Two initiation sites detected in the small s1 species of reovirus mRNA by dipeptide synthesis *in vitro*

(translation initiation/*in vitro* protein synthesis)

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ABSTRACT Reovirus mRNAs directed the synthesis of fMet dipeptides in a translation initiation system reconstituted from rabbit reticulocyte initiation and elongation factors, *Artemia salina* 80S ribosomes, yeast fMet-tRNA$_i^{Met}$ and *Escherichia coli* ^{3}H-labeled aminoacyl tRNAs. As predicted from the GC(U,G) codon that follows the 5'-proximal AUG in half of the viral mRNA species, fMet-Ala was the predominant dipeptide product obtained in response to a mixture of mRNAs or to the separated size classes of medium (m) and small (s) mRNA. The four individual small mRNA species each directed the synthesis of an fMet dipeptide that was consistent with the utilization of the 5'-proximal AUG for initiation. In addition to fMet-Asp, the s1 mRNA also directed fMet-Glu synthesis indicative of initiation in a second reading frame at the 5'-penultimate AUG. The tripeptide fMet-Glu-Tyr was also synthesized from s1 mRNA, which further verified this second initiation site. mRNAs containing 5'-terminal GpppG were 10–15% as active as the corresponding m^{7}G-capped templates. The dipeptide assay provides a rapid method for determining initiation sites in individual mRNAs or in mixtures of mRNAs.

Human reovirus type 3 is the prototype of a diverse group of viruses and virus-like elements that contain double-stranded RNA genomes (1). In reoviruses, the double-stranded RNA is organized into 10 unique segments (2), each consisting of a mRNA-like (+)-strand base-paired end-to-end with its complement (3). Expression of the genetic information in the duplexes is initiated by a virion-associated RNA polymerase that transcribes one strand of each segment to form 10 viral mRNAs (4–6). In addition to the transcriptase, reovirions contain several other enzymatic activities that modify the 5' ends of nascent transcripts (7). This results in mRNAs that contain a common 5'-terminal sequence, m^{7}GpppG^m-CUA, and are indistinguishable from the duplex (+)-strands, which are also "capped" (8).

Reoviruses have been particularly useful for analyzing eukaryotic transcription/translation mechanisms because the virion transcriptase and mRNA-modifying enzymes are stable and highly active *in vitro*. Consequently, large amounts of the multiple viral mRNA species—large (l), medium (m), and small (s)—can be prepared for functional studies. In a series of experiments with cell-free systems, it was found that at least 8 of the 10 reovirus mRNAs yielded a single initiation site as defined by ribosome protection against ribonuclease digestion (9, 10). At the level of 40S ribosomal subunits, each protected site included the cap and the 5'-proximal AUG located 12–32 nucleotides from the 5'-terminal m^{7}G. The same AUG and 10–15 nucleotides on either side were protected in each of the mRNA species by 80S ribosomes. These results make it likely that reovirus mRNAs, like most other eukaryotic messages, usually initiate protein synthesis at the 5'-proximal AUG (11). However, the s1 spe-

cies of reovirus mRNA possesses two overlapping initiation sites as determined by a ribosome protection assay. They included the 5'-proximal AUG and the next AUG, which is situated in a different reading frame 70 residues from the 5'-terminal m^{7}G (12). This suggested that a single reovirus mRNA species might contain two initiation sites for protein synthesis. Direct demonstration of reovirus initiation sites by correlation of mRNA and polypeptide sequences has been difficult because most of the proteins in reovirions contain a blocked NH$_2$ terminus (13). As an alternative approach, we used a modified eukaryotic translation system for measuring mRNA initiation sites based on the synthesis of the first dipeptide of the protein (14). By this procedure, it is possible to map any individual messenger-specific initiation sequence in a mixture of eukaryotic mRNAs, provided the second amino acids of the gene product are different.

