of the translation start codon at the 5′ end and 2 vector-derived nucleotides at the 3′ end. Plasmid pHST215 (37) contained human interleukin-1β (IL-1β) cDNA (encoding the 17.5-kDa mature form of the protein) where the AMV CP RNA 5′ UTR replaced the native leader sequence. The cDNA templates used to transcribe RNAs containing nucleotide substitutions in the first and second downstream AUG triplets (dAUG1 and dAUG2) were prepared by PCR mutagenesis.

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 mMessage mMachine) as described previously (27).

In vitro translations and protein quantitation. HeLa cell extracts were prepared and used as described previously (27). A 15.0-μl wheat germ extract translation reaction mixture contained 6.25 μl of commercially prepared wheat germ extract (Promega), 1 μCi L-[^{35}S]methionine, 133 μM amino acids minus methionine, 127 mM potassium acetate (KOAc), and mRNA as indicated in the legends to Fig. 1 to 3. Translation was initiated by transfer from ice to the incubation temperature (30°C for HeLa extracts; 25°C for wheat germ extracts). In RNA titration experiments, aliquots of the reaction mixture were removed during the period in which incorporation of [^{35}S]methionine was linear with respect to time. Protein synthesis was monitored by analyzing an aliquot (2 μl) of the translation reaction mixture via sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (45). Quantitation of radioactive methionine in the specific protein bands was performed on a Molecular Dynamics PhosphorImager. Values (PhosphorImager units) obtained were corrected for the number of methionines in the AMV coat protein (three methionines per mol of coat protein).

Analysis of RNA stability by Northern blot hybridization. mRNA integrity in the in vitro translation reactions was determined with 2-μl aliquots as described previously (27). RNA that had been microinjected into *Xenopus* oocytes was extracted essentially as described by Galili et al. (13). Frozen oocytes were suspended in a solution (50 μl/oocyte) containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.5% SDS, and proteinase K (150 μg/ml) and homogenized by gentle repeated pipetting with a Rainin P1000 pipette tip. The homogenate was centrifuged for 4 min at 10,000 × *g* at 4°C, and the supernatant was removed with care taken to avoid the top lipid layer. Supernatants were incubated at 37°C for 60 min, extracted twice with 1 volume of phenol equilibrated with 250 mM NaOAc (pH 4.8 to 5.2) and chloroform (1:1) and once with an equal volume of chloroform alone, and the RNA was ethanol precipitated from 2.5 M NH_4OAc at −80°C. The RNA pellets were collected by centrifugation at top speed in a microcentrifuge for 30 min, air dried, resuspended in urea gel sample buffer (7 M urea, 0.05% bromophenol blue, and 0.05% xylene cyanol in 1× TBE [89 mM Tris-borate–2 mM EDTA, pH 8.0]), heated to 65°C for 5 to 8 min, and loaded onto a 4% polyacrylamide-7 M urea gel. Current was applied until the xylene cyanol migrated approximately three-fourths of the length of the gel. The separated RNA was transferred electrophoretically overnight to a Zeta Probe membrane (Bio-Rad) at 20 V in 0.5× TBE at 4°C with a Hoefer transfer apparatus. RNA was cross-linked to the membrane with a Bio-Rad GS Gene Linker UV chamber. Northern blot hybridization using QuikHyb (Stratagene) was performed and quantitated essentially as described for the analysis of in vitro-translated RNA, except that the blot was prehybridized for 3 h and the final wash was done at 60°C in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% SDS for 60 min. To reprobe the blots, the hybridized probe was stripped from the Zeta Probe membranes by incubation for ~1.5 h in 0.1× SSC–0.5% SDS at 95°C.

***X. laevis* oocyte injections.** Stage VI *X. laevis* oocytes were prepared as previously described (13) except that the frogs were anesthetized with 1% ethyl *m*-aminobenzoate (150 μl/10 g of body weight) before removal of ovarian lobes. Oocytes were microinjected with an approximately 15-nl solution of RNA containing 9.6 fmol of test RNA (AMV CP RNA, AMV CP RNAΔ3′, TSV CP RNA, or TSV CP RNAΔ3′) and an equimolar amount of chimeric IL-1β RNA containing the AMV CP RNA 5′ UTR to serve as an internal control. Microinjected oocytes were incubated in Barth's medium [2 mM HEPES (pH 7.5), 88 mM NaCl, 0.2 mM K_2SO_4, 0.8 mM $MgCl_2$, 0.33 mM $Ca(NO_3)_2$, 0.40 mM $CaCl_2$] at 25°C. To monitor translation of the microinjected transcripts, oocytes were injected with 20 nl (40 pmol) of [^{3}H]leucine (New England Nuclear) (adjusted to a specific activity of 145 nCi/nmol) 5 h after transcript injection and incubated for an additional 45 min. Oocytes were gently rinsed three times in Barth's medium, snap frozen, and stored at −80°C prior to immunoprecipitation or Northern blot analysis. For polysome analysis, oocytes were injected as above and incubated in Barth's medium prior to homogenization as described below.

