Mammalian mitochondrial mRNAs are basically leaderless, having few or no untranslated nucleotides prior to the 5′-start codon. We demonstrate here that mammalian mitochondrial 55 S ribosomes preferentially form initiation complexes at a 5′-terminal AUG codon over an internal AUG. The preferential use of the 5′-start codon is also seen on mitochondrial 28 S small subunits, which suggests that mitochondrial translation initiation on leaderless mRNAs does not require the large ribosomal subunit. The selection of the 5′-AUG is dependent on the presence of fMet-tRNA and is enhanced by the presence of the mitochondrial initiation factor IF2mt. In prokaryotes, IF3 is believed to antagonize initiation on leaderless mRNAs. However, IF3mt stimulates initiation complex formation on leaderless mRNAs when tested with 55 S ribosomes. The addition of even a few nucleotides 5′ to the AUG codon significantly reduces the efficiency of initiation, highlighting the importance of the leaderless or nearly leaderless nature of mitochondrial mRNAs. In addition, very few initiation complexes could form on a hybrid mRNA construct consisting of tRNAMet attached at the 5′-end of a mitochondrial protein-coding sequence. This observation demonstrates that post-transcriptional processing must occur prior to translation in mammalian mitochondria.

In contrast to other genomes, the mammalian mitochondrial genome is very compact, containing ~16 kilobase pairs of DNA with very few noncoding nucleotides. This DNA encodes 2 rRNAs, 13 proteins, and 22 tRNAs (1). The 13 proteins are all essential components of the electron transport chain and ATP synthase. They are synthesized by the mitochondrial translational machinery of nine monocistronic mRNAs and two dicistronic mRNAs with overlapping reading frames. Mitochondrial mRNAs contain few or no noncoding nucleotides prior to the 5′-terminal start codon and are largely unstructured at their 5′-ends (2, 3). The exact number of nucleotides prior to the 5′-start codon in mature mitochondrial mRNAs has been determined in both humans and fruit flies. Direct analysis of the 5′-ends of the 11 open reading frames located at the 5′-ends of the human mitochondrial mRNAs demonstrated that post-transcriptional processing completely eliminates the 5′-leader in all but three mRNAs (4). These three mRNAs have one, two, and three nucleotides 5′ to the start codon. None of the 5′-cistrons of Drosophila melanogaster mitochondrial mRNAs begin with noncoding nucleotides (5).

Translation of mitochondrial mRNAs is accomplished by mitochondrial ribosomes, which sediment as 55 S particles and dissociate into 28 S and 39 S subunits. The translation process in mitochondria occurs with the help of two initiation factors: IF2mt promotes the binding of fMet-tRNA to the 28 S small ribosomal subunit, and IF3mt stimulates initiation complex formation by facilitating the dissociation of mitochondrial 55 S ribosomes. No factor homologous to prokaryotic IF1 has been found in mitochondria, and the role of this factor is thought to be carried out by an insertion found in IF2mt (6).

Prokaryotic mRNAs commonly contain a Shine-Dalgarno sequence just upstream of independent open reading frames that hydrogen bonds with nucleotides near the 3′-end of the 16 S rRNA (7). This interaction helps position the mRNA properly on the ribosome for initiation complex formation. Because mitochondrial mRNAs do not contain Shine-Dalgarno sequences, it is unclear how these mRNAs enter the ribosome or how the start codon is positioned in the P-site of the ribosome for initiation.

Cryo-electron microscopy images of the mitochondrial ribosome indicate that mRNAs may enter the 28 S small ribosomal subunit using a unique mRNA entrance gate composed of one or more mitochondrion-specific proteins and the homolog of bacterial ribosomal protein S2 (MRPS2) (8). These proteins come together to form a triangular mRNA entrance site, which may play a role in binding the leaderless, or nearly leaderless, mitochondrial mRNAs.

Prokaryotes also translate leaderless mRNAs, although they are much less common than mRNAs containing Shine-Dalgarno sequences. Although it is generally accepted that prokaryotic translation involving Shine-Dalgarno sequence-containing mRNAs is initiated on the 30 S small subunit, it is believed that initiation of leaderless mRNAs occurs on intact 70 S monosomes in bacteria (9–11). Although bacterial IF3 stimulates initiation complex formation on mRNAs that contain Shine-Dalgarno sequences, it has been shown to reduce translational efficiency on leaderless mRNAs when present in excess (12).