MATERIALS AND METHODS

L-[^{3}H]Alanine (82.7 Ci/mmol; 1 Ci = 37 GBq), L-[^{3}H]aspartic acid (14.3 Ci/mmol), L-[^{3}H]glycine (44.2 Ci/mmol), L-[^{3}H]serine (16.8 Ci/mmol), L-[^{3}H]valine (50.9 Ci/mmol), and L-[^{3}H]leucine (110 Ci/mmol) were from New England Nuclear, and L-[^{3}H]glutamic acid (38 Ci/mmol), [^{3}H]ATP (21 Ci/mmol), S-adenosyl-L-[*methyl*-^{3}H]methionine (76 Ci/mmol), and L-[^{3}H]tyrosine (45 Ci/mmol) were from Amersham. Rabbit blood enriched in reticulocytes was purchased from Pel-Freez, and rabbit globin mRNA was either obtained from Bethesda Research Laboratories or prepared according to published procedures (15). *Artemia salina* cysts were purchased from San Francisco Brand (Newark, CA). *Escherichia coli* tRNA^{Asp}, tRNA$_2^{Glu}$, tRNA$_2^{Tyr}$, tRNA$_3^{Ala}$, tRNA$_1^{Val}$, and tRNA$_1^{Ser}$ were purchased from Subriden RNA (Rolling Bay, WA) and yeast tRNA$_i^{Met}$ was initially obtained from P. Sigler (University of Chicago, IL) and later prepared according to a modification of a published procedure (16). Unfractionated *E. coli* tRNA was from Sigma, and crude brewers yeast tRNA was from Boehringer Mannheim.

The preparation of *A. salina* ribosomes, rabbit reticulocyte initiation factors, eukaryotic elongation factors 1 and 2 (EF-1 and -2), and the acylated tRNA species are described or referred to in a previous report (14). In the experiments reported in Table 3 and Fig. 3, the eukaryotic initiation factors (eIFs) isolated from the heparin-Sepharose column (14) were further fractionated by phosphocellulose chromatography as follows. The eIFs eluted from the heparin-Sepharose column were precipitated with (NH$_4$)$_2$SO$_4$ (70% saturation), and the precipitate was dissolved in 20 mM Tris·HCl, pH

7.6/2 mM dithiothreitol/0.1 mM EDTA/10% glycerol (buffer A) containing 150 mM KCl. After dialysis against buffer A containing 100 mM KCl, the preparation was applied to a phosphocellulose column (Whatman P-11, 0.5 × 2 cm). The column was eluted stepwise with 0.15 M, 0.45 M, and 1 M KCl in buffer A to yield fractions A, B, and C, respectively. The protein in each fraction was precipitated with $(NH_4)_2SO_4$ (70% saturation), the mixture was centrifuged, and the pellets were dissolved in and dialyzed against buffer A containing 100 mM KCl. The three fractions designated A, B, and C were supplemented with eukaryotic initiation factor 4A (eIF-4A) and together were used as the source of eIFs.

Preparation and Separation of Reovirus mRNAs. Radiolabeled reovirus mRNA (average final specific activity = 1800 cpm/μg) was synthesized with viral cores in the presence of [³H]ATP or [³H]UTP and separated into the l, m, and s size classes by glycerol gradient centrifugation as described (17). To isolate the individual species of s mRNA, a mixture of transcriptase products was dissolved in buffer (0.09 M Tris borate, pH 8.3/25 mM EDTA/7 M urea) and separated by electrophoresis in 1.5-mm thick gels containing 5% polyacrylamide and 8 M urea (room temperature, 18 hr, 700 V). RNA bands visualized by UV light after ethidium bromide staining were eluted (18) and recovered after precipitation with ethanol. The RNAs were further purified by precipitation from 1.6 M LiCl (20 hr, 5°C) and reprecipitated with 70% ethanol.

Hybrid Selection of s1 mRNA. A *Bam*HI-cleaved, pBR322-derived plasmid containing an insert of s1 cDNA (provided by Y. Furuichi of this Institute), was covalently linked to cellulose as described (19). Mixtures of mRNA were annealed with cellulose-bound DNA at 50°C for 5 hr in 1.2 ml of 50% formamide containing 0.6 M NaCl, 60 mM trisodium citrate, 100 mM sodium phosphate (pH 7.0), 0.1% NaDoDSO₄, and 200 μg each of poly(U) and yeast tRNA per ml. About 120 μg of cellulose-bound plasmid DNA was annealed with 1 mg of ³H-labeled reovirus mRNA. After incubation, the cellulose was pelleted and washed in 0.4 ml of hybridization solution at 50°C for 3 min followed by two washes in 1 ml of 0.3 M NaCl/30 mM trisodium citrate at 25°C. Hybridized RNA was eluted by heating the DNA-cellulose to 95°C for 2 min in 0.2 ml of 99% formamide. This was repeated, and the eluates were combined and concentrated by the addition of potassium acetate to 0.2 M and two volumes of ethanol. The pellet was collected by centrifugation, reprecipitated in 70% ethanol, and the 50 μg of recov-

ered RNA (estimated on the basis of radioactivity) was dissolved in 50 μl of H_2O.

