Effects of Length and mRNA Secondary Structure on the Interaction of Bovine Mitochondrial Ribosomes with Messenger RNA*

Hua-Xin Liao and Linda L. Spremulli†

From the Department of Chemistry, University of North Carolina, Chapel Hill, North Carolina 27599-3290

The mRNA for cytochrome oxidase subunit II (CoII) from bovine mitochondria binds to the small subunit of the mitochondrial ribosome in the absence of auxiliary factors. The synthetic polymer poly(U) is effective in competing with CoII mRNA for binding, although the polymer poly(A,U,G) competes very weakly. The effects of mRNA length on the interaction between the 28 S ribosomal subunit and mRNA have been examined using truncated derivatives of CoII mRNA. These results indicate that there is a minimum length of ~400 nucleotides required for the efficient binding of the mRNA to the small subunit. Shorter mRNAs will bind, but do so with much lower association constants. mRNAs of various lengths but with reduced secondary structure were prepared by substituting ITP for GTP during in vitro transcription reactions. These derivatives show the same effects of length as do the normal mRNA, indicating that mRNA secondary structure is not a critical factor in subunit-mRNA interaction. The binding of the mRNA to the 28 S subunit is not influenced by the presence of guanine nucleotides or by the presence of a triphosphate at the 5’ end of the RNA.

The mechanism of protein biosynthesis in mammalian mitochondria is currently not well understood. The ribosomes from these organelles are quite distinct from those found in bacterial or eukaryotic cytoplasmic systems. In general, they are 55-60 S particles and are composed of 28 S and 39 S subunits (1). These ribosomes have a low RNA:protein ratio and are composed of only ~25% RNA (2). They contain a large variety of proteins, many of which are larger and somewhat less basic than the ribosomal proteins from other systems (2).

Little information is available on the mechanism of chain initiation in this organelle, and no individual initiation factors have been described to date. However, it has been shown that Escherichia coli initiation factor 3 will bind bovine mitochondrial ribosomes and will facilitate their dissociation (3, 4). Recently, it has been reported (5) that, unlike other ribosomes, animal mitochondrial 28 S subunits have a binding site for guanine nucleotides. It is possible that GTP bound to these subunits may play a direct role in the initiation of translation.

The mechanism for selection of the translational start site on animal mitochondrial mRNAs must be distinct from that observed in prokaryotes or in the eukaryotic cell cytoplasm.

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† To whom correspondence should be addressed.

These mRNAs lack significant leader sequences 5’ to the translational start signal (6, 7). Hence, they cannot use a Shine-Dalgarno type of hydrogen bonding between rRNA and mRNA for the selection of the start codon. These mRNAs are not capped, and initiation in animal mitochondria does not involve a cap recognition and scanning mechanism analogous to that found in cytoplasmic systems (8). Our laboratory has recently observed (9) that mitochondrial 28 S ribosomal subunits bind mRNAs tightly, forming a 1:1 complex. Recently, Denslow et al. (10) reported a similar observation. This interaction is specific for the small subunit, and no binding is observed with 39 S subunits or with 55 S monosomes. The binding occurs readily in the absence of initiation factors and does not appear to occur at a specific sequence in the mRNA (9). It has been suggested (10) that recognition of the 5’ end of the mRNA may require the presence of initiation factors, although additional work will be required to explore this possibility. In this report, we have examined the interaction of bovine mitochondrial 28 S ribosomal subunits with mRNAs of different lengths and degrees of secondary structure. In addition, we have tested the effects of guanine nucleotides on mRNA binding to these ribosomal subunits.

EXPERIMENTAL PROCEDURES

Materials—T4 DNA ligase, T7 RNA polymerase, T1 RNase, T4 polynucleotide kinase, restriction enzymes (Rsal, EcoRI, HindIII, DdeI, and BglII), the Kleenow fragment of E. coli DNA polymerase, and a Sequenase kit were obtained from United States Biochemical Corp. Ribonucleotide triphosphates, poly(U), and polynUC(A,G) were from Pharmacia LKB Biotechnology Inc. Sp6 polymerase was obtained from Bethesda Research Laboratories, [5,6-3H]UTP (40 Ci/mmole), [α-32P]UTP (800 Ci/mmole), and [γ-32P]ATP (3000 Ci/mmole) were obtained from Du Pont-New England Nuclear. Calf intestinal alkaline phosphatase was from Boehringer Mannheim. RNA was from Promega Biotech. β-Filter blotting membranes for Southern transfers were obtained from Bio-Rad. The synthetic oligonucleotide primers for oligonucleotide-directed mutagenesis and DNA sequencing were prepared in the Department of Microbiology and Immunology, University of North Carolina at Chapel Hill.

