MECHANISMS OF TRANSLATIONAL INITIATION AND ELONGATION IN MAMMALIAN
MITOCHONDRIA

by
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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Chemistry.

Chapel Hill
2010

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ABSTRACT

Brooke E. Christian. Mechanisms of Translational Initiation and Elongation in Mammalian Mitochondria
(Under the direction of Linda L. Spremulli)

Initiation of protein synthesis in mammalian mitochondria involves two protein factors: initiation factor 2 (IF2_{mt}) and initiation factor 3 (IF3_{mt}). Mutations were designed in various domains of IF3_{mt} to identify the functionally important regions. Residues 170-171, and 175 in the C-terminal domain nearly abolished activity in initiation complex formation and in 55S ribosomal dissociation. However, these mutated proteins bound to the 28S ribosomal subunit with K_d values similar to the wild-type factor. The results suggest that IF3_{mt} plays an active role in initiation of translation.

Mammalian mitochondrial mRNAs have few or no nucleotides prior to the 5’ start codon. Both the mammalian mitochondrial 55S ribosome and 28S subunit preferentially formed initiation complexes at a 5’ terminal AUG codon over an internal AUG. The selection of the 5’ AUG depended on the presence of fMet-tRNA and was enhanced by the presence of IF2_{mt}. Addition of even a few nucleotides 5’ to the AUG codon significantly reduced the efficiency of translation. In addition, very few initiation complexes could form on a hybrid mRNA construct consisting of the tRNA^{Met} attached at the 5’ end to a mitochondrial protein coding sequence. This observation demonstrates that post-transcriptional processing must occur prior to translation in mammalian mitochondria.
Mutations in the nuclear genes for mammalian mitochondrial translational elongation factors are generally lethal shortly after birth. A mutation of EF-Ts\textsubscript{mt} (R325W) resulted in a significant reduction in the ability of EF-Ts\textsubscript{mt} to bind EF-Tu\textsubscript{mt}. A mutation of EF-Tu\textsubscript{mt} (R336Q) causes infantile encephalopathy arising from defects in mitochondrial translation. The R336Q mutation caused a two-fold decrease ternary complex formation with \textit{E. coli} aminoacyl-tRNA but completely inactivated EF-Tu\textsubscript{mt} for binding to mitochondrial aminoacyl-tRNA.

Polyamines are important in both prokaryotic and eukaryotic translational systems. Spermine stimulated fMet-tRNA binding to mammalian mitochondrial 55S ribosomes in a manner independent of the identity of the mRNA and to the same degree at all concentrations of IF2\textsubscript{mt} and IF3\textsubscript{mt}. The major effect of spermine in promoting initiation complex formation appears to be on the interaction of fMet-tRNA with the ribosome.
Dedicated to C. Clayton Christian
ACKNOWLEDGEMENTS

I will never be able to thank Dr. Linda Spremulli enough for the endless hours she patiently dedicated to my graduate career. Her rigorous analysis of all science taught me to question, and her knowledge of just about everything encouraged me to read. Her perseverance through multiple manuscript drafts taught me that I can always improve on what I’ve already said. Her humility taught me the conservative approach to interpretation of data. It is a true honor to say that I have worked with her.

I would also like to thank the current and former members of the Spremulli lab for their help in this process. Specifically, I want to thank Christie Jones, Emdadul Haque, Domenick Grasso, and Kevin Elmore for providing a great lab atmosphere, for teaching me so much about how to work in the lab, and for their intellectual contributions to my papers and presentations.

Finally, I would like to thank my family (the Agners AND Christians) for their love and support. I am grateful for my wonderful parents, who happily shared both my greatest achievements and pitfalls throughout this journey.

“Listen to the MUSTN’TS, child,
Listen to the DON’TS
Listen to the SHOULDN’TS
The IMPOSSIBLES, the WON’TS
Listen to the NEVER HAVES
Then listen close to me –
Anything can happen, child, ANYTHING can be.”
- Shel Silverstein
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LIST OF ABBREVIATIONS