Methylation of Reovirus RNAs with Vaccinia Virus Enzymes. Reovirus mRNAs containing unblocked (ppG) or blocked but unmethylated (GpppG) 5' termini were synthesized by using published conditions (20). RNAs containing GpppG termini were methylated by incubation with S-adenosyl-L-[*methyl*-³H]methionine and methyltransferase that had been extracted from purified vaccinia virus and passed through DEAE-cellulose to remove DNA (21, 22). The [³H]methylated RNA was digested with P1 nuclease and calf intestine alkaline phosphatase, and the products were analyzed by paper chromatography using isobutyric acid/0.5 M NH₄OH, 10:6 (vol/vol) (7). It was found that 95% of the radioactivity comigrated with marker m^7GpppG^m, and the remainder migrated with m^7GpppG. The calculated extent of conversion of GpppG to m^7GpppG^m was 31%.

mRNA-Directed Di- and Tripeptide Synthesis. The preparation of the various components used in the *in vitro* di- or tripeptide system and the characteristics of the system have been presented in detail elsewhere with globin mRNA as template (14). Each incubation mixture (60 μl) contained 17 mM Hepes·KOH at pH 7.5, 125 mM KOAc, 2.7 mM Mg(OAc)₂, 1 mM dithiothreitol, 0.5 mM GTP, 1 mM ATP, 17 mM creatine phosphate, creatine kinase at 0.4 mg/ml, 2.5% polyethylene glycol 6000, 10 pmol of unlabeled fMet-tRNA$_i^{Met}$, 10 pmol of ³H-labeled aminoacylated tRNA (3000–7000 cpm/pmol), 0.4 A_{260} unit of 80S ribosomes, 1 μg of eIF-4A, 0.27 μg of EF-1, various amounts of reovirus mRNA as indicated, and either 20–35 μg of eIFs purified by heparin-Sepharose (14) or phosphocellulose fractions A (20 μg), B (2 μg), and C (1 μg). For tripeptide synthesis (14), 0.15 μg of EF-2 and the second (unlabeled) and third (³H-labeled) aminoacyl-tRNAs were added also. Reaction mixtures were incubated for 60 min at 37°C. The reaction was stopped by the addition of 4.5 μl of 1 M NaOH, and the mixture was incubated for an additional 10 min at 37°C to hydrolyze any peptidyl tRNA. The mixture was then acidified with 0.5 ml of 0.5 M HCl and extracted with 3 ml of ethyl acetate (23). After a brief centrifugation, an aliquot of the ethyl acetate layer was removed and counted in a liquid scintillation spectrometer. Under these conditions, fMet di- and tripeptides containing a neutral or acidic second and third amino acid are extracted into the organic solvent. The values reported have been corrected for any aliquots removed, the extraction coefficient of the di- and tripeptides, and any extractable radioactivity in the absence of mRNA.

Table 1. NH₂-terminal dipeptides predicted from 5' sequences of reovirus mRNA species

mRNA	Position of presumptive initiation site(s)	Initial dipeptide
s1	m^7G . . . (12) . . . AUG GAU (52) . . . . . . AUG GAG	Met-Asp, Met-Glu
s2	m^7G . . . (18) . . . AUG GCU	Met-Ala
s3	m^7G . . . (27) . . . AUG GCU	Met-Ala
s4	m^7G . . . (32) . . . AUG GAG	Met-Glu
m1	m^7G . . . (13) . . . AUG GCU	Met-Ala
m2	m^7G . . . (29) . . . AUG GGG	Met-Gly
m3	m^7G . . . (18) . . . AUG GCU	Met-Ala
l1	m^7G . . . (18) . . . AUG UCA	Met-Ser
l2	m^7G . . . (5) . . . AUG GCG . (2) . . . . . . AUG GCG	Met-Ala, Met-Ala
l3	m^7G . . . (13) . . . AUG AAG	Met-Lys

The NH₂-terminal dipeptides were predicted from mRNA 5'-terminal sequences and, with the exception of l2 mRNA (24), from ribosome binding studies (9–12). The numbers in parentheses indicate the number of bases spanning the indicated region.