Analysis of proteins translated in vivo. AMV or TSV coat protein levels in the oocytes were determined by immunoprecipitation of radiolabeled protein. Frozen oocytes (5 to 12) were resuspended in immunoprecipitation homogenization buffer (20 mM Tris-HCl [pH 7.6], 0.1 M NaCl, 1% Triton X-100) (13) containing 1 mM AEBSF [4-(2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride; Calbiochem] instead of 1 mM phenylmethylsulfonyl fluoride (50 μl of buffer/oocyte), homogenized by gentle repeated pipetting on ice with a Rainin P1000 pipette tip, and then centrifuged for 4 min at 10,000 × *g* at 4°C. The supernatant was removed with care taken to avoid the top lipid layer and used immediately or stored at −20°C. The amount of radiolabeled amino acids incorporated into protein was determined by acid precipitation (51). Equivalent amounts of radioactivity (200,000 cpm) from the oocyte homogenate were added to NET-gel buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% Nonidet P-40, 0.25% gelatin, 0.02% sodium azide) (66) for a final volume of 500 μl. Rabbit polyclonal anti-AMV coat protein (10 μl) or polyclonal rabbit anti-TSV antibody (50 μl of immunoglobulin G fraction [2 mg/ml]; Agdia) were added to the appropriate tubes and incubated overnight at 4°C on a tube rotator. The resulting immune complexes were incubated with 100 μl of Protein G Sepharose 4 Fast Flow (Pharmacia) (suspended in NET-gel buffer; 3:1) for 3 h at 4°C. The protein G-antibody complexes were collected at 4°C by microcentrifugation at 12,000 × *g* for 20 s, washed twice with 1 ml of NET-gel buffer for 1 h and once with 10 mM Tris-Cl (pH 7.5)–0.1% Nonidet P-40 for 1 h, resuspended in 30 μl of Laemmli sample buffer, and incubated at 100°C for 3 min (45). The immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis, and the radiolabeled proteins were detected by fluorography.

To confirm the translational activity of the oocytes, IL-1β mRNA was coinjected with CP RNAs, and IL-1β protein levels in the oocyte homogenate were determined by a sensitive IL-1β enzyme-linked immunosorbent assay (ELISA) (Cistron Biotechnology, Pine Brook, N.J.) according to the manufacturer's instructions.

Polysome isolation and analysis. Polysomes were isolated from the microinjected oocytes as previously described (13) with some minor modifications. Oocytes (approximately 15) were homogenized as described above in 0.5 ml of ice-cold PB buffer (0.2 M sucrose, 0.2 M Tris-HCl [pH 8.5], 35 mM $MgCl_2$, 25 mM EGTA, 0.1 M KCl, 100 μg of cycloheximide per ml, and 0.5% Nonidet P-40). After microcentrifugation for 5 min, supernatants were removed and 150 μl of cleared homogenate from each of the two oocyte groups (i.e., injected with either full-length AMV CP RNA or AMV CP RNAΔ3′) was mixed and layered onto a 15 to 40% linear sucrose gradient. Gradients were centrifuged at 45,000 rpm for 75 min in a Beckman L8-80M ultracentrifuge fitted with an SW Ti50.1 rotor. Analyzing polysomes from the two oocytes groups on the same gradient permitted separation of the full-length and truncated RNA polysomes under identical conditions, thereby controlling for variations in possible sample loss during processing and Northern blotting. To distinguish polysomes from large ribonucleoproteins, polysomes were dissociated by adjusting the oocyte homogenate to 100 mM EDTA and 400 mM NaCl and incubated on ice for 15 min prior to centrifugation. Absorbance at 254 nm was monitored continuously as fractions (0.25 ml) were collected into 0.1-volume 10% SDS with an ISCO model 183