It is generally assumed that protein synthesis in mitochondria, performed using leaderless mRNAs, is comparable with translation of leaderless mRNAs in prokaryotes. However, a recent study on the translation of leaderless mRNAs in prokaryotes indicated that a specialized ribosome lacking a number of proteins (61 S particles) is used in the bacterial system to translate leaderless mRNAs (13). This reduced ribosome shares only three homologous proteins with the mitochondrial ribo-
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some. Thus, the process of translating leaderless mRNAs in mitochondria must differ from that in prokaryotic translation.

In this work, we demonstrate for the first time the preferential selection of a start codon located at the 5'-end of an mRNA by mitochondrial ribosomes and examine the influence of sequences 5' to the start codon in its selection. We also show that initiation of mitochondrial translation can occur on 28S subunits and that IF3<sub>mt</sub> stimulates initiation complex formation using leaderless mRNAs on 55S ribosomes. Taken together, our data indicate that the mechanism start site selection during mitochondrial translational initiation differs significantly from that observed in prokaryotic initiation.

**EXPERIMENTAL PROCEDURES**

**Materials**—General chemicals were purchased from Sigma or Fisher. Bovine mitochondrial ribosomes (55S), ribosomal subunits (28S and 39S), and yeast [35S]<sub>f</sub>Met-tRNA were prepared as described (8, 14–16). IF2<sub>mt</sub> and IF3<sub>mt</sub> were cloned, expressed, and purified as described previously (17). Both proteins were purified by high performance liquid chromatography following nickel-nitrioltriacetic acid chromatography as described.

Cloning Cytochrome Oxidase Subunit I, NADH Dehydrogenase Subunit 2, and tRNAMet/NADH Dehydrogenase Subunit 2 Fragments—The first 151 nucleotides of cytochrome oxidase subunit I (COI)<sup>2</sup> DNA, the first 150 nucleotides of NADH dehydrogenase subunit 2 (ND2) DNA, and the entire tRNAMet coding sequence plus the first 150 nucleotides of ND2 (tRNA<sub>Met</sub>/ND2) were amplified by PCR from plasmid p11-1 (18), which contains bovine mitochondrial DNA from nucleotides 15,743 to 8,788. Primers used for amplification incorporated the sequence for tRNAMet attached at the 5' end of the DNA sequence. The hammerhead ribozyme system was used to generate leaderless mRNAs via self-cleavage prior to the first nucleotide of each sequence (19). The primers also incorporated the XbaI restriction site on the 5'-end of the DNA sequence. The sequences for all primers used and the PCR amplification conditions are provided in supplemental Tables S1 and S2.

Ten different constructs were prepared using the sequence for COI DNA, including 5'-AUG mRNA, AUG<sup>68</sup> mRNA. Both AUG mRNA, 0 AUG mRNA, 1-mer AUG mRNA, 2-mer AUG mRNA, 3-mer AUG mRNA, 6-mer AUG mRNA, 9-mer AUG mRNA, and 12-mer AUG mRNA (see Fig. 1). Three different constructs were prepared using the sequence for ND2, including ND2 5'-AUG mRNA, ND2 AUG<sup>91</sup> mRNA, and ND2 0 AUG mRNA (see Fig. 1). A hybrid mRNA was prepared using the sequence for tRNA<sub>Met</sub> attached at the 5'-end to the first 150 nucleotides of ND2 (see Fig. 1). The resulting PCR products were digested with HindIII and XbaI and ligated into the linearized plasmid pUC19. The University of North Carolina at Chapel Hill Genome Analysis Facility was used to confirm all sequences. Mutations and/or additions to the DNA sequences were made using the QuikChange site-directed mutagenesis kit (Stratagene).

**Synthesis of mRNAs**—Plasmid DNA was linearized by digestion with XbaI, extracted using phenol/chloroform, and precipitated with ethanol before use. In vitro transcription reactions were prepared basically as described (20). Prior to hammerhead cleavage, transcription reactions were diluted five time in cleavage buffer containing 40 mM Tris-HCl (pH 7.6) and 30 mM MgCl<sub>2</sub>. Hammerhead cleavage was allowed to proceed for 1 h at 60°C, at which time 2× RNA load dye (Ambion) was added to the reactions, and the transcribed mRNAs were separated from the cleaved hammerhead fragments by gel electrophoresis using a 7 M urea-8% polyacrylamide gel. Full-length mRNA transcripts were excised from the gel and eluted in RNase free H<sub>2</sub>O (Ambion) for 48 h at 4°C. The concentration of each mRNA was determined by measuring the absorbance at 260 nm. The sequences of all mRNA constructs used are summarized in Fig. 1B.

