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Relationship between structure of the 5′ noncoding region of viral mRNA and efficiency in the initiation step of protein synthesis in a eukaryotic system

(eukaryotic mRNA/plant virus RNA/initiation complex of protein synthesis)

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ABSTRACT **To determine whether the rate of protein synthesis is controlled by the structure of mRNA near its 5′ terminus, protein-synthesizing ability, especially in its initial stage, was compared among RNAs of plant viruses. Those viruses used here contain several definite pieces of single-stranded RNA. Each of these RNAs acts as a messenger. Cucumber mosaic virus (CMV) RNA 5 synthesizes a small amount of a protein, M_r 7000, in an *in vitro* protein-synthesizing system from wheat germ or reticulocyte. Brome mosaic virus (BMV) RNA 4 synthesizes a large amount of a coat protein under the same conditions. Both RNAs have the same 5′-cap structure and a short noncoding region (10 nucleotides in CMV RNA 5 and 9 in BMV RNA 4) between the 5′ terminus and the initiation codon AUG. A sequence complementary to the 3′ terminal of 18S ribosomal RNA is contained in BMV RNA 4 but is not apparent in CMV RNA 5. Formation of the initiation complex for protein synthesis by the 5′-terminal-labeled mRNA of cytoplasmic polyhedrosis virus was inhibited by the addition of unlabeled BMV RNA 4 whereas it was only slightly inhibited by unlabeled CMV RNA 5. BMV RNA 4, which has a sequence complementary to rRNA, can form the initiation complex more easily than CMV RNA 5. It is concluded that an apparent complementary sequence to the 3′ terminal of 18S rRNA in the 5′ noncoding region of eukaryotic mRNA and the 5′-cap structure enhance the rate of initiation complex formation in protein synthesis.**

Usually, prokaryotic mRNA is polycistronic whereas eukaryotic mRNA is monocistronic. The latter carries the cap structure at the 5′ terminal and protein synthesis begins from the initiation codon AUG (1). In prokaryotic mRNA, a sequence complementary to the 3′-terminal part of 16S rRNA is located in front of the initiation codon of protein synthesis, and it is considered to bind to ribosomes to form an initiation complex as the first step in protein synthesis (2–5).

The nucleotide sequences of the 3′-terminal region of 18S eukaryotic rRNA have been determined for mouse, rat, rabbit, hen, silkworm, wheat, and barley (6–8). These are almost identical to one another and similar to prokaryotic 16S rRNA sequences. Thus, this part of eukaryotic rRNA should have a secondary structure similar to the prokaryotic one, taking a hairpin structure containing the m_2^6A-m_2^6A sequence in the loop part (6–8). According to the psolaren crosslinking experiment of Nakashima *et al.* (9), the 5′-terminal part of eukaryotic mRNA interacts with the 3′-terminal part of 18S rRNA as seen in prokaryotes. But the eukaryotic sequence of 18S rRNA near the 3′ terminus lacks the sequence C-C-U-C-C, which is complementary to mRNA in prokaryotes. Therefore, the mechanism of initiation interaction of mRNA with ribosomes should be different in prokaryotes and in eukaryotes. The 5′-terminal noncoding regions of the different eukaryotic mRNAs vary not only in length and secondary structure but also in nucleotide sequence. Some contain a nucleotide sequence complementary to the 3′-terminal part of 18S rRNA whereas some others do not contain such a clearly complementary sequence (10, 11). To determine the relationship between the structure of the 5′-terminal leader sequence of eukaryotic mRNA and the efficiency of protein synthesis, especially in the initial stage, the protein syntheses of a few kinds of plant viral RNAs, in which the leader sequences are short, were studied.

Cucumber mosaic virus (CMV) strain Y contains five single-stranded RNA species. RNA 5, the shortest one, is related to the expression of the characteristic symptoms. As some strains of CMV do not contain it, RNA 5 is considered to be a satellite component (12). This RNA carries a cap structure at the 5′ terminus, and the nucleotide sequence of the 5′ noncoding region is only 10 nucleotides long and simple (13, 14). Brome mosaic virus (BMV) contains four single-stranded RNA species. The shortest one, BMV RNA 4, can act as a coat protein mRNA. It carries a cap at the 5′-terminus, and its 5′ noncoding region is only nine nucleotides long (15). In this region, BMV RNA 4 contains a sequence complementary to the 3′-terminal part of 18S rRNA, but CMV RNA 5 does not contain such a sequence. The protein-synthesizing ability and the formation of the initiation complex for protein synthesis were studied by using these RNAs as mRNAs in *in vitro* protein-synthesizing systems.