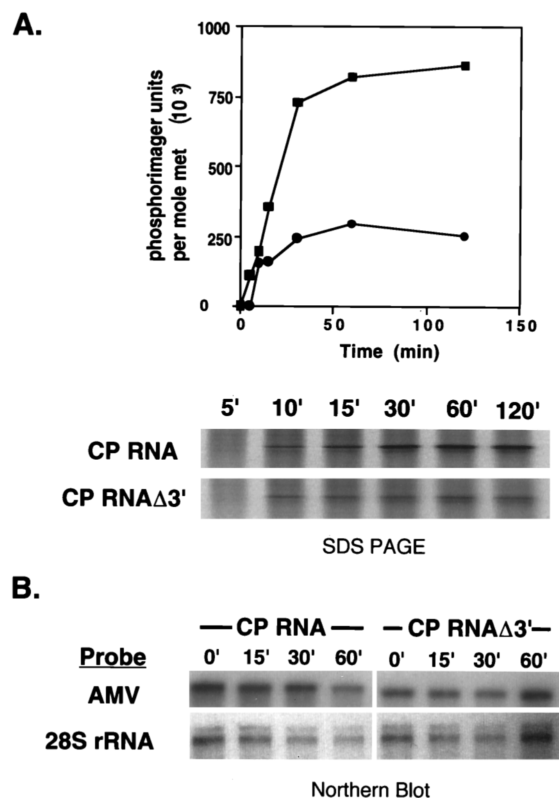

FIG. 1. Translational profiles of AMV CP RNA and AMV CP RNAΔ3′ transcripts in HeLa extracts. (A) Translational kinetics of AMV CP RNA (filled squares) and AMV CP RNAΔ3′ (filled circles). Translation reactions were performed and analyzed as described in Materials and Methods. The panel below the graph is an autoradiogram of the SDS-polyacrylamide gel used to generate the data. PAGE, polyacrylamide gel electrophoresis. (B) Northern blot hybridization of AMV CP RNA and AMV CP RNAΔ3′ extracted from translation reaction mixtures. Aliquots (2 μl) were removed at the times indicated and processed as described in Materials and Methods. To control for equal RNA loading, the blots were stripped and rehybridized with a probe recognizing 28S rRNA. Time is shown above the lanes, in minutes.

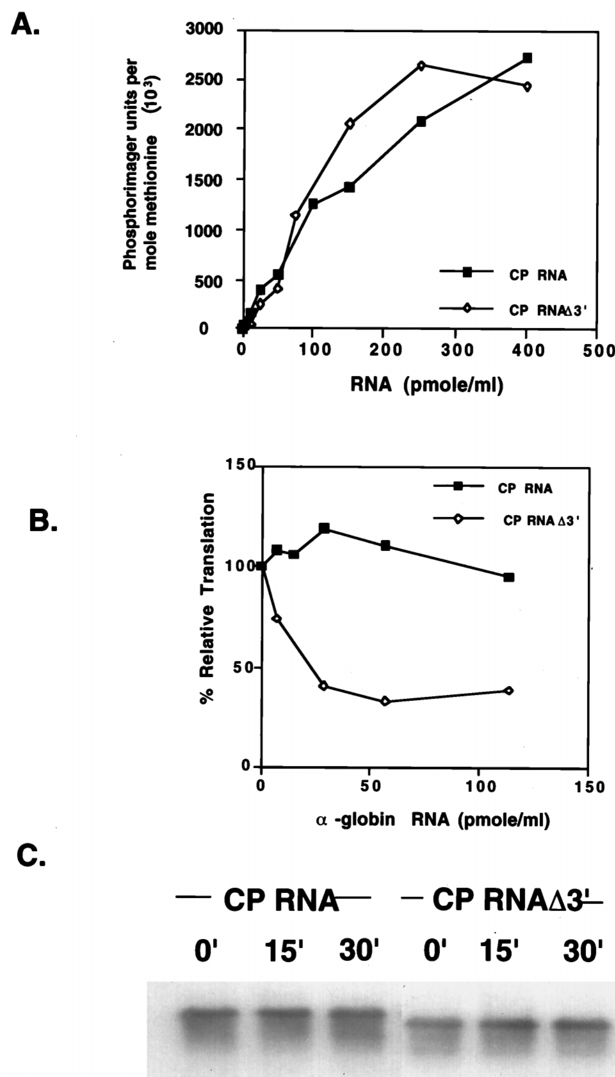

FIG. 2. Translation of AMV CP RNA and AMV CP RNAΔ3′ in wheat germ extracts. (A) Titration of AMV CP RNA and AMV CP RNAΔ3′. RNA was added to 12.5-μl translation reaction mixtures at the concentrations indicated. Following a 30-min incubation at 25°C, 2-μl aliquots were removed and analyzed for protein levels as described in Materials and Methods. (B) Competitive translation of AMV CP RNA or AMV CP RNAΔ3′ with α-globin RNA. Test RNAs (AMV CP RNA or AMV CP RNAΔ3′) were added to the translation reaction mixtures at a 50-pmol/ml concentration in the presence of increasing amounts of α-globin RNA. Following a 30-min incubation at 25°C, 2-μl aliquots were removed and analyzed for protein levels as described in Materials and Methods. (C) Northern blot hybridization of AMV CP RNA and AMV CP RNAΔ3′ extracted from translation reaction mixtures. Aliquots (2 μl) were removed at the time indicated and processed as described in Materials and Methods. Time is shown in minutes.

density gradient fractionator. The fractionated polysomal RNA was treated with proteinase K (200 μg/ml; Boehringer Mannheim) for 0.5 to 1 h at 37°C and stored at −20°C prior to processing. Samples were extracted and precipitated from 0.3 M NaCl in the presence of 50 μg of carrier yeast tRNA as described for Northern blot analysis. The pelleted RNA was dissolved in urea sample buffer for separation and analysis with Zeta Probe membranes as described above.