$A_{260}$, $A_{280}$, or $A_{595}$ absorbance at the indicated wavelength

aa-tRNA amino-acyl tRNA

ADP adenosine diphosphate

ATP adenosine triphosphate

ASD anti-Shine-Dalgarno

BME $\beta$-mercaptoethanol

BSA bovine serum albumin

CD circular dichroism

CoI Cytochrome oxidase subunit I

CoIII Cytochrome oxidase subunit III

cpm counts per minute

cryo-EM cryo-electron microscopy

ddNTP dideoxynucleotide triphosphate

DTT dithiothreitol

EDTA ethylenediaminetetraacetic acid

EF-G elongation factor G

EF-G1<sub>mt</sub> mitochondrial elongation factor G1

EF-G2 elongation factor G2

EF-G2<sub>mt</sub> mitochondrial elongation factor G2

EF-Tu elongation factor Tu

EF-Tu<sub>mt</sub> mitochondrial elongation factor Tu
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
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<tr>
<td>EF-Ts</td>
<td>elongation factor Ts</td>
</tr>
<tr>
<td>EF-Ts&lt;sub&gt;mt&lt;/sub&gt;</td>
<td>mitochondrial elongation factor Ts</td>
</tr>
<tr>
<td>fMet-tRNA</td>
<td>formylated methionyl-tRNA</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine 5’-diphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine 5’-triphosphate</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxy-ethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IF1</td>
<td>prokaryotic translational initiation factor 1</td>
</tr>
<tr>
<td>IF2</td>
<td>prokaryotic translational initiation factor 2</td>
</tr>
<tr>
<td>IF3</td>
<td>prokaryotic translational initiation factor 3</td>
</tr>
<tr>
<td>IF2&lt;sub&gt;mt&lt;/sub&gt;</td>
<td>mitochondrial translational initiation factor 2</td>
</tr>
<tr>
<td>IF3&lt;sub&gt;mt&lt;/sub&gt;</td>
<td>mitochondrial translational initiation factor 3</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>K&lt;sub&gt;d&lt;/sub&gt;</td>
<td>equilibrium dissociation constant</td>
</tr>
<tr>
<td>LB</td>
<td>lauria broth</td>
</tr>
<tr>
<td>LSU</td>
<td>large subunit</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>ND2</td>
<td>NADH dehydrogenase subunit 2</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEP</td>
<td>phospho(enol)pyruvate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>poly(A,U,G)</td>
<td>poly(adenylic, uridylic, guanylic) acid</td>
</tr>
<tr>
<td>RF1</td>
<td>release factor 1</td>
</tr>
<tr>
<td>RF1a</td>
<td>release factor 1a</td>
</tr>
<tr>
<td>RF2</td>
<td>release factor 2</td>
</tr>
<tr>
<td>RRF</td>
<td>ribosome recycling factor</td>
</tr>
<tr>
<td>RRF&lt;sub&gt;mt&lt;/sub&gt;</td>
<td>mitochondrial ribosome recycling factor</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>s</td>
<td>seconds</td>
</tr>
<tr>
<td>SD</td>
<td>Shine-Dalgarno</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SSU</td>
<td>small subunit</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>Tris</td>
<td>tris-(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
</tr>
<tr>
<td>UDP</td>
<td>uridine diphosphate</td>
</tr>
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CHAPTER 1

MECHANISM OF PROTEIN SYNTHESIS

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INTRODUCTION

Mitochondria are small, membrane-bound organelles found in eukaryotic cells that are responsible for the generation of 90% of the cell’s energy in the form of ATP. Mitochondria range in size from 0.5 – 10 µM in diameter. Mitochondria contain an inner membrane that is highly invaginated (Figure 1-1). The folds of the inner membrane create internal compartments known as christae and produce an additional compartment known as the intercristal space. The boundary of the inner membrane is connected to the cristae membrane by narrow, tube-like structures that serve as barriers between the intracristal space and the intermembrane space (2). The production of ATP occurs along the inner membrane, using the electron transport chain and ATP synthase. The electron transport chain uses oxidation/reduction carriers to create a proton gradient across the inner mitochondrial membrane to drive the synthesis of ATP (Figure 1-2). Four multi-subunit protein complexes and several mobile carriers (coenzyme Q and cytochrome c), compose the electron transport chain: Complex I (NADH: ubiquinone oxidoreductase, or NADH dehydrogenase), Complex II (Succinate: ubiquinone oxidoreductase, or succinate...
dehydrogenase), Complex III (ubiquinone: cytochrome c oxidoreductase, or cytochrome reductase) and Complex IV (cytochrome c: oxygen oxidoreductase, or cytochrome oxidase). Although most proteins involved in the synthesis of ATP are synthesized in the cell cytoplasm and imported into the mitochondrion, thirteen are mitochondrially encoded and synthesized (3). These proteins are very hydrophobic, and thus import into the mitochondrion would be difficult.