MATERIALS AND METHODS

Preparation of RNAs. Cytoplasmic polyhedrosis virus (CPV) mRNA was prepared *in vitro* as described by Shimotohno and Miura (16). BMV RNAs were prepared as by Bockstahler and Kaesberg (17). CMV RNAs were prepared as described (13). The RNA segments of BMV and CMV were separated by gel electrophoresis according to Floyd *et al.* (18). The gel was composed as follows: 2.3% acrylamide/0.11% bisacrylamide/6 M urea/0.1% $NaDodSO_4$/Loening's buffer/0.6% agarose/0.1% EDTA/0.1% persulfate. The acrylamide/agarose solution was poured into Perspex gel tubes (inside diameter, 6.5 mm) to a height of 14 cm. The gels were kept at 20°C for 2 to 3 hr and then overnight at 4°C, when the agarose solidified. Samples were dissolved in Loening's buffer/0.1% $NaDodSO_4$/6 M urea to which was added 1/20 vol of 0.1% xylene cyanol tracking dye. Gel electrophoresis was done in Loening's buffer at 120 V.

Extraction and Recovery of RNAs. The positions of the RNAs separated by electrophoresis were determined by staining with ethidium bromide. The gel bands containing RNA were then cut out and broken into small pieces, and the RNAs were

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Abbreviations: CMV, cucumber mosaic virus; BMV, brome mosaic virus; CPV, cytoplasmic polyhedrosis virus.

eluted by electrophoresis, treated with an equal volume of 80% phenol, and precipitated with ethanol. The mixture was left overnight at −20°C, and then the precipitate was recovered by centrifugation. The pellet was rinsed twice with ethanol, dried at reduced pressure, and dissolved in distilled water.

Cell-Free System for Protein Synthesis. A cell-free system was prepared from wheat germ as described by Roberts and Peterson (19). A cell-free system from reticulocytes was purchased from Radiochemical Centre, Amersham, England.

Identification of Peptides Produced. The *in vitro* products were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis as described (20, 21). The distribution of protein bands was analyzed by fluorography using Kodak RP/S X-Omat or FUJI RX film (22).

The molecular weights of the peptide products were determined by comparison with marker proteins of known molecular weights (calibration kit from Boehringer Mannheim and a [^{14}C]methylated protein mixture from The Radiochemical Centre). Electrophoresis was in a 15% acrylamide/0.1% $NaDodSO_4$ slab gel. After electrophoresis, proteins were stained with 0.25% Coomassie brilliant blue in 5% methanol/7.5% acetic acid, and the gels were dried and fluorographed as described above.

Formation of the Initiation Complex for Protein Synthesis in Wheat Germ Extract. *Methyl*-^{3}H-labeled CPV mRNA was added to a cell-free protein-synthesizing system containing 0.1 mM cycloheximide to inhibit the elongation of peptide chains; the mixture was incubated at 30°C for 15 min. Then, the reaction mixture was put on a 10–30% glycerol density gradient in 20 mM Tris acetate, pH 7.4/75 mM KCl/3 mM magnesium acetate/0.2 mM EDTA and centrifuged at 27,000 rpm for 4 hr in a Beckman Spinco ultracentrifuge using an SW 40 rotor. From the bottom of the tube, 0.4-ml fractions were collected (23).

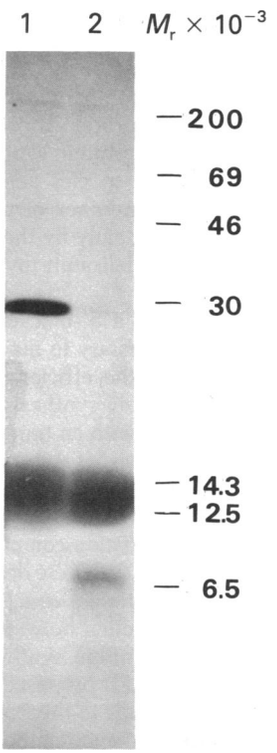

FIG. 1. Molecular weights of *in vitro* translation products of CMV RNAs 4 and 5. The translation mixtures (total vol, 11 μl) contained 8 μl of reticulocyte lysate, 2 μCi of [^{3}H]leucine (1 Ci = 3.7×10^{10} becquerels), 2 μCi of [^{35}S]methionine, and 0.1 μg of either CMV RNA 4 (lane 1) or CMV RNA 4 (lane 2) and were incubated at 30°C for 90 min. A common thick protein band is produced without addition of exogenous viral RNA to the reticulocyte lysate.