RESULTS

Translation of AMV CP RNA and AMV CP RNAΔ3′ in HeLa cell and wheat germ extracts. Because differences in mRNA translational efficiency are most pronounced when translational components are limiting (49), we translated AMV CP RNA and AMV CP RNAΔ3′ both in HeLa extracts, which are known to be relatively inefficient in protein synthesis (27, 36, 76), and in more efficient wheat germ extracts. The results shown in Fig. 1A are evidence that AMV CP RNAΔ3′ was translated with significantly lower efficiency than AMV CP RNA in the HeLa cell extracts. The reduced translation of AMV CP RNAΔ3′ was independent of mRNA concentration (data not shown) and could not be explained by differential mRNA stability (Fig. 1B). In contrast, deleting the 3′ UTR did not diminish translation of AMV CP RNA in wheat germ extracts over a wide range of RNA concentrations (Fig. 2A); in fact, AMV CP RNAΔ3′ often translated better than AMV CP RNA in the wheat germ extracts (Fig. 2A and data not shown).

The differential translation of AMV CP RNAΔ3′ in the HeLa and wheat germ extracts could be interpreted either as a translational characteristic of the HeLa cell extract or as an inherent property of the RNAs. To distinguish between these two possibilities, we translated AMV CP RNA and AMV CP RNAΔ3′ in wheat germ extracts under competitive conditions, i.e., where the total mRNA concentration saturated the translational apparatus. To assess mRNA competitive activity, a fixed amount of AMV CP RNA was cotranslated with increasing concentrations of competitor mRNA. Protein synthesis directed by competitive mRNAs is relatively unaffected by

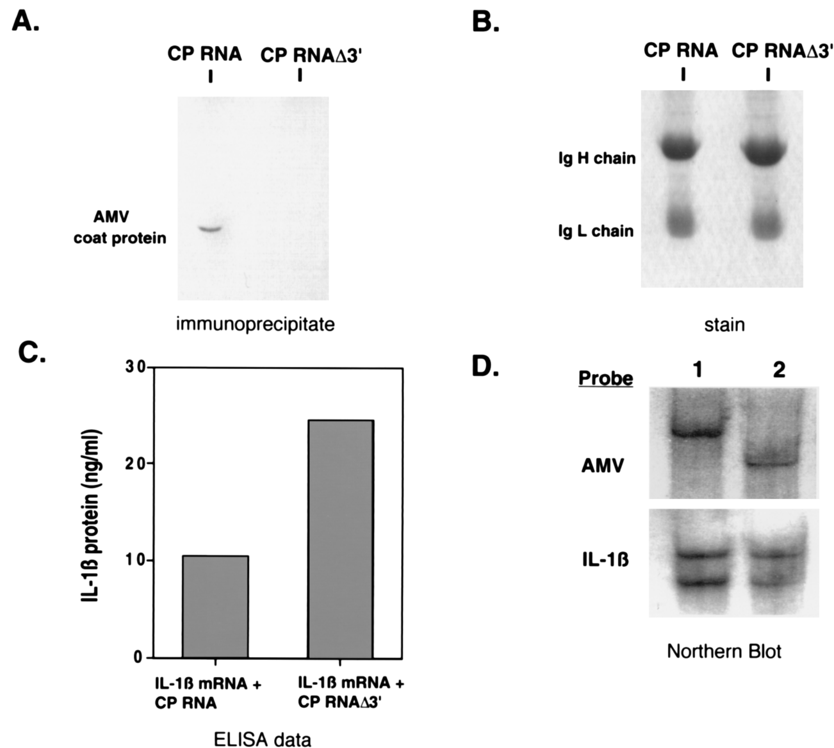

FIG. 3. Translation of AMV CP RNA and AMV CP RNAΔ3′ in *X. laevis* oocytes. Stage VI oocytes were microinjected with approximately 15 nl of solution containing 10 fmol of AMV CP RNA or AMV CP RNAΔ3′ (10 to 15 oocytes/group) and an equimolar amount of IL-1β RNA. Translated proteins were radiolabeled and immunoprecipitated as described in Materials and Methods. (A) Fluorograph of immunoprecipitated oocyte extracts resolved by SDS-polyacrylamide gel electrophoresis. The position of the AMV coat protein (apparent molecular mass of 28 kDa) is indicated. (B) Coomassie blue staining of the gel in panel A indicating equal protein loading. The positions of the immunoglobulin (Ig) heavy (H) and light (L) chains are indicated. (C) IL-1β expression from translation of the coinjected transcript. IL-1β levels were determined by ELISA as described in Materials and Methods. (D) Northern blot hybridization of RNA purified from AMV CP RNA- and AMV CP RNAΔ3′-microinjected oocytes. Lane 1, RNAs isolated from oocytes injected with both full-length CP RNA and the IL-1β control transcript; lane 2, RNAs isolated from oocytes injected with both CP RNAΔ3′ and the IL-1β control transcripts. After transfer, the membrane was probed separately with AMV or IL-1β probes. The ratios of the AMV-specific transcript to the IL-1β RNA transcript, determined by phosphorimaging analysis, were 0.79 for CP RNA and 0.82 for CP RNAΔ3′. Other experimental details may be found in Materials and Methods.

added competitors, while translation of noncompetitive mRNAs diminishes as the competitor mRNA concentration increases (3, 30, 77). The results shown in Fig. 2B demonstrate that when a fixed amount of AMV CP RNA was translated in the presence of increasing concentrations of competitor α-globin mRNA, the amount of coat protein synthesized remained relatively constant. AMV CP RNAΔ3′ translation was, however, diminished significantly (Fig. 2B) under the same conditions. The data presented in Fig. 2C show that the diminished protein synthesis directed by AMV CP RNAΔ3′ is not due to mRNA instability. The results shown in Fig. 2 are evidence that the AMV CP RNA 3′ UTR contributes to competitive translational activity in vitro.