**Phosphorylation of mRNAs**—COI 5'-AUG and COI AUG<sup>68</sup> mRNAs were phosphorylated in reactions (500 µl) that contained 100 units of T4 polynucleotide kinase (New England Biolabs), 50 µl of 10× T4 polynucleotide kinase buffer (New England Biolabs), 50 µg of bovine serum albumin, 1 mM ATP, and 1.25 nmol of mRNA. Reactions were incubated at 37°C for 1 h and extracted using phenol/chloroform, and the RNA was precipitated with ethanol. Excess ATP was removed from the phosphorylated mRNAs using mini Quick Spin™ RNA columns (Roche Applied Science).

**Initiation Complex Formation on Mitochondrial Ribosomes**—Stimulation of [35S]<sub>f</sub>Met-tRNA binding to mitochondrial 55S ribosomes or 28S subunits was examined using a filter binding assay. Reaction mixtures (50 µl) were prepared as described previously (16, 21) and contained the indicated amounts of mRNA, 70 nM yeast [35S]<sub>f</sub>Met-tRNA, 0.25 mM GTP, 1.25 mM phosphoenolpyruvate, 0.04 units of pyruvate kinase, 80 nM 55S ribosomes or 80 nM 28S ribosomal subunits, and, unless indicated otherwise, saturating amounts of IF3<sub>mt</sub> (0.25 µM) and IF2<sub>mt</sub> (0.15 µM). IF3<sub>mt</sub> was not present in assays using 28S subunits.

The goal of this study was to investigate the translation of naturally occurring mitochondrially encoded mRNAs. To do this, we set out to modify the sequences of the mRNAs as little as possible. Yeast fMet-tRNA<sub>mt</sub> was used because it decodes only AUG as an initiation codon, whereas mitochondrial fMet-tRNA<sub>mt</sub> decodes both AUA and AUG. Mitochondrial mRNAs are very AU-rich and use AUA 80% of the time for methionine codons during elongation. This leads to the presence of many internal AUA codons in the mRNA. To determine the position of the ribosome on the mRNA, it was important to be able to precisely direct the initiator tRNA to a defined position on the mRNA. The use of the mitochondrial initiator tRNA would require alteration of numerous nucleotides in the mRNA. Changing all of the in-frame and out-of-frame AUA codons to other codons would lead to an increase in the GC content of the mRNA and could introduce secondary structure to the otherwise weakly structured mitochondrial mRNAs.
Competition Assays—The ability of COI AUG68 mRNA to compete with COI 5′/H11032-AUG mRNA for binding to mitochondrial 55 S ribosomes was tested in a competition assay. Initiation complex assays using 55 S ribosomes were performed as described above except that mixtures of COI 5′/H11032-AUG and COI AUG68 mRNAs were prepared using 0.1 M 5′/H11032-AUG mRNA at ratios to AUG68 mRNA of 1:0, 1:1, 1:1.5, 1:2, 1:5, and 1:10 prior to addition to the reaction. Reactions were incubated at 37 °C for 10 min and processed as described above.