Figure 1-2. Electron transport chain. Figure obtained from commons.wikipedia.org as a free public domain file. Complexes are indicated by Roman numerals. Q is coenzyme Q, and Cyt c is cytochrome c.
Mitochondria are unique organelles, containing their own genome and protein synthetic machinery. Mammalian mitochondria contain about 16 kilobase pairs of DNA (4). This genetic information encodes 2 ribosomal RNAs (rRNAs), 22 transfer RNAs (tRNAs), and 13 proteins (Figure 1-3). The DNA is circular and continuous; it lacks significant non-coding regions. The proteins encoded in this genome include subunits ND1, ND2, ND3, ND4, ND4L, ND5, and ND6 of the NADH dehydrogenase complex, subunits Col, ColI, and ColII of the cytochrome oxidase complex, cytochrome b from complex III, and ATPase6 and ATPase8 of the ATP synthase complex.

The mitochondrial genome codes for one tRNA for every amino acid and two tRNAs for serine and leucine. The rRNAs coded for by the mitochondrial genome include the 12S rRNA of the small ribosomal subunit and the 16S rRNA of the large ribosomal subunit. Mitochondrial DNA is transcribed into a long, polycistronic RNA, which is then cleaved into the appropriate mRNAs, tRNAs, and rRNAs. The mRNAs are subsequently polyadenylated. DNA sequences that code for the mitochondrial tRNAs exist between
almost all of the mitochondrial protein coding genes. The “tRNA punctuation” model of mRNA processing states that cleavage of the tRNAs from between mitochondrial protein coding sequences results in mRNAs that are ready for translation (5). This model accurately predicts the 5’ ends of human mitochondrial mRNAs that lie in between tRNA sequences, but it does not address the question of how the mRNAs for other proteins are processed. For example, the gene encoding CoIII is preceded by the gene encoding ATP6 instead of a tRNA. Thus, cleavage of the polycistronic RNA precursor to release the CoIII mRNA requires a mechanism distinct from tRNA punctuation.

PROKARYOTIC PROTEIN SYNTHESIS

Mitochondria have many features in common with prokaryotes, including antibiotic sensitivity, and structures and functions of protein factors, tRNAs, and ribosomes. The endosymbiotic theory of mitochondrial origin suggests that a bacterial cell was taken in by another cell, and that the endocytosis of that bacterial cell provided the evolutionary advantage of respiration. Since mitochondria are thought to have arisen from a bacterial cell, the process of translation in that organelle is often compared to translation in prokaryotes.

Structure of the Prokaryotic Ribosome

*Escherichia coli* ribosomes (70S) are complexes of RNA and protein containing 2/3 RNA and 1/3 protein. These ribosomes are 2.3 MDa in molecular mass and are composed of two subunits, a small and large subunit, which sediment at 30S and 50S, respectively.
While the crystal structure of the Thermus thermophilus 70S ribosome has been solved at 2.80 Å resolution (6), the *E. coli* 70S ribosome has only been solved at 3.5 Å resolution (7). The small ribosomal subunit (SSU) has an overall shape similar to that of a bird and contains body, head, neck, beak, and platform regions (Figure 1-4). The SSU consists of 16S rRNA (1542 nucleotides) and 21 proteins, while the large subunit (LSU) consists of 5S rRNA (120 nucleotides), 23S rRNA (2904 nucleotides), and 33 proteins. The SSU is responsible for binding the mRNA and anticodon loop of the tRNA. The LSU is shaped like a glove with three fingers extended. Two of the “fingers” are composed of the LSU proteins L1 and L7/L12, respectively, and the middle “finger” (the central protuberance) is composed of several proteins and the 5S rRNA. The LSU binds the aminoacyl end of the tRNA and is where the peptidyl transferase reaction is catalyzed. The ribosome contains three tRNA binding sites: the A-site, P-site, and E-site. The A-site (aminoacyl-site) is the decoding site where the aminoacyl-tRNAs (aa-tRNAs) bind in response to codons exposed on the mRNA. The P-site (peptidyl-site) holds the peptidyl-tRNA prior to its transfer to the incoming amino
acid. The E-site (exit-site) binds the deacylated tRNAs until their final release from the ribosome.