Translation of AMV CP RNA and AMV CP RNAΔ3′ in microinjected *X. laevis* oocytes. In vitro translation data (Fig. 2) suggested that removing the AMV CP RNA 3′ UTR diminished its competitive translational activity. To extend the in vitro analysis, we translated the mRNAs in microinjected stage VI *X. laevis* oocytes. Because the oocyte translational capacity is saturated (46, 60), microinjected mRNAs must compete with endogenous mRNAs for translational components, thereby providing a useful assay for differential mRNA competitive activity. It has been shown previously that AMV CP RNA is stable in *X. laevis* oocytes (33) and that coat protein can be immunoprecipitated from oocyte homogenates (50). Oocytes were microinjected with a solution containing approximately 10 fmol of AMV CP RNA or AMV CP RNAΔ3′ (10 to 15 oocytes/group) and an equimolar amount of a chimeric human IL-1β mRNA containing the unstructured AMV RNA 4 untranslated leader sequence (37), which served as an internal control. Five hours after RNA injection, the oocytes were injected with [^{3}H]leucine for protein labeling and incubated for an additional 45 min prior to homogenization. The extracts were either immunoprecipitated with a polyclonal rabbit anti-AMV coat protein antibody to monitor coat protein translation and then subjected to Northern blot analysis to assess mRNA stability or assayed with an IL-1β ELISA to determine the expression levels of the coinjected control transcript.

The results presented in Fig. 3A (left lane) show that AMV coat protein was readily detected (within 2 to 3 days of exposure) in immunoprecipitates from AMV CP RNA-injected oocytes but not in oocytes that received the AMV CP RNAΔ3′ RNA (Fig. 3A, right lane). A greatly extended exposure period (~1 month) was needed to detect coat protein synthesized in the oocytes injected with the 3′-truncated RNA (data not shown). The coinjected IL-1β mRNA transcript is translated in both sets of injected oocytes (Fig. 3C), indicating that transcripts were present and that protein synthesis was not selectively inhibited in the AMV CP RNAΔ3′-injected oocytes. The 50% reduction in expression of coinjected IL-1β mRNA transcript in AMV CP RNA-injected oocytes compared to oocytes injected with AMV CP RNAΔ3′ (Fig. 3C) suggests that full-length AMV CP RNA was translated at the expense of IL-1β mRNA, while the 3′-truncated RNA did not similarly outcom-

pete IL-1β mRNA translation. Quantitation of AMV CP RNA and IL-1β mRNA transcript levels by Northern blot and phosphorimaging analyses (Fig. 3D) shows that the transcript ratios are similar, indicating that the steady-state levels of AMV CP RNA and AMV CP RNAΔ3′ were not significantly different. These results are consistent with the in vitro translation data and provide additional evidence that the viral RNA 3′ UTR is a positive determinant of translational efficiency and competitive activity.

As a further test, we also injected oocytes with TSV CP RNA transcripts. TSV has a genomic organization similar to that of AMV, and its 3′ UTR nucleotide sequence is related to that of AMV. Injection of oocytes with TSV CP RNA or TSV CP RNAΔ3′ gave the same overall results as those obtained with AMV CP RNA; that is, immunoprecipitates from oocytes injected with full-length TSV CP RNA contained detectable TSV coat protein, while no corresponding protein was observed in immunoprecipitates of oocytes injected with TSV CP RNAΔ3′ (data not shown). These data indicate that the competitive translational advantage provided by the AMV CP RNA 3′ UTR is a characteristic of a related RNA (TSV) and may be common to other ilarviruses.

Polyribosome distribution of full-length and 3′-truncated AMV CP RNA. Polyribosomes were analyzed to assess the mechanism of the 3′-UTR effect. Oocytes were injected with AMV CP RNA or AMV CP RNAΔ3′ and incubated for 5 h. Subsequently, cytoplasmic extracts were prepared and polyribosome distribution analyzed by sucrose density gradient centrifugation (13) and Northern blot hybridization to localize CP RNA transcripts. The results presented in Fig. 4A show that 61% of full-length AMV CP RNA sedimented as polysomes, compared to 39% for AMV CP RNAΔ3′. This result shows that the proportion of large AMV CP RNAΔ3′ polysomes is significantly reduced compared to that in full-length CP RNA. Furthermore, a reproducible and distinct peak (fraction 17) of large polysomes is evident in extracts from oocytes injected with AMV CP RNA but absent in the AMV CP RNAΔ3′ sample (Fig. 4A). Polysome disruption by adding sodium chloride and EDTA prior to gradient centrifugation (Fig. 4B) demonstrated that the mRNAs were indeed bound to ribosomes, ruling out the possibility that the absorbance and hybridization patterns were due to large cosedimenting nonpolysomal ribonucleoprotein complexes. The results presented in Fig. 3 and 4 show that low coat protein levels correlate directly with oocyte polysome profiles in that more ribosomes are observed bound to full-length AMV CP RNA than to truncated RNA, and a greater percentage of the total RNA sediments as polyribosomes.