RESULTS

CMV RNA 5 is a small single-stranded RNA, M_r 100,000, and its leader sequence is short. The first initiation codon AUG appears at the 11th nucleotide from the 5′ terminus (13, 14). The protein coded for by this RNA in an *in vitro* system was characterized. The newly synthesized protein was labeled with [^{3}H]leucine/[^{35}S]methionine in a reticulocyte cell-free system, adding CMV RNA 5 as mRNA. The labeled protein was analyzed by gel electrophoresis in a 15% acrylamide gel slab and fluorography. As shown in Fig. 1, a protein of $M_r \approx 7000$ is produced by CMV RNA 5. However, the amount was very small. When CMV RNA 4, which carries the coat protein cistron, was used as mRNA in the same system, a protein of M_r 28,000, corresponding to the coat protein, was produced in large amount.

BMV RNA 4 is a small RNA carrying the coat protein cistron (24). Its leader sequence, before the first initiation codon AUG, is only 9 nucleotides long (15), whereas the leader sequence of CMV RNA 4 is 76 nucleotides long (unpublished). The protein-synthesizing capabilities of CMV RNA 5 and BMV RNA 4 were compared in the *in vitro* reticulocyte system, using the same (mol/mol) amounts of RNAs. Analysis of the products encoded by these RNAs is shown in Fig. 2. BMV RNA 4 produces a much greater amount of protein than CMV RNA 5. This tendency was also seen in the *in vitro* system for protein synthesis from wheat germ in which CMV RNA 5 produced less protein than BMV RNA 4. To express these facts quantitatively, the amount of specific product was measured by tracing the radioautograms as shown in Table 1. Although both CMV RNA 5 and BMV RNA 4 have short leader sequences with similar lengths, the latter synthesizes protein ≈100 times as efficiently as the former.

As the leader sequence preceding the cistron participates in the formation of an initiation complex for protein synthesis, the

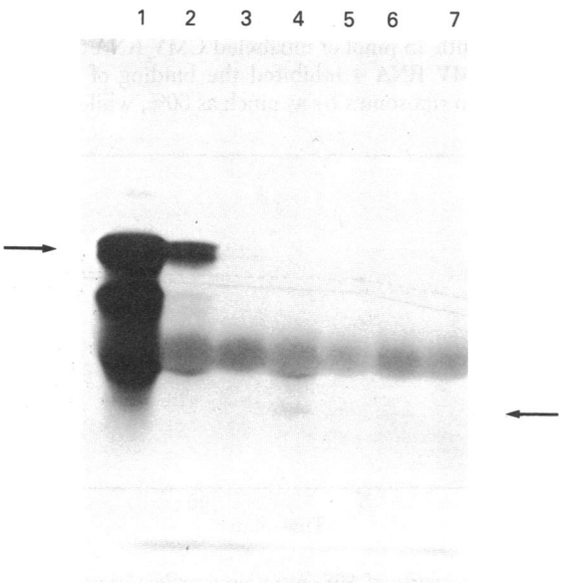

FIG. 2. Analysis of peptides produced in the reticulocyte cell-free system. The *in vitro* products were prepared as described in Fig. 1 except for the RNAs. Lanes: 1–3, 0.5, 0.05, and 0.005 μg, respectively, of BMV RNA 4; 4–6, 0.5, 0.05, and 0.005 μg, respectively, of CMV RNA 5; 7, blank (no mRNA). Arrows indicate specific products of BMV RNA 4 and CMV RNA 5.