**Translational Initiation**

Prokaryotic translation can be divided into three stages: initiation, elongation, and termination/ribosome recycling. Initiation of translation in *E. coli* involves three protein factors: initiation factor 1 (IF1), initiation factor 2 (IF2), and initiation factor 3 (IF3). IF1 is an essential 71 amino acid protein whose exact function is unknown (8). IF2 promotes the binding of the initiator tRNA (fMet-tRNA) to the P-site of the 30S ribosomal subunit and promotes the joining of the 30S and 50S ribosomal subunits (9). In *E. coli*, IF3 is thought to have a number of roles. These include (1) dissociating 70S monosomes into subunits by the preferential binding of IF3 to the small ribosomal subunit, (2) promoting the formation of initiation complexes, (3) proofreading initiation complexes by dissociating those with non-canonical start codons, (4) promoting the shift of the start codon on the mRNA into the P-site of the SSU, and (5) mediating the codon/anticodon interactions between the initiator tRNA and the mRNA (10-18).

Many bacterial mRNAs contain a Shine-Dalgarno (SD) sequence in their 5’ untranslated regions. This sequence (AGGAGG), or a variation of this sequence, is located about 8 nucleotides upstream of the start codon, and has been shown to hydrogen bond with the anti-Shine-Dalgarno (ASD) region near the 3’ end of the 16S rRNA. The ASD consists of nucleotides complementary to the SD sequence. The SD/ASD interactions help
specify the start codon and correctly position the mRNA on the ribosome for translation initiation.

In the current model of bacterial initiation, *E. coli* 70S ribosomes are in equilibrium with their subunits, but the equilibrium strongly favors the 70S particle (Figure 1-5). IF3 binds the 30S subunit, preventing its association with 50S subunits. In a random order of binding, IF1 and IF2:GTP, along with the fMet-tRNA and mRNA, bind to the 30S subunit of the ribosome (19). A pre-initiation complex is formed when the SD sequence of the mRNA is recognized and bound by the SSU carrying the fMet-tRNA and all three initiation factors. When codon-anticodon interactions between the AUG start codon of the mRNA and the fMet-tRNA occur, a stable initiation complex is formed. Efficient translation depends on the correct formation of this initiation complex, and initiation complex formation is the rate-limiting step of translation. IF1 and IF3 are released prior to, or at the time of, 50S subunit
binding. The release of IF2 is coupled to GTP hydrolysis triggered by the 50S subunit, and leads to the formation of the 70S initiation complex. This complex then proceeds to the elongation phase of translation.

**Translational Elongation**

Elongation in *E. coli* involves several factors and is represented by three basic steps: selection of the aa-tRNA, peptide bond formation, and translocation. Initially, elongation factor Tu (EF-Tu) binds GTP and the aa-tRNA to form the ternary complex (20). The ternary complex then binds to the A-site of the ribosome. This process is preceded by the binding of EF-Tu to GTP and the aa-tRNA to form the ternary complex. Selection of the correct ternary complex is carried out by codon:anticodon interactions in the A-site. After EF-Tu delivers the aa-tRNA to the ribosome, it is released as an EF-Tu:GDP complex. Elongation factor Ts (EF-Ts) binds to the EF-Tu:GDP complex and promotes the exchange of GDP for GTP to form the regenerated EF-Tu:GTP complex (21). The peptidyl transferase center of the ribosome catalyzes peptide bond formation by transferring the growing polypeptide chain to the amino function of the aa-tRNA in the A-site (21). Elongation factor G (EF-G) catalyzes the translocation step in which the deacylated tRNA and the peptidyl-tRNA are moved from the P-site to the E-site and the A-site to the P-site, respectively, to expose the ribosome to the next codon in the mRNA sequence (1). The activities of *E. coli* EF-G are well studied, and, in most bacteria, this protein acts both during polypeptide chain elongation and termination.
Translational Termination and Recycling

Translational termination begins when the ribosome encounters one of three stop codons (UAA, UAG, or UGA) in the A-site. The stop codon is recognized by one of two release factors: release factor 1 (RF1) recognizes UAA and UAG, and release factor 2 (RF2) recognizes UAA and UGA. Either RF1 or RF2 binds to the A-site of the ribosome and triggers transfer of the peptide chain from the tRNA in the P-site to a water molecule, thus ending protein synthesis. Release factor 3 (RF3) then binds to the ribosome and stimulates the release of the polypeptide chain and either RF1 or RF2.

After translation termination, the ribosomes must be recycled in order to be used for another round of protein synthesis. In prokaryotes, ribosome recycling involves a protein factor termed ribosome recycling factor (RRF). RRF together with EF-G split the 70S ribosome into its subunits, releasing the deacylated tRNA and mRNA (1).