Identification of specific downstream elements required for competitive translational activity. The 3′ UTR of AMV CP RNA contains an unusually large number of AUG triplet nucleotides (9 AUG triplets/180 nucleotides) and thus 9 potential downstream open reading frames (dORFs) (Fig. 5). While the observed frequency of AUG triplets in a random sampling of mRNA 3′ UTRs is approximately that predicted by random probability (i.e., 1.5 AUG triplets per 100 nucleotides), the value is significantly higher (i.e., 4.4 AUG triplets per 100 nucleotides) for AMV and related ilarvirus RNAs (Table 1). AUG triplets are found within the 3′ UTR of other AMV and ilarvirus RNAs (Fig. 5) but not among other members of the bromovirus family (data not shown). In order to begin to localize sequence or structural elements responsible for the competitive translational effect, we hypothesized that the dAUG triplets might be important for competitive translational activity.

To test the potential role of dAUG1 or dAUG2 in defining CP RNA competitive activity, nucleotide substitutions (underlined) were introduced into full-length CP RNA (dAUG1, GGGAUG→GGUAUA and dAUG2, UUGAUG→UUGAUA [Fig. 6A]). RNAs containing substitutions in dAUG1, dAUG2, or both dAUG1 and dAUG2 were coinjected with an equimolar amount of chimeric IL1-β mRNA transcripts into *Xenopus* oocytes as described above. As shown in Fig. 6B, CP RNA transcripts containing nucleotide substitutions in dAUG1 were translated very poorly in the oocytes, similar to the 3′-truncated mRNAs. Alternatively, mutations in dAUG2 had little effect on CP RNA translation. The doubly substituted RNA variant translated poorly, in a manner similar to that of the disrupted dAUG1 and the 3′-truncated transcripts. The coinjected IL1-β mRNA was translated in all oocytes, and Northern blot analysis indicated that the injected transcripts had similar stabilities. These data strongly suggest that the nucleotide element GAUG, at positions 721 to 724, contains important determinants of translational efficiency and competitive activity.

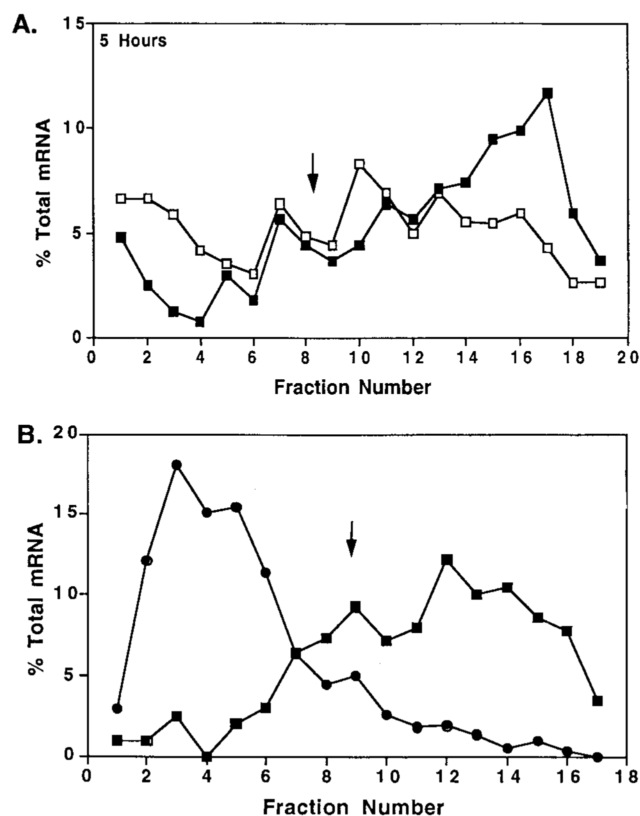

FIG. 4. Polysome distribution of AMV CP RNA and AMV CP RNAΔ3′ in microinjected *Xenopus* oocytes. (A) Distribution of AMV CP RNA and AMV CP RNAΔ3′ in oocyte polysomes 5 h following microinjection. RNA present in the fractions was measured by phosphorimaging of Northern blots. The top of the gradient is to the left; the arrow marks the position of the monosome peak. Open squares, AMV CP RNAΔ3′; closed squares, AMV CP RNA. (B) Polysome disruption by treatment with 100 mM EDTA and 400 mM NaCl. The top of the gradient is to the left; the arrow marks the position of the monosome peak. Homogenates from oocytes injected with either full-length AMV CP RNA or AMV CP RNAΔ3′ were pooled and adjusted in 100 mM EDTA-400 mM NaCl (circles) or maintained in PB buffer (squares) for 15 min prior to sucrose gradient centrifugation. The experiment was performed as described above.