RESULTS

Table 1 lists the reovirus l, m, and s mRNAs and the position of the 5'-proximal AUG in each. For 8 of the 10 viral mRNA species, this codon forms part of the single ribosome binding site in each species, suggesting strongly that it corresponds to the initiator AUG (10). The NH_2-terminal dipeptides shown in the table can be predicted on the basis of these findings. In the case of the s1 mRNA species, however, ribosome protection studies have indicated that there may be two start sites for translation (12). In addition, although the ribosome binding site in the l2 mRNA species has not been characterized (25), the putative initiation region at the 5' end has been reported to contain two closely spaced A-U-G-G-C-G sequences (ref. 24 but see also ref. 26). Accordingly, two possible initiation sites are listed for the s1 and l2 mRNAs. From the mRNA 5'-terminal sequences, five virus-encoded proteins are expected to begin with Met-Ala and two with Met-Glu.

Dipeptide synthesis (14) was used in these studies to ascertain the initiation site of the various reovirus mRNAs *in vitro*. Although eukaryotic initiation does not require that the initiator Met-tRNA be formylated, in these studies we used fMet-tRNA (which can be recognized by eIFs) because fMet dipeptides can be extracted readily with ethyl acetate. Thus, fMet-Ala should be the predominant dipeptide formed in the *in vitro* initiation system directed by a mixture of reovirus mRNAs. As shown in Fig. 1, fMet-Ala synthesis was linear for 60 min in the presence of [^{3}H]Ala-tRNA$_3^{Ala}$ (anticodon = GGC) in response to unfractionated reovirus mRNAs. About 2 pmol of the dipeptide was obtained with an input of ≈2 pmol of total message, if one assumes an average chain length of ≈2000, the size of the m class of reovirus mRNAs. Under the same conditions, there was no significant incorporation of valine or leucine into an ethyl acetate-extractable product, from [^{3}H]Val-tRNA$_1^{Val}$ or [^{3}H]Leu-tRNA^{Leu} (data not shown).

Reovirus mRNAs were resolved into three size groups by glycerol gradient centrifugation, and the s and m classes of mRNA, which comprise about 63 and 31 mol % of the total mRNA, respectively, were tested separately as templates. The l class of mRNAs was <10 mol % of the total transcripts and was not studied. From the 5'-terminal sequences (see Table 1) and the relative abundance of the individual mRNA species as determined by agarose gel electrophoresis (17), fMet-Ala and fMet-Glu should be the major dipeptides directed by the s class of mRNA and fMet-Ala but not fMet-Glu by the m class. Fig. 2 shows the effect of mRNA concentration on dipeptide formation for the mixture of mRNAs as
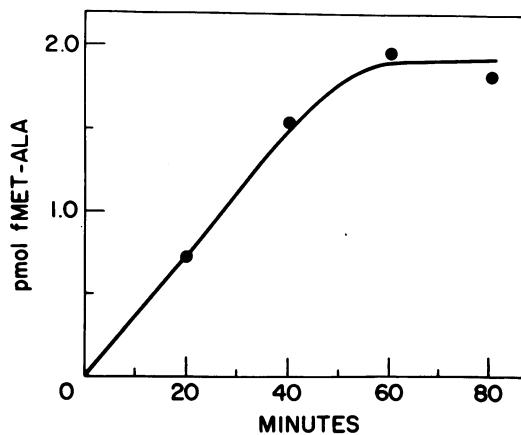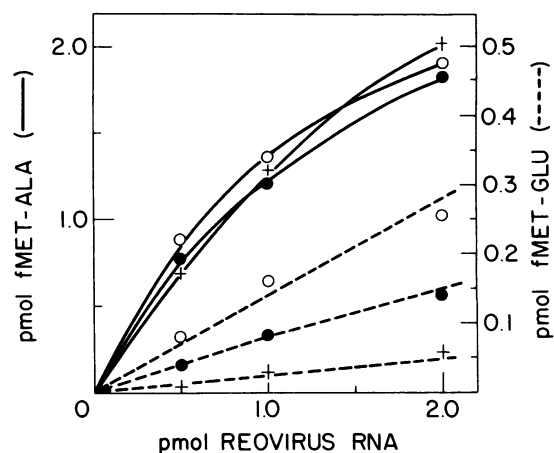

FIG. 2. Formation of fMet-Ala and fMet-Glu in response to an unfractionated mixture of total reovirus RNA species (●) or the separated s (○) and m (+) size classes of mRNA. Details of the assay are described in the text.