MITOCHONDRIAL PROTEIN SYNTHESIS

Structure of the Mitochondrial Ribosome

The structure of the bovine mitochondrial ribosome has been solved by cryo-electron microscopy to a resolution of 13.5 Å (22). The overall architecture of the mitochondrial ribosome is similar to its prokaryotic counterpart. It is 2.7 MDa in molecular mass and is composed of a large (39S) and small (28S) subunit, which associate to make an intact (55S) ribosome. Mitochondrial ribosomes are composed of 2/3 protein and 1/3 RNA, a ratio that is reversed from that of the prokaryotic ribosome. The mitochondrial rRNA is coded for in the mitochondrial genome, but all mitochondrial ribosomal proteins are
encoded in the nuclear DNA and are subsequently imported into the mitochondria.

Although the mitochondrial ribosome is larger in molecular weight than the prokaryotic ribosome, it has a lower sedimentation value, reflecting the lower rRNA content and a more porous structure. Approximately half of the mitochondrial ribosomal proteins are homologs of those in the prokaryotic system, while the other half are unique to mitochondria.

The SSU is composed of 12S rRNA (950 nucleotides) and 29 proteins (Figure 1-6). It is also larger in size than the prokaryotic SSU. Of the 29 proteins, S2, S5-S7, S9-S12, S14-18, and S21 have sequence homology with the SSU proteins in the bacterial ribosome. Much of the rRNA found in the prokaryotic ribosome is missing, including the ASD region of the 16S rRNA.

The LSU is composed of 16S rRNA (1560 nucleotides) and approximately 50 proteins and is larger in size than its prokaryotic counterpart. It is missing several segments of RNA.
that are present in the bacterial ribosome (Figure 1-6). Twenty-eight of the LSU proteins have sequence homology with the LSU proteins of the bacterial ribosome. Like the bacterial LSU, it is shaped like a glove with the L1 stalk, L7/L12 stalk, and the central protuberance. However, the mitochondrial LSU has an additional protein feature, named the LSU handle that extends from the central protuberance to the main body of the subunit. It is located on the solvent side of the subunit and is hypothesized to function in place of the 5S rRNA found in the bacterial ribosome.

**Mitochondrial mRNAs**

An important difference between bacterial and mitochondrial translation initiation is the process by which the start codon on the mRNA is placed in the P-site of the ribosome. In prokaryotes, the SD sequence upstream of the start codon directs the small subunit to the correct AUG codon as described above (23). Mitochondrial mRNAs are largely leaderless; therefore, the mechanism of the selection of the translational start site must be different from that in prokaryotes.

Leaderless mRNAs, in addition to being translated in mitochondria, are also translated in bacteria and archaea, although each system has unique mechanisms reported for the initiation of leaderless messages. In prokaryotes, it is unclear whether initiation of leaderless mRNAs occurs on intact 70S monosomes or on dissociated 30S subunits (24-26). It is important to note, however, that most of the studies performed in prokaryotes on “leaderless” mRNAs used sequences beginning with “GAUG,” since transcription by T7 RNA
polymerase is substantially more effective when G is the first nucleotide in the transcript. These “GAUG” mRNAs are arguably not actually leaderless.

In the archaeal system, 2/3 of all mRNAs are leaderless, and of the mRNAs that contain leaders, few have SD sequences (27). In contrast to other systems where alternative initiation codons can be used, archaeal leaderless mRNAs are only translated when an AUG start codon is present (28). In addition, introduction of an internal AUG to a leaderless mRNA resulted in translation from both the 5’ AUG and the internal AUG, although it was not determined that initiation occurred on both AUG’s simultaneously (28).

**Mitochondrial Translation Initiation**

Translation of the mRNAs encoded by mitochondrial DNA requires the presence of a protein biosynthetic system that is distinct from that of the cell cytoplasm. Not only are the ribosomes composed of more protein and less rRNA (Figure 1-6), but the initiation factors and tRNAs have some important differences. *E. coli* has separate initiator and elongator tRNAs for methionine, while mitochondria only have one that functions in both processes. Mitochondria also have the ability to use alternative start codons for translation initiation: AUA, AUC, and AUU, in addition to the canonical AUG start codon.