Toeprints—Binding of mitochondrial 55 S ribosomes to mRNA was examined using a toeprint assay. Reaction mixtures (20 μl) contained 50 mM Tris-HCl (pH 8), 10 mM dithiothreitol, 7 mM MgCl₂, 40 mM KCl, 0.1 mM spermine, 0.25 mM GTP, 1.25 mM phosphoenolpyruvate, 0.04 units of pyruvate kinase, 0.5 mM dNTPs, 0.4 μM fMet-tRNA, 0.4 μM IF2mt, 0.4 μM IF3mt, 0.3 μM mitochondrial 55 S ribosomes, 50 nM 32P-labeled primer, and 50 nM mRNA (COI 5′-AUG, AUG68, Both AUG, or 0 AUG mRNA). The labeled primer (CGTCTCCGAGCA-GAG) was prepared as described previously (3) and gel-purified using a 7 M urea-20% polyacrylamide gel). Toeprint reactions were incubated at 37 °C for 10 min, at which time 200 units of SuperScript III reverse transcriptase (Invitrogen) was added, and reverse transcription was allowed to proceed at 37 °C for 10 min. The reactions were stopped by the addition of 2 μl of 2 M NaOH and then heated for 5 min at 95 °C. The reactions were neutralized by the addition of 29 μl of acid stop mixture (4:25 v/v) mixture of 1 M unbuffered Tris-HCl and stop dye (85% formamide, 0.5× Tris borate/EDTA, 50 mM EDTA (pH 8), bromphenol blue, and xylene cyanol)). One-half of each reaction was then loaded onto a 7 M urea-10% polyacrylamide gel and run at 1550 V for 3 h. At this time, the other half of each reaction was loaded onto separate lanes, and the gel was run at 1550 V for an additional 3 h. The gel was exposed to a PhosphorImager screen overnight and then scanned using a Typhoon Trio⁺ variable mode imager (GE Healthcare). Images were analyzed using ImageQuant software. Control reactions to determine nonspecific enzyme stops were performed in the absence of 55 S ribosomes. Sequencing reactions were performed using 0.5 mM dNTPs, 2 μl of 5× Superscript III reverse transcription buffer (Invitrogen), 0.5 mM dNTP (A or C), 50 nM 32P-labeled primer, and 50 nM mRNA (COI 5′-AUG mRNA). Sequencing reactions were incubated at 52 °C for 10 min, at which time the reactions were stopped by the addition of 1 μl of 2 M NaOH, heated for 5 min at 95 °C, and neutralized by the addition of 14.5 μl of acid stop mixture.

RESULTS

The Mitochondrial Ribosome Discriminates between a 5′-AUG Codon and an Internal AUG Codon—Initial experiments to examine the selection of the start codon at the 5′-ends of mitochondrial mRNAs were carried out using transcripts from the first 150 nucleotides of COI mRNA (Fig. 1). To precisely position the AUG at the 5′-end of this mRNA, transcription was coupled with hammerhead ribozyme cleavage to generate an mRNA with the AUG at the 5′-end (5′-AUG mRNA). Additional variants of this mRNA were prepared containing an internal AUG at position 68 (AUG68), two AUG codons (one at the 5′-end and one at position 68 (Both AUG mRNA), or 0 AUG mRNA). The labeled primer (CGTCTCCGAGCA-GAG) was prepared as described previously (3) and gel-purified using a 7 M urea-20% polyacrylamide gel). Toeprint reactions were incubated at 37 °C for 10 min, at which time 200 units of SuperScript III reverse transcriptase (Invitrogen) was added, and reverse transcription was allowed to proceed at 37 °C for 10 min. The reactions were stopped by the addition of 2 μl of 2 M NaOH and then heated for 5 min at 95 °C. The reactions were neutralized by the addition of 29 μl of acid stop mixture (4:25 v/v) mixture of 1 M unbuffered Tris-HCl and stop dye (85% formamide, 0.5× Tris borate/EDTA, 50 mM EDTA (pH 8), bromphenol blue, and xylene cyanol)). One-half of each reaction was then loaded onto a 7 M urea-10% polyacrylamide gel and run at 1550 V for 3 h. At this time, the other half of each reaction was loaded onto separate lanes, and the gel was run at 1550 V for an additional 3 h. The gel was exposed to a PhosphorImager screen overnight and then scanned using a Typhoon Trio⁺ variable mode imager (GE Healthcare). Images were analyzed using ImageQuant software. Control reactions to determine nonspecific enzyme stops were performed in the absence of 55 S ribosomes. Sequencing reactions were performed using 0.5 mM dNTPs, 2 μl of 5× Superscript III reverse transcription buffer (Invitrogen), 0.5 mM dNTP (A or C), 50 nM 32P-labeled primer, and 50 nM mRNA (COI 5′-AUG mRNA). Sequencing reactions were incubated at 52 °C for 10 min, at which time the reactions were stopped by the addition of 1 μl of 2 M NaOH, heated for 5 min at 95 °C, and neutralized by the addition of 14.5 μl of acid stop mixture.
5′-AUG and Both AUG mRNAs was stimulated as mRNA levels were increased (Fig. 2A). Initiation on the Both AUG mRNA was slightly higher than that on the 5′-AUG mRNA, possibly due to a slight difference in binding affinity to the 55 S ribosome. However, because initiation complex formation was the same within error for both mRNAs using 28 S subunits (see below) and because initiation of translation in mitochondria is thought to occur on 28 S ribosomal subunits, we concluded that the difference in initiation on 55 S ribosomes between the 5′-AUG and Both AUG mRNAs was not significant. In contrast, no initiation complexes were formed on 55 S ribosomes using either the AUG68 or 0 AUG mRNA. This observation indicates that the ribosome discriminates between mRNAs that contain start codons at their 5′-end and those that do not and preferentially forms initiation complexes at AUG codons positioned at the 5′-end.