Table 1. Relative amounts of *in vitro* translation products in the reticulocyte system

Column*	mRNA, μg	Relative amount of product: BMV RNA 4	CMV RNA 5
1 or 4	0.5	129.9	1.35
2 or 5	0.05	16.0	0.21
3 or 6	0.005	1.0	—

The autoradiograms of Fig. 2 were traced by microdensitometer (Joyce–Loebl model 3CS). Relative amounts of *in vitro* products were calculated on the basis of paper weight of a scanned pattern from the autoradiogram taking the amount produced by 0.005 μg of BMV RNA 4 as 1.0.
* See Fig. 2.

rate of its formation was compared quantitatively by the following method. 5′-Terminal-labeled native mRNA was prepared by *in vitro* transcription of CPV, which contains double-stranded RNA as a genome, as described above and in ref. 16. [^{3}H]Methyl was incorporated into the 5′-terminal cap structure of CPV mRNA. As the labeled CPV mRNA forms the initiation complex for protein synthesis, unlabeled BMV RNA or CMV RNA was added to the reaction mixture to compete with it. ^{3}H-Labeled CPV mRNA was added to the *in vitro* wheat germ protein-synthesizing system as described above, but cycloheximide was added to prevent peptide chain elongation and thus permit detection of the initiation complex in protein synthesis. Formation of the initiation complex was analyzed by glycerol density-gradient centrifugation (see Fig. 4). A significant proportion of the CPV mRNA was recovered as the 80S initiation complex. As shown in Fig. 3, formation of the 80S initiation complex reaches a plateau after 15 min of incubation at 30°C. The experiments were carried out under this condition. CPV mRNA consists of 10 molecular species (M_r, 0.17–1.27 × 10^6) having a mean M_r of 0.61 × 10^6 (25). Molar concentrations of CPV mRNA were calculated based on this value. Formation of the initiation complex was compared when 25 pmol of ^{3}H-labeled CPV mRNA was incubated with 25 pmol of unlabeled BMV RNA 4 (Fig. 4*A*) or with 25 pmol of unlabeled CMV RNA 5 (Fig. 4*B*). Unlabeled BMV RNA 4 inhibited the binding of ^{3}H-labeled CPV mRNA to ribosomes by as much as 60%, while unlabeled

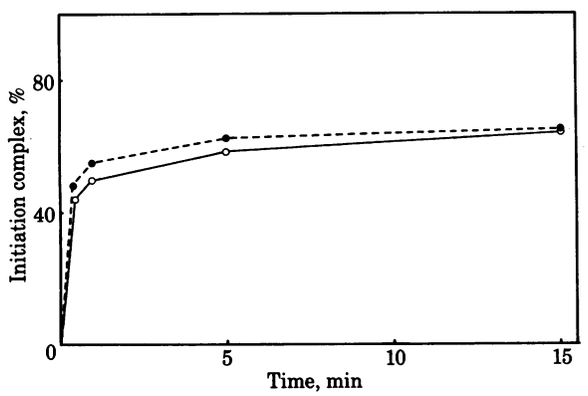

FIG. 3. Time course of 80S initiation complex formation. Intact CPV mRNA (*methyl*-^{3}H labeled) was incubated with wheat germ extract in the presence of cycloheximide. The reaction mixture was placed on a 10–30% glycerol density gradient and centrifuged in a Beckman Spinco SW 40 rotor at 27,000 rpm for 4 hr, and 0.4-ml fractions were collected from the bottom of the centrifuge tube. Radioactivity of each fraction was measured, and the percentage of ^{3}H-labeled mRNA bound to the 80S initiation complex was calculated. Reaction mixtures were incubated at 20°C (○) or at 30°C (●).

CMV RNA 5 did so only slightly. Even if 3 times the amount (75 pmol) of unlabeled CMV RNA 5 was added, the inhibition was slight (Fig. 4*C*). These results indicate that BMV RNA 4 can bind to ribosomes to form an initiation complex for protein synthesis much more efficiently than CMV RNA 5. Comparing the structures of these two RNAs, both have the same 5′ cap, m^7G^5pppG, and a short leader sequence in front of the initiation codon AUG (9 nucleotides for BMV RNA 4 and 10 for CMV RNA 5) as shown in Fig. 5. However, the complementary nucleotide arrangement to the 3′-terminal part of 18S rRNA in the leader sequence of BMV RNA 4 has a higher potential energy [ΔG = 3.0 kcal (1 cal = 4.18 J)] than that of CMV RNA 5 (−0.2 kcal), according to the method of Tinoco *et al.* (26).