Translation initiation factors have similarities in the bacterial and mitochondrial systems, but several key differences are apparent. Three essential translation initiation factors have been identified in *E. coli*, while only two have been identified in the mitochondrial system. In *E. coli*, IF2 promotes the binding of fMet-tRNA to the P-site of the 30S ribosomal subunit and promotes the joining of the 30S and 50S ribosomal subunits (9).
Mitochondrial IF2 (IF2\textsubscript{mt}) appears to have the same fundamental activities found in its bacterial counterpart. IF1 is an essential protein in \textit{E. coli}, but its precise function is unknown (8). No factor corresponding to IF1 has been identified in mitochondria. However, IF2\textsubscript{mt} has a 37 amino acid insertion that is believed to function in place of IF1 in translation (29). Mitochondrial IF3 (IF3\textsubscript{mt}), like bacterial IF3, stimulates initiation complex formation by providing a pool of free 28S subunits for initiation (Figure 1-7). IF3\textsubscript{mt} has the unique role not present in \textit{E. coli} IF3 of reducing the IF2\textsubscript{mt}-mediated binding of fMet-tRNA to 28S subunits in the absence of mRNA (30). This observation suggests that mRNA binding normally precedes fMet-tRNA binding in the mitochondrial system.

\textit{Mitochondrial Translation Elongation, Termination, and Ribosome Recycling}

Translation elongation and termination in mitochondria are very similar to the respective processes in \textit{E. coli}. Following translation initiation, the elongation step begins when EF-Tu\textsubscript{mt}:GTP delivers the aa-tRNA to the A-site of the ribosome (Figure 1-7). EF-Ts\textsubscript{mt} regenerates the EF-Tu\textsubscript{mt}:GTP from EF-Tu\textsubscript{mt}:GDP. EF-G (EF-G\textsubscript{1 mt} in the mitochondrial system) catalyzes the translocation of the tRNAs and the movement of the mRNA to expose the next codon in the A-site.

Mitochondrial elongation factor Tu (EF-Tu\textsubscript{mt}) is a 45.1 kDa protein that is 56% identical in sequence to \textit{E. coli} EF-Tu (31). EF-Tu\textsubscript{mt} is composed of three domains, of which domain I contains the guanine nucleotide binding site. All three domains are involved in binding to the aa-tRNA (32). Domain 1 of EF-Tu\textsubscript{mt} rotates with respect to domains II and III when GTP is bound in comparison to when GDP is bound, creating the conformation that is
Figure 1-7. Schematic diagram for protein synthesis in mammalian mitochondria. The figure represents the four stages of protein synthesis: initiation is carried out by IF2\textsubscript{mt} and IF3\textsubscript{mt}; elongation by EF-Tu\textsubscript{mt}, EF-Ts\textsubscript{mt}, and EF-G1\textsubscript{mt}; termination is facilitated by RF1\textsubscript{mt}; ribosome recycling requires RRF\textsubscript{mt} and EF-G2\textsubscript{mt}. A possible active role of IF3\textsubscript{mt} is also indicated as an alternative mechanism. Note that only P- and A-sites are shown, since cryo-EM studies suggest that there is no site structurally equivalent to the E-site in mammalian mitochondrial ribosomes (2). Figure obtained with permission from (1).

active in binding the aa-tRNA (33). The binding of EF-Tu\textsubscript{mt}:GTP to mitochondrial Phe-tRNA (76 nM) is not as tight as the interaction between \textit{E. coli} EF-Tu and bacterial Phe-tRNA (~1 nM) (34;35).

Mitochondrial elongation factor Ts (EF-Ts\textsubscript{mt}) is a 30.7 kDa protein that consists of an N-terminal domain and a core domain with N- and C-terminal subdomains (31). EF-Tu\textsubscript{mt} and EF-Ts\textsubscript{mt} interact through an extensive contact surface that includes domains I and III of EF-Tu\textsubscript{mt} and all three domains of EF-Ts\textsubscript{mt} (31;36). EF-Tu\textsubscript{mt} associates with EF-Ts\textsubscript{mt} with a binding constant of 5.5 nM (35).
In contrast to prokaryotic EF-G, which functions in both translation elongation and termination, two forms of EF-G are present in mitochondrial systems. Recent work has provided convincing evidence that the factor designated EF-G1_{mt} catalyzes translocation but is inactive in the ribosome recycling step (37). EF-G2_{mt} works with RRF_{mt} to release the tRNA and mRNA and to dissociate the monosome into subunits. In contrast to EF-G1_{mt}, EF-G2_{mt} is not active in translocation. Thus, EF-G2_{mt} is not biologically equivalent to a traditional translocase, and recently Tsuboi et al. (37) proposed that this factor be renamed RRF2_{mt} to reflect its role in ribosome recycling.