To examine whether the 28 S subunit alone has the intrinsic ability to select a 5′-AUG codon, initiation assays were also performed using 28 S subunits (Fig. 2B). IF3mt was not present in these assays because it does not affect mRNA-dependent initiation complex formation on 28 S subunits. Initiation on 28 S subunits was stimulated by increasing amounts of 5′-AUG and Both AUG mRNAs, as seen with 55 S ribosomes (Fig. 2B). However, no initiation complex formation on 28 S subunits was seen using the 0 AUG mRNA, and only a small level of initiation complex formation was observed with the AUG68 mRNA (10–15%). These observations indicate that the ability to preferentially use a 5′-AUG over an internal AUG is an intrinsic property of the 28 S subunit. This idea is in agreement with the observation that mRNA binds exclusively to the small subunit (20). However, the ability to initiate on mitochondrial 28 S subunits contrasts sharply with initiation on bacterial leaderless mRNAs, which is believed to occur on intact ribosomes (10).

It is known that mitochondrial mRNAs bind to the 28 S subunit in a sequence-independent manner (20). One model for this binding is that the 28 S subunit binds the mRNA at the mRNA entrance gate, and the mRNA is fed through the gate beginning at the 5′-end. In the absence of a 5′-AUG start codon and fMet-tRNA to trap and stabilize the initiation complex, the mRNA continues to slide through the gate and eventually exits the small subunit. If this model is correct, one might expect that the AUG68 mRNA would be capable of binding the small subunit in a transient fashion and could thus inhibit initiation complex formation on the 5′-AUG mRNA. To test this idea, increasing amounts of AUG68 mRNA were added to initiation complex formation assays using the 5′-AUG mRNA. As indicated in Fig. 2C, the addition of the AUG68 mRNA led to inhibition of [35S]fMet-tRNA binding to 55 S ribosomes in response to the 5′-AUG mRNA. The high levels of the AUG68 mRNA required to compete with the 5′-AUG mRNA for ribosome binding indicate that, whereas mRNAs that lack a start codon are able to bind mitochondrial ribosomes, the binding is not...
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FIGURE 3. Toeprints showing the position of the 55 S ribosome on the mRNA. Sequencing reactions were carried out using ddCTP and ddATP. Nucleotides indicated with arrows are numbered from the 5′-end of the mRNA. A, toeprints showing the position of the 55 S ribosome on the 5′-AUG mRNA. Toeprint assays were performed as described under “Experimental Procedures.” Control reactions were performed in the absence of fMet-tRNA, IF2mt, or 55 S ribosomes as indicated. Asterisks indicate the toeprint signal at nucleotide 17. 8, toeprints illustrating the region of the mRNA where the expected signal from ribosome binding to the internal AUG at position 68 for the AUG68 mRNA would appear. All signals observed in this region either were present in the absence of 55 S ribosomes or were not repeatable. C, toeprints showing the position of the 55 S ribosome on the 5′-AUG mRNA in the presence and absence of IF2mt and fMet-tRNA. Control reactions were performed in the absence of fMet-tRNA, IF2mt, or 55 S ribosomes as indicated. The asterisk indicates the toeprint signal at nucleotide 17.

stable. In contrast, the presence of fMet-tRNA on a 5′-AUG codon strengthens the initiation complex on the ribosome, leading to a very stable complex that cannot be disrupted by the AUG68 mRNA.