Oligonucleotide-directed Mutagenesis—The plasmid pTZ19RCoII, which carries the CoII gene oriented for transcription by T7 RNA polymerase, was constructed as previously described (9). In this construct, there are no convenient restriction enzyme sites immediately following the translational stop codon of the CoII gene. Hence, ~100 nucleotides 3’ to the translational stop codon are present in RNA transcripts from this plasmid when it is linearized by digestion with BamHI. To obtain transcripts without additional nucleotides 3’ to the stop codon, a BglII site was introduced at a position corresponding to residue 8057 (immediately past the CoII translational stop codon) on the original mitochondrial DNA map (11) by oligonucleotide-directed mutagenesis (12). This construct provided a convenient restriction enzyme site on the 3’ side of the CoII gene without altering nucleotides in the actual coding sequence. The mRNA transcribed from this construct will resemble the in vivo mRNA which lacks a significant 3’-untranslated sequence (11). The sequence at the 3’ end

The abbreviation used is: CoII, cytochrome oxidase subunit II.
of the CoII gene was confirmed both by restriction enzyme digestion and by DNA sequencing. This construct has been designated pTZ19RCoII, and the designation CoII mRNA will be used here to designate RNA transcribed from this vector.

In Vitro Transcription Reactions—Transcription reactions were prepared basically as described (9). Plasmid DNA, purified by centrifugation in CsCl/EthBr gradients, was linearized by appropriate restriction enzymes, extracted with phenol/chloroform, and precipitated with ethanol before use. CoII mRNA was routinely prepared using a template linearized with BglII. Different length CoII mRNA derivatives were prepared by using templates linearized with BamHI, BglII, Sphl, Ddel, HindIII, EcoRI, and Rsal, which gave transcripts of 768, 680, 349, 246, and 114 bases in length, respectively. These CoII mRNA derivatives had specific activities of 5,000-30,000 cpm/pmol depending on their lengths. Inosine-containing CoII mRNA transcripts were prepared by using TTP instead of GTP as described above. Preparation of CoII mRNA for use in hybridization reactions was carried out as described (9) except that 0.1 mm [α-32P]UTP (final specific activity of 35 Ci/mmol) was used and the other ribonucleotide triphosphates were added at a final concentration of 0.33 mM.

Preparation of Bovine Mitochondrial Ribosomes and Ribosomal Subunits—Digitonin-treated bovine mitochondria and mitochondrial ribosomes were prepared from 4-kg samples of fresh liver as described by Matthews et al. (2) with modifications as described (13). Ribosomal subunits were partially dissociated into subunits in buffer (50 mM Tris-HCl, pH 7.6, 5 mM β-mercaptoethanol, 5 mM MgCl2, and 100 mM KCl), and the particles were separated by centrifugation through linear 10-30% sucrose gradients prepared in the same buffer. CoII RNA was purified by centrifugation in CsCl/EthBr gradients, was linearized by appropriate restriction enzymes, extracted with phenol/chloroform, and precipitated with ethanol before use. CoII mRNA was routinely prepared using a template linearized with BglII. Different length CoII mRNA derivatives were prepared by using templates linearized with BamHI, BglII, Sphl, Ddel, HindIII, EcoRI, and Rsal, which gave transcripts of 768, 680, 349, 246, and 114 bases in length, respectively. These CoII mRNA derivatives had specific activities of 5,000-30,000 cpm/pmol depending on their lengths. Inosine-containing CoII mRNA transcripts were prepared by using TTP instead of GTP as described above. Preparation of CoII mRNA for use in hybridization reactions was carried out as described (9) except that 0.1 mm [α-32P]UTP (final specific activity of 35 Ci/mmol) was used and the other ribonucleotide triphosphates were added at a final concentration of 0.33 mM.

Mitochondrial Ribosome-mRNA Interactions

Bovine mitochondrial 28 S ribosomal subunits can bind mRNAs to form a 1:1 complex, although neither tRNA nor rRNA can form a similar complex with these subunits (9). The interaction observed does not appear to occur at a defined sequence in the mRNA. We have examined the ability of poly(U) to compete in ribosome binding with the mRNA for CoII mRNA. This mRNA has been synthesized in vitro from a copy of the gene cloned behind the T7 transcriptional promoter (see "Experimental Procedures"). As indicated in Fig. 1, poly(U) competes very effectively with CoII mRNA for binding to 28 S subunits. Assuming that the average length of poly(U) is ~100 nucleotides, we calculate that this synthetic RNA binds to the small subunit with equal or greater efficiency than does CoII mRNA. This observation is in agreement with that of others (10) and suggests that this synthetic polynucleotide is binding to the same site on the ribosome as is CoII mRNA. It should be noted that poly(U) is known to bind very efficiently to ribosomes from a variety of sources. In contrast to poly(U), poly(A,G,U) is not very effective in competing with CoII mRNA for subunit binding (Fig. 1). Gel electrophoretic analysis indicates that the average chain length in our poly(A,G,U) preparations is ~100 nucleotides. Based on this length, we calculate that the binding of poly(A,G,U) to 28 S subunits must be at least 1000-fold weaker than that of CoII mRNA.