We have confirmed that CMV RNA 5 itself is able to form an initiation complex. Furthermore, the 5′-terminal fragment, ≈20 nucleotides long, of CMV RNA 5 including the leader sequence and the first initiation codon AUG was prepared, and it also was able to form an initiation complex. The details of these data will be reported elsewhere. It is clear that a short and simple leader sequence (10 nucleotides) before the first initiation codon of CMV RNA 5 participates in binding to ribosomes to form the initiation complex, although the binding efficiency is less than that of the mRNA carrying a distinct complementary sequence to the 3′-terminal part of 18S rRNA.

DISCUSSION

There are many kinds of mRNAs specific for each differentiated cell. However, the quantities of protein molecules produced by the different kinds of mRNAs vary (27). For example, the mRNAs for the α- and β-chains of hemoglobin are similar in size, 550 nucleotides in length and the amount of α-chain mRNA is 1.5 times that of β-chain mRNA, but the α- and β-peptides are produced in the same total amounts (28).

In CMV Y strain containing five RNA segments, a large amount of RNA 5 is contained compared with other segments but a large amount of the coat protein is produced by RNA 4 (29). Thus, the total amount of a protein synthesized under the direction of a particular mRNA species depends on both the translation efficiency and the number of mRNA molecules. Gene expression is controlled mainly by the rate of transcription, but it is also controlled delicately by the efficiency of translation.

Eukaryotic mRNA possesses a 5′-blocked structure—i.e., the 5′ cap (30, 31)—that is necessary to maintain the stability of the mRNA and to enhance the efficiency of complex formation for the initiation of protein synthesis. When a cap-deleted mRNA (23, 32) or mRNA with an unmethylated cap (33) was added to the protein-synthesizing system, formation of the 80S initiation complex decreased remarkably. Addition of cap analogue or 7-methylguanylic acid to the *in vitro* protein-synthesizing system also inhibits initiation-complex formation of an intact mRNA competitively (34, 35). These data suggest that the cap structure participates in initiation-complex formation but not obligatorily. Actually, interaction between the mRNA cap and some initiation factor for protein synthesis was revealed (36, 37). Kozak and Shatkin (1, 11) proposed the idea that, in a eukaryote cell, mRNA first binds to the small ribosome subunit at the 5′ terminus through the aid of the cap-binding protein and then moves until the initiation codon AUG is encountered.

CMV RNA 5 and BMV RNA 4 carry the same cap structure. However, the efficiency of initiation-complex formation and rate of protein synthesis are significantly different. This difference should depend on the structure of the leader sequence. The length of the 5′-noncoding region of BMV RNA 4 is only