Two forms of the EF-G (EF-G1 and EF-G2) are present in a variety of microorganisms, and both proteins show significant sequence homology to each other. Recent studies suggest that the roles of EF-G2 in bacteria vary from one organism to another. For example, *Mycobacterium smegmatis* EF-G2 shows no ribosome dependent GTPase activity and is unable to catalyze translocation or ribosome recycling (38), while EF-G2 from *T. thermophilus* retains GTPase activity and behaves similarly to EF-G1 (39).

Possible roles of EF-G1_{mt} and EF-G2_{mt} have been investigated in yeast. In this organism, mutations of EF-G1_{mt} lead to impaired activity in mitochondrial protein synthesis *in vivo*, but mutations in EF-G2_{mt} failed to show any clear phenotype (40). Defects in mitochondrial translation have been observed in human patients due to mutations in EF-G1_{mt}, and these defects are generally lethal shortly after birth. No human defects in EF-G2_{mt} have yet been found (41).

When the translation machinery encounters a stop codon, a release factor (RF1a_{mt}) induces the hydrolysis of the newly formed polypeptide from its tRNA (42). Following the
action of the release factor, the 5S complex carrying the deacylated tRNA and mRNA is targeted by RRF\textsubscript{mt} and EF-G2\textsubscript{mt} (43). How the tRNA and the mRNA dissociate from the ribosome is not clear.
REFERENCES


CHAPTER 2

EVIDENCE FOR AN ACTIVE ROLE OF IF3\textsubscript{mt} IN THE INITIATION OF TRANSLATION IN
MAMMALIAN MITOCHONDRIA

The majority of the work reported in this chapter has been published (1;2) and reprinted
with permission.
INTRODUCTION

Mitochondria are involved in a number of human diseases. In particular, dysfunctions in mitochondria and mutations in mitochondrial DNA have been linked to genetic diseases, Alzheimers disease, Parkinsons disease, and other age-related neurodegenerative diseases (3). Before the relationship between mitochondria and disease states can be fully understood, a number of fundamental questions about mitochondrial processes, including mitochondrial gene expression, must be answered.

The mammalian mitochondrial genome contains 16 kilobase pairs of DNA (4), encoding two ribosomal RNAs, 22 transfer RNAs, and 13 proteins. Translation of the mRNAs encoded by mitochondrial DNA requires the presence of a protein biosynthetic system that is distinct from that of the cell cytoplasm. Mitochondrial ribosomes are 55S particles that have about half the rRNA content and twice the protein content of bacterial ribosomes (5). Mitochondrial ribosomal subunits have sedimentation coefficients of 28S and 39S, while bacterial ribosomal subunits have sedimentation coefficients of 30S and 50S and form 70S monosomes.

Following removal of the mitochondrial import signal, IF3_{mt} is a 29 kDa protein composed of three regions that have homology to the bacterial factor: the N-terminal domain, the linker, and the C-terminal domain (Figure 2-1). The N-terminal homology domain is preceded by an extension of 30 amino acids, and the C-terminal domain is followed by an extension of 33 amino acids. Most of the functions of *E. coli* IF3 and IF3_{mt} tested *in vitro* have been localized to the C-terminal domain.
As mentioned in Chapter 1, translation initiation factors have similarities in the bacterial and mitochondrial systems, but several key differences are apparent. Although IF2<sub>mt</sub> seems to serve similar functions as its prokaryotic counterpart, it has a 37 amino acid insertion that is believed to function in place of IF1 in translation (6). Like <i>E. coli</i> IF3, the majority of the functions of IF3<sub>mt</sub> have been localized to the C-terminal domain. Functions of <i>E. coli</i> IF3 include: inducing the dissociation of monosomes by preferentially binding to the small ribosomal subunit; promoting the formation of initiation complexes; proofreading formed initiation complexes and dissociating those with non-canonical start codons; and mediating the interactions between the codon and anticodon. While IF3<sub>mt</sub> is known to promote initiation complex formation, the other functions above have not been demonstrated for IF3<sub>mt</sub>. However, IF3<sub>mt</sub> is thought to contain an additional proofreading function which reduces fMet-tRNA binding in the absence of mRNA. This function is thought to be localized to the C-terminal extension and linker (7;8). This observation
suggests that mRNA binding normally precedes fMet-tRNA binding in the mitochondrial system.