Denslow et al. (10) have observed that there is a minimum length of ~18 nucleotides for efficient binding of poly(U) to 28 S subunits. We have tested whether a similar length requirement is observed with other RNAs by preparing transcripts containing CoII mRNA sequences of different lengths and comparing their abilities to bind mitochondrial small subunits. These transcripts were made by cutting the cloned CoII gene at different positions prior to transcription by T7 RNA polymerase (Fig. 2A). Using this method, we obtained a series of RNAs ranging in size from 114 to 785 bases. They are identical at the 5' end, but extend for different distances into the CoII RNA sequence. When these transcripts were tested for their abilities to bind 28 S subunits, the results indicated in Fig. 3 were obtained. Messages of greater than ~500 nucleotides long all bound equally well to ribosomal subunits. However, mRNAs of less than ~400 nucleotides in length showed reduced binding to 28 S subunits, and substantially reduced binding was observed with the 114-base mRNA. Similar results were observed at a variety of mRNA and 28 S subunit concentrations.

The observation described above suggests that there may be a minimum length required for the efficient binding of a natural mRNA such as CoII mRNA to 28 S subunits. Alternatively, one might suggest that there is a recognition site for ribosomal subunits located somewhere between positions 250 and 400 on CoII mRNA. This site might facilitate the initial interaction of the ribosomal subunit with the mRNA. Following entry at this site, the subunit could then slide on the mRNA, giving the final appearance of a random interaction. To distinguish between these two possibilities, we prepared mRNA from a construct that contains 178 nucleotides 5' to the normal start site for the CoII gene (Fig. 2B). mRNA (424 nucleotides) was synthesized from this vector after lineariza-
Tran*cript

the shorter RNA has a lower association constant for binding both short and long mRNAs.

binding is observed. If the mRNA concentration is increased

stant for a shorter derivative (114 nucleotides long) is -1.2 x 10^7 M^-1, whereas the binding con-

on the interaction between the 28 S subunits and mRNAs,

suggests that there is a minimum length for efficient binding

results obtained were precisely those expected for a mRNA of

A

FIG. 2. Origin and structures of CoII mRNA transcripts of different lengths. A, restriction sites used to linearize the cloned CoII gene (9) prior to in vitro transcription are shown along with the sizes of the mRNAs arising from these templates; B, a plasmid carrying sequences 5' to the normal CoII gene oriented for transcription by Sp6 polymerase (9) was restricted at the EcoRI site to produce a message containing 424 bases in length.

B

reaction with EcoRI. This RNA does not contain the region downstream from position 250 in the original CoII message. When this RNA was tested for binding to 28 S subunits, the results obtained were precisely those expected for a mRNA of ~400 nucleotides in length (data not shown). This observation suggests that there is a minimum length for efficient binding of mRNA to 28 S subunits and argues against the idea of an entry site for the initial interaction between the RNA and the ribosomal subunit.

To develop a better understanding of the effects of length on the interaction between the 28 S subunits and mRNAs, we have tested the mRNA binding obtained at a variety of concentrations. As indicated in Fig. 4, when the amount of a short mRNA added to the incubation is increased, enhanced binding is observed. If the mRNA concentration is increased enough, comparable levels of binding can be observed with both short and long mRNAs. This observation indicates that the shorter RNA has a lower association constant for binding to 28 S subunits but that it does have the ability to bind. We estimate that the K_a for the binding of CoII mRNA (686 nucleotides long) is ~5 x 10^8 M^-1, whereas the binding constant for a shorter derivative (114 nucleotides long) is ~1.2 x 10^7 M^-1. These values indicate that this short derivative of

CoII mRNA is ~40-fold less efficient in subunit interaction than is the normal message. The poor binding obtained with poly(A,U,G) described above (Fig. 1) is probably a reflection of this length effect. Since the average chain length in this synthetic RNA is only ~100 nucleotides, it falls short of the optimal length observed with CoII mRNA. It should be noted that the length requirements observed with CoII mRNA and presumably with poly(A,U,G) are quite distinct from those observed with poly(U), where chains of 18 nucleotides or greater are active in binding (10). These observations suggest that poly(U) may bind exceptionally well to mitochondrial 28 S subunits and that its binding may have some characteristics which are not representative of the binding of other RNAs to these subunits.