Toeprint Analysis of the Mitochondrial 55 S Initiation Complex—To directly analyze the position of the ribosome on the mRNA in the initiation complex, a series of toeprint reactions was performed using COI mRNAs (Fig. 3). In the absence of 55 S ribosomes, a reverse transcriptase stop was seen at position 20 on the mRNA due to a nonspecific stop by the enzyme (Fig. 3A, lane 1). When initiation complexes were assembled with the 5′-AUG mRNA, a clear and repeatable toeprint was seen 17–19 nucleotides from the 5′-end of the mRNA (Fig. 3A, lane 2). This position corresponds to the distance from the edge of the mRNA entrance tunnel to the P-site. This observation agrees with previous data that indicate that prokaryotic ribosomes produce a toeprint signal ~15 nucleotides from the P-site (22). The mitochondrial ribosome is slightly larger in size than its prokaryotic counterpart and is expected to cover a slightly larger section of the mRNA (8). Furthermore, in non-specific complexes between 28 S subunits and mitochondrial mRNAs, a region of ~45 nucleotides of the mRNA was protected from RNase T1 (20). This value represents the distance between the mRNA entrance tunnel and the point where the mRNA exits the small subunit beyond the platform. Because the P-site is close to the center of the small subunit, the toeprint signal observed here is at a reasonable position for a ribosome positioned at the 5′-end of the mRNA.

When the AUG68 mRNA was used in the initiation complex mixture, no toeprint signal was observed from positions 18 to 19 on the mRNA, but a toeprint signal was observed at nucleotide 17 (Fig. 3A, lane 4). This signal depends on the presence of 55 S ribosomes and appears to arise from an intrinsic interaction between the mRNA and the ribosome. The signal may represent the formation of a transient complex in which the 5′-end of the mRNA has been fed into the mRNA entrance gate with the first codon of the mRNA temporarily positioned at or near the P-site. This brief pause may allow the ribosome to inspect the codon at the 5′-end of the mRNA, looking for the AUG triplet.

If the ribosome was bound at the internal AUG at position 68, a toeprint signal would be observed in the region around nucleotide 85. We closely analyzed the toeprint in the region of nucleotides 85–88 for a band pattern that would correspond to the formation of an initiation complex at the internal AUG. At position 87, a small signal was observed with both the 5′-AUG and AUG68 mRNAs. This signal was also present in the absence of 55 S ribosomes, indicating that it is not relevant to initiation complex formation. The signal at position 88 is dependent on the presence of 55 S ribosomes but is present using both the 5′-AUG mRNA, which does not have an AUG at position 68, and the AUG68 mRNA, again indicating that it does not represent the formation of an initiation complex. Thus, the signals observed in this region cannot be attributed to initiation complex formation (Fig. 3B). The lack of a toeprint signal in this region agrees with the lack of initiation complex formation on the AUG68 mRNA. The toeprint with the Both AUG mRNA was the same as that observed with the 5′-AUG mRNA, and a toeprint at nucleotide 17 only was seen using the 0 AUG mRNA (data not shown).

The importance of fMet-tRNA and IF2mt to the ability of the 55 S ribosome to bind specifically to the 5′-end of the mRNA was also examined using the toeprint assay. In the absence of IF2mt and fMet-tRNA, the toeprint signal was drastically reduced (Fig. 3C, lane 3), indicating that both are required to stably position the ribosome at the 5′-end of the mRNA. However, a weak signal was observed at position 17. This signal was identical in appearance to that obtained with the AUG68 mRNA (Fig. 3C, lane 4) and reinforces our hypothesis that the ribosome pauses for inspection of the first codon of the mRNA. The addition of fMet-tRNA in the absence of IF2mt, (Fig. 3C, lane 4) strengthened the toeprint signal and caused a weak signal to be
whether the 5′-phosphate might influence the selection of the 5′-AUG codon, the COI 5′-AUG and AUG68 mRNAs were phosphorylated using polynucleotide kinase and then tested for activity in initiation complex formation. The mitochondrial ribosome showed the same discrimination between the 5′-AUG and AUG68 mRNAs, regardless of the presence of a 5′-OH or a 5′-phosphate (supplemental Fig. S2), suggesting that the presence of a 5′-phosphate on the mRNAs does not affect the preferential use of the 5′-AUG codon.