DISCUSSION

The in vitro and in vivo translation data presented here strongly indicate that the AMV CP RNA 3′ UTR is a positive

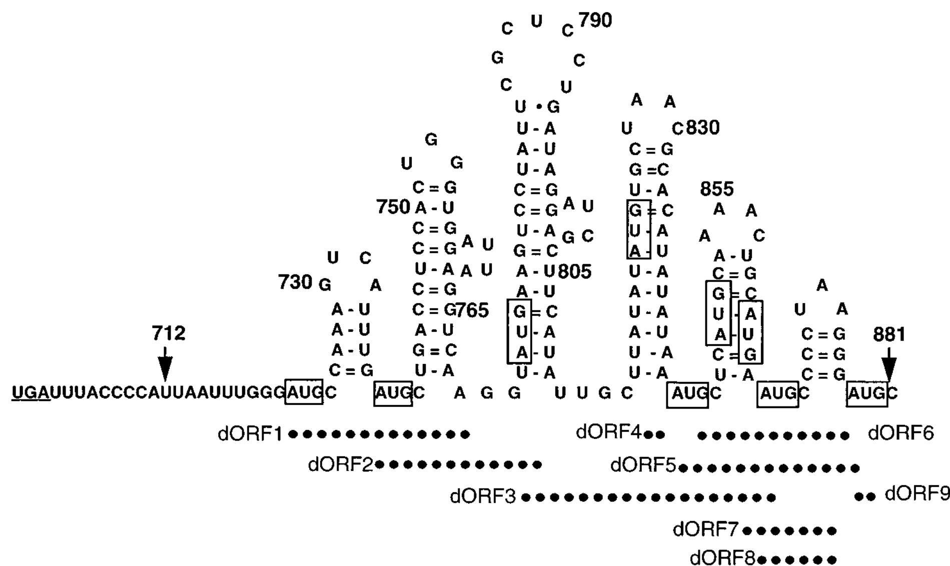

FIG. 5. Putative dORFs in the 3′ UTR of AMV CP RNA. The termination codon of the coat protein open reading frame is found at nucleotides 700 to 702, and the truncation site used in our experiments is found at position 712. There are nine AUG triplets in the 3′ UTR; each circle beneath the sequence indicates one amino acid in the respective dORF.

determinant of competitive translational activity and that the nucleotides in the element GAUG at positions 721 to 724 are necessary for this function. At issue is how the AMV CP RNA 3′ UTR influences competitive translational activity, and we propose two interpretations of the results that form a basis for further experimentation. One interpretation is that protein-RNA interactions in the 3′ UTR increase translational efficiency and competitive activity. Indeed, there are a number of examples of RNA-protein interactions in the 3′ UTR, although most correlate with translational repression (7, 10, 11, 24, 29, 39, 44, 55). Among examples of positive translational control, it has been suggested that selective protein binding to the 3′ UTR or poly(A) tail may enhance translation (20, 54, 65). It is clear that AMV coat protein binds specifically to the 3′ termini of viral RNAs (32); however, we (26) and others (17, 22, 53, 63, 75) have reported that coat protein binding does not specifically affect AMV CP RNA translation. Moreover, in vitro translation of full-length AMV CP RNA was not affected significantly by excess AMV CP RNA 3′ UTR fragments (~170 nucleotides) added in *trans* (28). Dependence on a cellular RNA binding protein for enhanced coat protein expression would seem to offer little advantage to the virus, particularly since virus encapsidation requires large amounts of coat protein. We cannot rule out the possibility that cellular proteins bind to the AMV CP RNA 3′ UTR to increase competitive translational activity, but other models seem more attractive.

A second interpretation is that the AMV CP RNA 3′ UTR increases competitive translational activity by facilitating ribosome-mRNA interactions. Prior evidence suggests that following translation termination, 40S ribosomal subunits can remain associated with the mRNA by continued scanning and/or reinitiation on the 3′ UTR (6, 25, 52). Both the polyribosome data (Fig. 4) and the lack of competitive activity of the coat protein transcript containing the nucleotide substitution in dAUG1 (Fig. 6) are consistent with a reinitiation model. The large polyribosomes reported in Fig. 4 may indicate that full-length CP RNA initiates translation more efficiently than 3′-truncated RNA, but it is also possible that part of the increase is due to ribosomes that are bound to the 3′ UTR. A prediction from this model is that after terminating coat protein translation at nucleotides 700 to 702, a population of 40S subunits could continue scanning and reinitiate translation, via dAUG1, on the 3′ UTR. The net result of the reinitiation would include increased sedimentation of full-length CP RNA in sucrose gradients. This proposed mechanism is supported by published data. In related studies, Purvis et al. (58) showed that yeast pyruvate kinase mRNA lacking the 3′ UTR was translated very poorly and sedimented as small polysomes. Similar to that of AMV and ilarvirus mRNAs, the 3′ UTR of yeast pyruvate kinase mRNA also contains a relatively large number of AUG triplets (58), with one in close proximity to the termination codon and in good translational context (42).