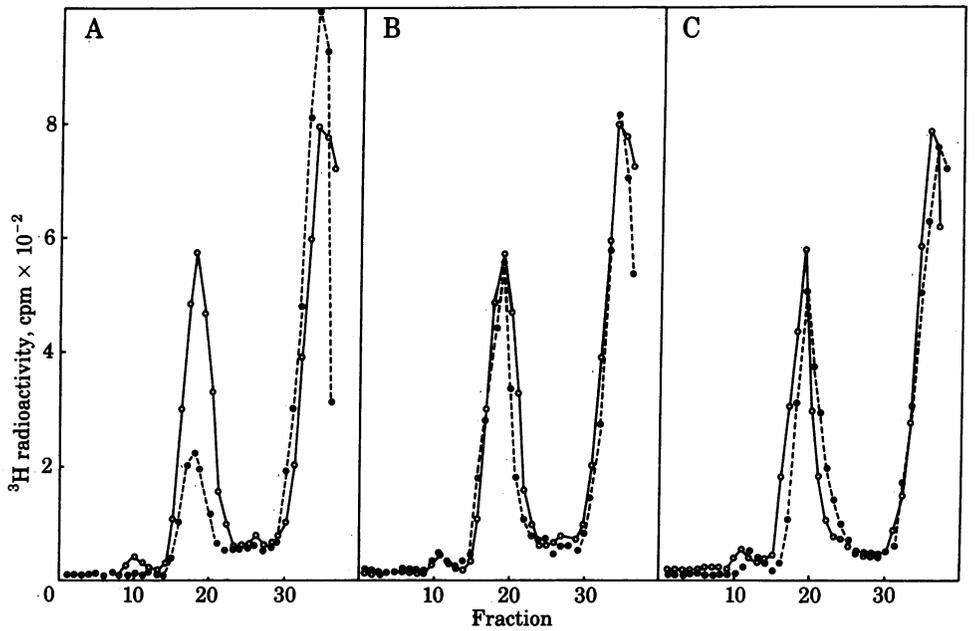

FIG. 4. Binding of mRNA to 80S ribosomes in wheat germ extract. *Methyl*-^{3}H-labeled CPV mRNA and various amounts of BMV or CMV RNAs were incubated with wheat germ extract in the presence of cycloheximide. Samples were sedimented through 10–30% glycerol density gradients. (*A*) ○, 25 pmol of *methyl*-^{3}H-labeled CPV mRNA; ●, 25 pmol of labeled CPV mRNA and 25 pmol of BMV RNA 4. (*B*) ○, 25 pmol of labeled CPV mRNA; ●, 25 pmol of labeled CPV mRNA and 25 pmol of CMV RNA 5. (*C*) ○, 25 pmol of labeled CPV mRNA and 25 pmol of CMV RNA 5; ●, 25 pmol of labeled CPV mRNA and 75 pmol of CMV RNA 5.

9 nucleotides and that of CMV RNA 5 is 10. These are the shortest leader sequences known in eukaryotic mRNAs. Other short leader sequences are known in the coat protein cistron of tobacco mosaic virus (9 bases) (38) and the NS protein cistron of vesicular stomatitis virus (10 bases) (10), but a leader sequence shorter than 8 bases has not been discovered.

The difference in the leader sequences of BMV RNA 4 and CMV RNA 5 seems to affect affinity to the ribosomal RNA. BMV RNA 4 has the sequence U-A-A-U, which is complementary to the 3′-terminal part of 18S rRNA, whereas CMV RNA 5 does not carry a strong continuous complementary sequence. Calculation of the affinity between the leader sequence and the rRNA by the method of Tinoco *et al.* (26) showed that the free energy of a possible pairing structure between the leading sequence in BMV RNA 4 and the 3′-terminal part of 18S rRNA is apparently greater than that between CMV RNA 5 and rRNA. Thus, BMV RNA 4 should have a stronger affinity to ribosomes than CMV RNA 5. CMV RNA 5 can form the initiation complex for protein synthesis but the efficiency is much lower than that of BMV RNA 4. Therefore, a possible complementary sequence to rRNA ahead of the initiation codon would enhance the first binding of mRNA to ribosomes.

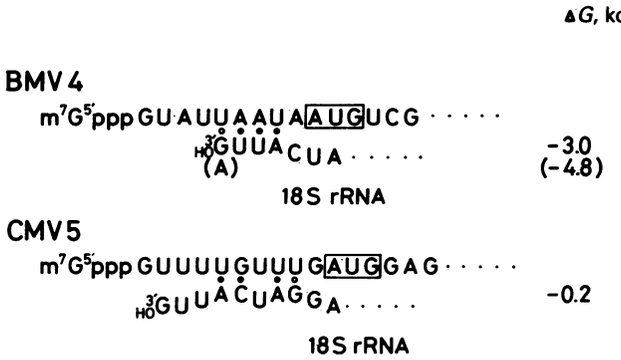

FIG. 5. Possible base pairing between the 3′-terminal part of eukaryotic 18S rRNA and the leader sequence in viral RNAs. The free energies (Δ*G*) for the base-paired regions were calculated according to Tinoco *et al.* (26). The 3′-terminal nucleoside of 18S rRNA is guanosine in wheat germ but adenosine in animals. Value in parentheses represents the latter case.

The distance between the sequence complementary to ribosome and the initiation codon is usually several bases long in a prokaryote mRNA. It is known that, if this distance is increased by adding bases, the efficiency of the protein-synthesizing capability of a mRNA decreases (39). In eukaryotes, however, the distance between the ribosome-binding sequence, if any, and the initiation codon is different for each species of mRNA. The distance may be >100 bases—for example, the 128-base-long sequence in β-lipotropin mRNA (40). In this case, however, the leader sequence has the ability to base pair within the long sequence itself to bring the ribosome-binding sequence closer to the initiation codon, and similar cases are observed elsewhere.

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