Effect of Additional Nucleotides 5′ to the AUG on Initiation Complex Formation—In humans, the maximum number of nucleotides preceding a start codon on a 5′-cistron is three, as observed in COI mRNA (4). In the case of the AUG68 mRNA, where the start codon is located 68 nucleotides into the mRNA, no initiation complexes were formed on 55 S ribosomes. Because 68 nucleotides prior to the AUG codon are prohibitive to initiation complex formation, it was interesting to examine exactly how many nucleotides prior to the 5′-AUG could be tolerated. A series of 5′-extended mRNAs was prepared containing 1, 2, 3, 6, 9, and 12 nucleotides prior to the 5′-AUG (Fig. 1). The nucleotides added correspond to the 12 present at positions 18 and 19. In the presence of IF2mt alone, only the signal at position 17 was observed (Fig. 3C, lane 5). These data argue that IF2mt alone does not affect the interaction of the ribosome with the 5′-end of the mRNA. IF2mt does, however, enhance fMet-tRNA binding to the ribosome and stabilize the formation of the initiation complex at the 5′-AUG codon, leading to the strong toeprint at positions 18 and 19. The data also suggest that the additional stabilization of the initiation complex provided by codon/anticodon interactions between the fMet-tRNA and the mRNA is important for the discrimination of the 5′-AUG by the ribosome.

Effect of a 5′-Phosphate on Initiation Complex Formation at a 5′-AUG—Mammalian mitochondrial mRNAs are synthesized as long transcripts and are subsequently cleaved to produce mature tRNAs, tRNAs, and mRNAs (23). This post-transcriptional processing produces a free phosphate group at the 5′-end of the mRNA. All mRNAs studied above were synthesized using in vitro transcription and then subsequently exposed to hammerhead ribozyme cleavage. The mechanism of hammerhead cleavage leaves the mRNAs with a free 5′-hydroxyl group instead of a 5′-phosphate group. To address the question of nucleotides present in the human mitochondrial genome between the upstream cistron and the start codon of the COI mRNA. Three of these are present in the mature mRNA (2). As indicated in Fig. 4A, initiation complex formation was slightly reduced by the addition of a single nucleotide prior to the 5′-AUG. The presence of only three nucleotides preceding the AUG codon led to more extensive decreases in initiation complex formation (>40%). The presence of additional nucleotides prior to the AUG start codon led to further decreases in initiation complex formation, with an 80% reduction in initiation complex formation observed when 12 nucleotides were present 5′ to the start codon. This result indicates that the ribosome is very inefficient in recognizing the start codon of mRNAs with more than six nucleotides 5′ to the AUG.

Preferential Selection of the 5′-AUG Is Observed on Additional Mitochondrial mRNAs—All of the assays performed above used COI mRNA. To ensure that the results obtained were applicable to other mRNAs, a second mRNA, derived from the first 150 nucleotides of ND2, was tested in initiation complex formation. In mammalian mitochondria, both AUG and AUA serve as Met codons. The ND2 mRNA uses an AUA
start codon. Because yeast [35S]fMet-tRNA was used in the initiation complex formation assays performed here and because AUA is not recognized as a start codon in the yeast translational system, the AUG start codon present in ND2 was changed to AUG. Variations of the ND2 mRNA with the 5'-AUG, with an AUG at position 91, or without AUG codons (Fig. 1) were tested in initiation complex formation. The same preferential use of a 5'-start codon by 55 S ribosomes was observed with these mRNAs (Fig. 4B). The ND2 mRNA with the 5'-AUG readily formed initiation complexes; however, no initiation complexes were formed using the ND2 AUG91 or 0 AUG mRNA. This observation suggests that ribosomal discrimination in favor of a 5'-AUG is a general phenomenon for all mitochondrial mRNAs.

**Effect of IF3mt on Initiation Complex Formation Using Leaderless mRNAs**—In the prokaryotic system, IF3 is thought to inhibit initiation complex formation on leaderless mRNAs (12). However, in the mitochondrial system, IF3mt is known to be stimulatory to initiation complex formation using poly(A,U,G) as the mRNA (24). To test whether IF3mt has the same effect on initiation of mRNAs containing more than three to six nucleotides prior to the 5'-start codon, we showed that mitochondrial translation initiation can occur on 28 S subunits and that IF3mt is stimulatory to translation on the ND2 mRNA alone (Fig. 4D). As expected, very few initiation complexes were formed on the tRNA^Met/ND2 mRNA (Fig. 4D), indicating that translation of ND2 occurs following cleavage of tRNA^Met from its 5'-end.