The competitive role of the AMV CP RNA 3′ UTR may be influenced by other mRNA elements. CP RNA (the viral subgenomic RNA 4) is 3′ coterminal with genomic RNA 3, although coat protein is translated only from subgenomic RNA 4 (74). Despite the fact that RNAs 3 and 4 have identical 3′ termini, CP RNA outcompetes RNA 3 in wheat germ translation extracts (23, 30). Although the CP RNA 5′ UTR is unstructured in solution (21), the 5′ UTR of RNA 3 has significant potential for forming a stable secondary structure (23) and possibly masking the competitive effect (57). In contrast to results obtained with the tobacco mosaic virus 3′ UTR (20, 47),

TABLE 1. Frequency of occurrence of AUG triplets in the 3′ UTRs of selected organisms[a]

mRNA source[b]	n^c	No. of AUG triplets/ 100 nucleotides
AMV and ilarviruses	3,981	4.41
Other plant viruses	4,429	0.95
Mammals	9,595	1.62
Animal viruses	3,272	1.50

[a] The probability of an AUG triplet occurring randomly is 4^{-3}, or 1.56 AUG triplets per 100 nucleotides.
[b] cDNA sequences from each source group were extracted at random from nucleotide databases, and the 3′ UTRs were searched for AUG triplets.
[c] Total number of nucleotide triplets analyzed from each source.

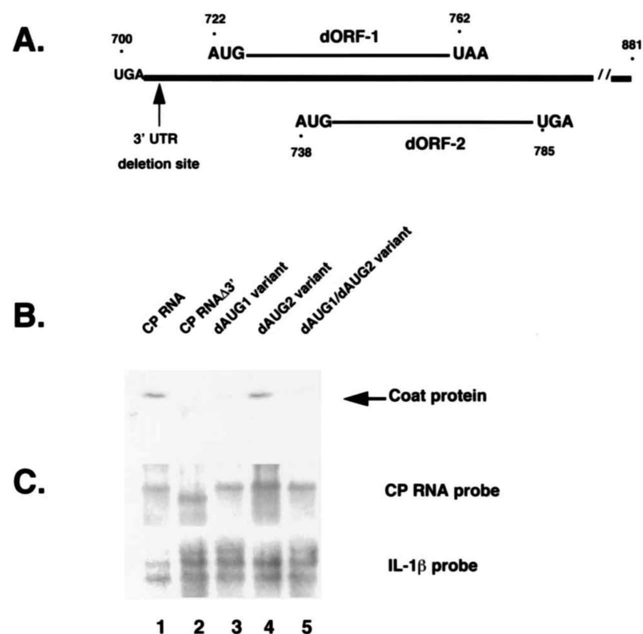

FIG. 6. Nucleotide substitutions in dORF1 impair competitive translation. (A) Schematic representation of the AMV CP RNA 3′ UTR showing the positions of dORFs 1 and 2, which utilize different reading frames. (B) Translation in *X. laevis* oocytes. The methods of mRNA transcription, injection, labeling, and immunoprecipitation are the same as those described in the legend to Fig. 3. The lanes represent translation of full-length CP RNA, CP RNAΔ3′ full-length CP RNA with nucleotide substitutions (underlined) in dAUG1 (GGGAUG→GGU AUA; positions 719 to 724), full-length CP RNA with nucleotide substitutions (underlined) in dAUG2 (UUGAUG→UUUAUA), and full-length CP RNA with nucleotide substitutions in both dAUG1 and dAUG2. (C) Northern blot analysis of injected RNAs isolated from extracts prepared at the end of the incubation period.

we have not observed generalized translational stimulation by fusing the AMV CP RNA 3′ UTR alone onto heterologous mRNAs. There is no evidence at this time for physical interactions via base pairing between the 5′ and 3′ UTRs of AMV CP RNA in a manner that has been suggested for satellite tobacco necrosis virus RNA (8, 71). Therefore, we propose that the 5′ and 3′ UTRs of AMV CP RNA act in concert to promote efficient coat protein expression, which is critical for the viral life cycle. During the early period of virus replication, the 5′ UTR of AMV CP RNA is likely to be a translational enhancer that facilitates efficient initiation (19, 38). Then, as viral transcripts accumulate and translational components become limiting in the infected cells, the competitive influence of the 3′ UTR becomes apparent and ribosome 3′→5′ recycling could maintain a high concentration of competent ribosomes (2, 31) in the local mRNA environment. As noted above, ribosome recycling is only one of the mechanisms that could explain the competitive effect, and other possibilities are being explored.

Until recently, the mRNA 3′ UTR was thought to possess little regulatory importance beyond perhaps providing physical protection from nucleases. However, it is becoming increasingly clear that the functional significance of the 3′ UTR has been underestimated (78). Both RNA-protein complexes (examples cited above) and RNA-RNA interactions (8, 48, 71, 79) are linked to translational regulation, but further study is required to elucidate the relevant mechanisms. The significance of 3′-UTR sequences in regulating gene expression may be implicated in a human disease state (34, 36a). An important challenge for future study in the field is to understand the interactions and communication between the mRNA 5′ and 3′ ends that define selective mRNA translation, competitive activity, and translational efficiency.

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