As indicated above, CoII mRNA and a synthetic polymer such as poly(A,U,G) must be longer than poly(U) for efficient interaction with mitochondrial small ribosomal subunits. One possible explanation for this observation might lie in the difference in the degree of secondary structure between poly(U) and these other RNAs. Poly(U) is thought to have very little secondary structure, whereas poly(A,U,G) and CoII mRNA would be expected to possess some secondary structure. Computer modeling (data not shown) and nuclease probing experiments (10) have indicated that CoII mRNA has a great deal of secondary structure. To test the idea that longer RNAs would be required for 28 S subunit binding if they contained higher degrees of structure, we prepared a series of different lengths of CoII mRNA using inosine triphosphate in place of GTP. Under these conditions, the mRNA produced would contain significantly less secondary structure than the original mRNA, and shorter inosine-containing mRNAs might be expected to bind efficiently to ribosomal subunits. However, as indicated in Fig. 5, inosine-containing CoII mRNA transcripts of various lengths show binding to 28 S subunits which is very similar to that of the corresponding guanosine-containing transcripts. Shorter inosine-containing mRNAs show a reduction in binding comparable to that observed with the normal guanosine-containing messages.
These observations indicate that a reduction in the degree of secondary structure of an mRNA does not significantly affect the length required for its efficient interaction with 28 S subunits.

Previous results (9) have indicated that a 1:1 complex is formed between the mRNA and the 28 S subunit. The apparent requirement for an mRNA of at least 300-400 nucleotides for efficient binding may account for the observation that only one ribosomal subunit is generally present on a mRNA of 700-800 nucleotides. We wished to investigate whether some alteration in an mRNA bound to a 28 S subunit prevented the subsequent binding of additional subunits. To examine this question, we prepared transcripts that were considerably longer than the normal CoII mRNA. These transcripts were obtained by cutting the vector carrying the cloned CoII gene well downstream of the CoII coding region, followed by in vitro transcription. The transcript prepared in this way has the CoII sequence in the 5' half and RNA sequences from the β-galactosidase α-peptide at the 3' end. The RNA produced from this plasmid is 1768 nucleotides long and might be expected to have the ability to bind two or perhaps three small subunits. The number of 28 S subunits binding to this mRNA was investigated in two ways. First, we examined the effect of limiting the 28 S subunit concentration on the amount of the mRNA bound to subunits using the nitrocellulose filter binding assay (9). If more than one ribosomal subunit was able to bind to each mRNA, we would expect that higher subunit concentrations would be required for binding the entire amount of RNA present in the incubation mixtures. However, as indicated in Fig. 6, the same concentration of 28 S subunits can bind both the elongated transcript and the normal mRNA. This observation suggests that only one 28 S subunit is binding to each mRNA. The second approach used was to examine the 28 S subunit-mRNA complexes on sucrose gradients. Complexes containing two or three 28 S particles would be expected to sediment further into the gradient than those containing only one subunit. Sedimentation profiles for complexes containing either 686-base CoII mRNA or the 1768-base elongated transcript were essentially the same (data not shown). Thus, we believe that only one 28 S subunit is capable of interacting with each mRNA. The mechanism by which other subunits are excluded remains an intriguing and perplexing problem that will require further investigation.

O'Brien and co-workers (5) have made the interesting observation that bovine mitochondrial 28 S subunits can bind GTP and GDP and that this binding appears to occur specifically on ribosomal protein S5. We have examined our preparations of 28 S subunits for this nucleotide binding ability and have obtained results comparable to those reported. In our hands, subunit preparations are generally 80-90% active in mRNA binding and 25-40% active in guanine nucleotide binding (data not shown). We have investigated the effects of the presence of GTP or GDP on the binding of CoII mRNA to 28 S subunits. mRNA binding is neither stimulated nor inhibited by the presence of either GTP or GDP (data not shown). In addition, using 32P-labeled GTP, we observed that mRNA binding had no effect on nucleotide binding to 28 S subunits (data not shown). These results suggest that nucleotide and mRNA binding to 28 S subunits are independent events.