**DISCUSSION**

The sequences of many mammalian mitochondrial genomes are known. DNA sequence analysis has revealed that few or no nucleotides exist prior to the start codon in animals. As mentioned in the Introduction, a maximum of three nucleotides in humans and zero nucleotides in D. melanogaster exist prior to the 5'-start codon. The mechanism of post-transcriptional processing in mitochondria has been proposed as a "tRNA punctuation" model. This theory predicts that mitochondrial DNA is transcribed as a long polycistronic mRNA. The tRNAs are removed from the transcript via post-transcriptional cleavage, and mature mRNAs that are ready for translation remain (25). This model accurately predicts that nucleotides 5' to the start codon for those mRNAs with tRNAs immediately preceding them in human mitochondrial DNA. In this study, we have clearly demonstrated that post-transcriptional processing in bovine mitochondria is necessary prior to translation because initiation of mRNAs containing more than three to six nucleotides prior to the 5'-AUG is very inefficient.

Eukaryotic ribosomes bind to mRNAs in a cap-dependent manner and scan to find the first initiation codon (26). Prokaryotic ribosomes find the AUG start codon with the help of Shine-Dalgarno interactions. In this work, we have demonstrated that mitochondrial ribosomes recognize and bind to the start codon at the 5'-end of mitochondrial mRNAs using primarily codon/anticodon interactions between the fMet-tRNA and the mRNA to drive start codon selection. These interactions are strengthened by the addition of IF2mt, which agrees with the proposed role of IF2mt in promoting the binding of fMet-tRNA to the ribosome. We also showed that mitochondrial translation initiation can occur on 28 S subunits and that IF3mt is stimulatory to initiation complex formation using leaderless mRNAs. Taken together, our data demonstrate that mitochondrial
translation initiation proceeds in a fashion that is distinct from that in the prokaryotic system.

In our current model for initiation in mammalian mitochondria (Fig. 5), IF3mt first interacts with the 55 S ribosome, forming a transient 28 S:IF3mt:39 S intermediate complex (step 1) (24). This transient complex dissociates into free 28 S subunits bound to IF3mt and free 39 S subunits (Fig. 5, step 2). Following the ribosome dissociation step, the mRNA feeds into the mRNA entrance gate on the 28 S subunit (Fig. 5, step 3). It is unclear whether IF2mt:GTP binds at this point or later. It is believed that mRNA binding precedes fMet-tRNA binding because IF3mt has been shown to destabilize the fMet-tRNA bound to 28 S subunits in the absence of mRNA (27). When the first 17 nucleotides have entered the ribosome, the ribosome pauses and inspects the codon at the 5′-end of the mRNA, giving rise to the weak toeprint 17 nucleotides from the 5′-end of the mRNA. This inspection step occurs regardless of the presence or absence of a 5′-AUG. During this pause, IF2mt:GTP can promote the binding of fMet-tRNA to the ribosome (Fig. 5, step 4), and the resulting codon/anticodon interactions between the fMet-tRNA and the 5′-AUG start codon lead to a stable initiation complex. The formation of this complex gives rise to the strong toeprint at positions 17–19. If no codon/anticodon interactions occur due to a lack of fMet-tRNA and/or the absence of a 5′-AUG start codon, the mRNA resumes sliding through the ribosome and eventually dissociates. This idea explains the lack of a toeprint signal at position 18, 19, or 85 on the AUG68 mRNA. If fMet-tRNA binds to the 5′-start codon, the large subunit joins the 28 S initiation complex, IF2mt, hydrolyzes GTP to GDP, and the initiation factors are released, resulting in a full 55 S initiation complex that is then free to move to the elongation phase of protein synthesis (Fig. 5, step 5).

In future work, it will be particularly interesting to determine what creates the pause as the mRNA is fed into the P-site. Because the mitochondrial ribosome is composed of many mitochondrion-specific proteins, it is possible that one or more of these proteins forms a physical barrier to prevent the 5′-end of the mRNA from sliding through the ribosome before the start codon inspection step. The pause for inspection of the codon present at the 5′-end of the mRNA creates a kinetic opportunity for the initiator tRNA to bind to the start codon in the P-site and to provide the codon/anticodon interactions necessary to prevent the mRNA from continuing to slide through the ribosome. Future work is needed to understand the kinetics involved in mRNA pausing and start codon recognition by mitochondrial tRNA Met.

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