compared to the separated s and m classes. The unfractionated mRNA directs the synthesis of both fMet-Ala and fMet-Glu. fMet-Ala is also produced when either s or m mRNA is used as template, but significant amounts of fMet-Glu are made only from the s class mRNA. It should be noted that synthesis of fMet-Glu directed by total or s class mRNA was only 8–13% of the fMet-Ala formed. Because in the mixture of s class mRNAs, the relative abundance of s4 (43%) was the same as the sum of the s2 plus s3 mRNA species (17), these results indicate that the individual mRNAs are utilized with different efficiencies in this assay, similar to that described for the translation of these mRNAs in a wheat germ extract (27). In agreement with the predicted products summarized in Table 1, a low but significant amount of fMet-Gly was also obtained with the m class of mRNA (data not shown). No fMet-Ser, fMet-Val, or fMet-Leu was synthesized using either the s or m class mRNAs as templates (data not shown).

The results obtained with the separated classes of mRNA indicated that the dipeptide assay could detect specific initiation sites in a mixture of mRNAs. This was confirmed by testing the four individual species of s mRNA separated by polyacrylamide gel electrophoresis. Each directed the formation of the fMet dipeptide predicted from the 5'-proximal sequence that constitutes the 80S ribosome binding site (Table 2). In addition, in agreement with the finding that there are two possible protein synthesis initiation sites in s1 mRNA (12), assay mixtures containing this messenger yielded both fMet-Asp and fMet-Glu. No fMet-Ala was synthesized from the s1 mRNA, showing that this species was not contaminated with s2 mRNA. About twice as much fMet-Asp was synthesized as fMet-Glu at various s1 mRNA concentrations (Fig. 3).

Although the s1 mRNA that was used in these experi-

FIG. 1. Time course of fMet dipeptide synthesis directed by a mixture of reovirus mRNAs using tRNA$_3^{Ala}$ (●). Dipeptide synthesis was assayed as described in the text.

Table 2. Synthesis of dipeptides from various reovirus mRNAs

mRNA	Acylated tRNA used	Dipeptide formed, pmol
s1	Asp	0.8
	Glu	0.4
	Ala	0
s2	Ala	0.2
s3	Ala	0.2
s4	Glu	0.4

One microgram of each mRNA was incubated under conditions described in the text and elsewhere (14) in the presence of the appropriate labeled and acylated tRNA as indicated above. The assay for the formation of the dipeptides is described in the text.
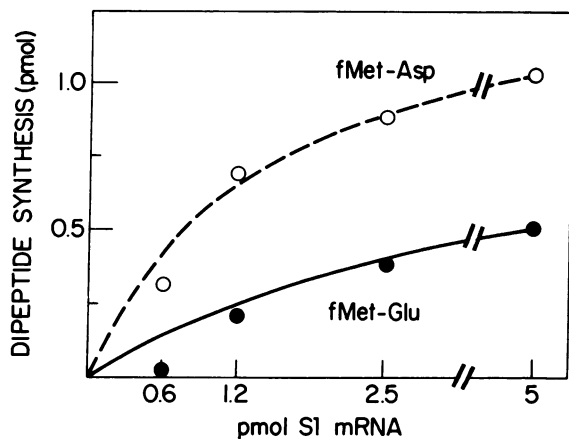
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FIG. 3. Effect of s1 mRNA concentration on the synthesis of fMet-Asp (o) and fMet-Glu (●). For these experiments, the eIFs were fractionated by phosphocellulose chromatography. s1 mRNA was selected by hybridization to cDNA-cellulose and assayed as described in the text.

ments was hybrid-selected and appeared to be highly purified by electrophoretic analysis, it was important to exclude the possible presence of s4 mRNA or its 5'-terminal fragments that also would direct the synthesis of fMet-Glu. The triplet following A-U-G-G-A-G in s1 mRNA is UAU (10) and in s4 mRNA is GUG (11). These triplets code for tyrosine and valine, respectively. This difference allowed us to verify that s1 mRNA contains two different initiation sites by demonstrating the synthesis of the specific tripeptide fMet-Glu-Tyr in response to the hybrid-selected s1 mRNA. Table 3 shows that between 0.25–0.40 pmol of fMet-Glu-Tyr was synthesized in the incubation and that the synthesis of the tripeptide, as measured by extractable radioactivity, was dependent on the presence of Glu-tRNA. Furthermore, when [^{3}H]Val-tRNA was substituted for [^{3}H]Tyr-tRNA, no fMet-Glu-Val could be detected, thus showing that the s1 mRNA was not contaminated with functional s4 mRNA (Table 3).