Previous results (9) have indicated that the complex formed between the mitochondrial small subunits and CoII mRNA does not occur at a specific site on the RNA. Mitochondrial RNA synthesis is initiated on both the heavy and light strands of DNA, producing primary transcripts that are processed into the appropriate mRNAs, rRNAs, and tRNAs. Hence, the 5' ends of these mRNAs presumably carry a 5'-phosphate (or 5'-OH) group. In contrast, the RNAs prepared by in vitro transcription are initiated with a 5'-triphosphate. We were concerned about the possibility that the presence of this highly charged structure at the 5' end of the mRNA might affect the ability of the small subunit to localize at the translational start codon which is located only 1 residue from the 5' end of CoII mRNA. We have therefore treated CoII mRNA transcripts with phosphatase to produce 5'-OH termini. A portion of this RNA was then incubated with polyribonuclease kinase and ATP to yield 5'-monophosphate groups. The abilities of these RNAs to bind to 28 S subunits were then tested and compared to the normal in vitro transcripts produced. As indicated in Fig. 7, all of these RNAs bind equally well to ribosomal subunits. In addition, we have examined the possibility that the localization of the subunit on the mRNA is affected by the precise structure at the 5' end. For these experiments, fragments of CoII mRNA (containing a 5'-triphosphate or a 5'-OH) that were protected from T1 RNase by the presence of the 28 S subunit were used to probe Southern blots of RsaI fragments of the plasmid containing the CoII gene as described previously (9). RNA-protected fragments appear to arise from a variety of sequences in the mRNA (data not shown). We interpret these results to indicate that the 28 S ribosomal subunit is binding to several and probably many sites on the mRNA. Essentially identical patterns are obtained with mRNA fragments from the 5'-triphosphate RNA and from the RNA containing a 5'-OH (data not shown). These results suggest that the ribosomal subunits are not being excluded from preferential interaction with the start codon at the 5' end of the RNA by the presence of the triphosphate group. Clearly, some other component must be required to position the small subunit at the start site on the mRNA.
DISCUSSION

Considerable work remains to be carried out on the mechanism of translational initiation in animal mitochondria. The data presented here indicate that the interaction between bovine mitochondrial 28 S subunits and mRNAs has a number of features that differ from those of other translational systems. Previous work (9) has shown that the 28 S subunit protects a region of the RNA ~40 nucleotides long from digestion by high levels of T1 RNase. The results provided here indicate that a more extensive stretch of RNA (greater than ~400 bases) is important for the tight association of an mRNA with the small subunit. One might imagine that there is a strong interaction between the 28 S particle and ~40 nucleotides of the mRNA, followed by a wrapping of the mRNA around the subunit. This wrapping process could be relatively independent of the secondary structure of the message, but would require a minimum length. Contacts between the 28 S subunit and portions of the mRNA wrapped around it could serve to stabilize the interaction of the RNA with the subunit and could also result in a compact structure. A highly organized compact structure might be important in mitochondria because of the low aqueous volume of this compartment (15). It has been estimated that there is room for only one to two layers of water molecules between the macromolecules in mitochondria. An interaction in which the mRNA is confined to a small volume near the ribosome by a series of contacts with the outside of the 28 S particle could facilitate the process of translation in this organelle.

The mRNAs in animal mitochondria range in size from ~350 bases to ~1500 bases. The results presented here indicate that mRNAs that are smaller than ~400 nucleotides will interact with 28 S subunits less readily than larger mRNAs. Both of the small open reading frames in animal mitochondria (<300 nucleotides) are present in dicistronic mRNAs (6, 11). We believe that these cistrons have evolved as a part of a dicistronic mRNA to overcome potential problems in the initiation of translation of short mRNAs. One of the most interesting observations emerging from these studies is the apparent exclusion of additional 28 S subunits from mRNAs already interacting with a ribosomal subunit. This phenomenon was observed with a transcript of ~1800 bases in length which corresponds rather well to the longest mRNA present in animal mitochondria. Thus, it appears that the 28 S subunit “marks” an mRNA in some way, perhaps by the wrapping process described above, and prevents the binding of subsequent subunits. This ability would serve to confine the message to the formation of monosomes. It should be noted that polysomes have not been observed in animal mitochondria. We believe that the initial interaction between the 28 S subunit and the mRNA occurs in a sequence-independent manner and is followed by the movement of the subunit to the 5' end of the message and the formation of the initiation complex. We would like to suggest that 28 S subunits must be excluded from mRNAs that are already in the process of translation to prevent the collision of the migrating subunit with the translating ribosome. A general conformation induced in the mRNA upon initial contact with the small subunit could be maintained during elongation and indicates that the mRNA present is not available for interaction with an additional ribosome. Work is currently in progress in an effort to define features of the mRNA that affect interaction with 28 S subunits and to identify translational initiation factors that may play a role in the assembly of the final initiation complex.

REFERENCES