The dipeptide assay was used also to test the effect of 5'-terminal guanylylation and methylation on mRNA function. The mixture of reovirus mRNAs containing predominantly either uncapped and unmethylated (ppG) ends or guanylylated and unmethylated (GpppG) 5' termini were 10–15% as active as the corresponding capped (m^{7}GpppG^m) mRNAs for directing fMet-Ala synthesis (Table 4). To demonstrate that the relative inactivity of the mRNA containing a GpppG end was due to the absence of a methylated terminus, the same RNA was retested after incubation with vaccinia virus methyltransferase. About half of the template activity was restored with the methylated mRNA in which an estimated 31% of the 5' ends had been converted from GpppG to m^{7}GpppG^m (Table 4).

DISCUSSION

Assays of the initiation of protein synthesis based on the formation of fMet dipeptides have directly confirmed that the

Table 3. Synthesis of fMet-Glu-Tyr and fMet-Glu-Val from s1 mRNA

System	Tripeptide, pmol	
	Exp. 1	Exp. 2
Complete	0.43	0.25
-Glu-tRNA	0.12	0
-Tyr-tRNA + Val-tRNA	0	—

The incubation conditions and the assay are described in the text and elsewhere (14). The eIFs after phosphocellulose fractionation were used in these experiments.

Table 4. Effect of capping and methylation on template activity of reovirus mRNAs

mRNA 5' structure	fMet-Ala formed, pmol
m^{7}GpppG^m	2.0
ppG	0.2
GpppG	0.3
m^{7}GpppG^m*	1.1

*Mixture of molecules containing m^{7}GpppG^m (31%) and GpppG after incubation of unmethylated, GpppG-terminated mRNA with vaccinia virus methyltransferase (see text).

5'-proximal AUG-containing sequences in reovirus mRNAs correspond to the sites of initiation of polypeptide synthesis. In addition, the strong dependence on the presence of a 5'-terminal m^{7}G-cap for reovirus mRNA template activity suggests that the dipeptide assay reflects the properties of eukaryotic translation initiation. The results are in excellent agreement with the reovirus mRNA initiation sites defined by ribosome binding and RNase protection studies (9–12). For two mRNAs in each of the s and m size classes of reovirus mRNA, the first AUG at the 5' end is followed by GCU. Accordingly, fMet-Ala was the predominant dipeptide formed in response to separated s or m class mRNAs or to an unfractionated mixture of viral mRNAs. No fMet-Val or fMet-Leu synthesis was detected by the dipeptide assay, although Met-Val, -Leu, and -Thr were the previously suggested NH$_2$-terminal assignments for the polypeptide products directed by s and m mRNAs in an ascites cell-free translation system (28).

The data also show that when an individual s mRNA species of reovirus was used as template, dipeptide synthesis yielded the predicted products. In addition, with s1 mRNA, the presence of two functional initiation sites in this mRNA species was detected by the di- and tripeptide assay. This result confirms a previous suggestion (12) that s1 mRNA has two protein synthesis initiation sites. As described by Kozak (29), most eukaryotic mRNAs initiate protein synthesis at the 5'-proximal AUG, which is part of a consensus sequence (A-G)-N-N-A-U-G-G. In the case of s1 mRNA, the 5'-proximal initiation sequence is C-G-G-A-U-G-G. According to the scanning model, some ribosomes would bypass the first AUG and initiate at the second AUG, which has the A-U-A-A-U-G-G consensus sequence (12). It will be of interest to determine if both AUGs are in open reading frames and initiate the synthesis of two different polypeptides, as described recently for a bicistronic RNA of influenza B virus (30). It also should be possible to determine the relative initiation efficiencies of other reovirus mRNAs by this procedure because cDNAs of all 10 genome double-stranded RNA segments have been cloned (31, 32).

The dipeptide system also can be used to examine competition between viral and cellular mRNAs and the possible effects of intramolecular structure on initiation. For example, some rotavirus (32) and reovirus mRNAs (33, 34) have been shown to contain complementary 5' and 3' termini, and in some cases the potential for forming a stable base-paired stem involving translation initiation and termination regions is high. If dipeptide synthesis, like the formation of initiation complexes (35), can be directed by 5'-terminal fragments of mRNA, this assay can be used to evaluate the influence of mRNA secondary structure on translation initiation.

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