Full length IF3mt is thought to bind on the interface side of the small subunit close to the platform with a $K_d$ of 30 nM (8). The binding constant was determined using both Surface Plasmon Resonance and Microcon centrifugation. The isolated C-terminal domain of IF3mt also has a strong affinity for the 28S subunit and binds with a $K_d$ of 95 nM (8). The isolated N-terminal domain of E. coli IF3 has no detectable binding to the 30S ribosomal subunit (9). This domain of IF3 is thought to increase the affinity of the intact IF3 protein for the 30S subunit by two orders of magnitude. In contrast, the isolated N-terminal domain of IF3mt binds to the 28S subunit with a $K_d$ of 390 nM (8). The N- and C-terminal extensions of IF3mt are not required for binding of the protein to the small subunit, and removal of the extensions has almost no effect on the binding constant (10). However, the C-terminal extension, along with the linker, plays a role in preventing fMet-tRNA binding to the 28S subunit in the absence of mRNA (8).

Native IF3mt has never been detected or purified from mammalian mitochondria. This observation probably reflects the low abundance of IF3mt in mitochondria. Further, detection of the cDNA for IF3mt in the EST databases is a challenge due to the relatively low sequence conservation of this factor (20-25 % identity to prokaryotic IF3) (11). Hence, MitoProtII was used to predict the cleavage site for the removal of the mitochondrial import signal (12). This program predicted an import signal of 31 amino acids, giving a 247 amino acid mature protein. This factor is predicted to have a molecular mass of 29 kDa after removal of the predicted mitochondrial import sequence.
MATERIALS AND METHODS

Materials

Laboratory supplies and chemicals were purchased from Sigma-Aldrich or Fisher Scientific. A rabbit polyclonal primary antibody to the region of IF3_{mt} homologous to the bacterial factors was prepared as previously described (7). Bovine mitochondrial ribosomes (55S), ribosomal subunits (28S and 39S), bovine IF2_{mt}, and yeast [{^35}S]{fMet-}tRNA were prepared as described (2;13;14).

Preparation of IF3_{mt}

An EST encoding human IF3_{mt} was obtained from American Type Culture Collection and the region encompassing the mature protein (amino acids 32 to 278) was previously cloned into pET-21c(+) using the NdeI and XhoI restriction sites (11). This vector provides a C-terminal His\textsubscript{6}-tag. This construct was transformed into E. coli BL21(DE3) carrying the pArgU218 plasmid to supply the isoacceptor of tRNA\textsuperscript{Arg\textsubscript{Arg}} recognizing the AGA and AGG codons. Twelve mutated derivatives of IF3_{mt}, designated IF3_{mt}:1-12 (Table 2-1), were prepared by site-directed mutagenesis using the primers listed in Table 2-2 and the QuikChange site-directed mutagenesis protocol (Stratagene). All mutations were verified by DNA sequencing. The mutated plasmids were transformed into E. coli BL21 RIL cells (Stratagene).
Mutation | Location | Residues Mutated | Residues Changed to |
--- | --- | --- | --- |
IF3<sub>mt</sub>:1 | N-domain | KKTKK (66-70) | AATAA |
IF3<sub>mt</sub>:2 | N-domain | TSTE (121-124) | AAAA |
IF3<sub>mt</sub>:3 | Linker | REMEK (143-147) | AAMAAA |
IF3<sub>mt</sub>:4 | C-domain | KKK (184-186) | AAA |
IF3<sub>mt</sub>:5 | C-domain | HD (170-171) | AA |
IF3<sub>mt</sub>:6 | C-domain | K (175) | A |
IF3<sub>mt</sub>:7 | C-domain | K (194) | A |
IF3<sub>mt</sub>:8 | C-domain | EE (207-208) | AA |
IF3<sub>mt</sub>:9 | C-extension | EE (247-248) | AA |
IF3<sub>mt</sub>:10 | C-extension | KE (252-253) | AA |
IF3<sub>mt</sub>:11 | C-extension | DT (261-262) | AA |
IF3<sub>mt</sub>:12 | C-extension | KD (265-266) | AA |

Table 2-1. Summary of the mutations prepared in IF3<sub>mt</sub>.

Cells were grown to an A<sub>595</sub> of 0.6 in Lauria Broth (LB) media containing ampicillin (100 µg/mL) and chloramphenicol (34 µg/mL), at which time the expression of IF3<sub>mt</sub> was induced using 50 µM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were allowed to induce either overnight at 25 °C or for six h at 37 °C. Similar yields were obtained from cells grown under either of these conditions. After induction, cells were harvested by centrifugation at 5,000 rpm (7,300 g) for 30 min at 4 °C in a Sorvall RC3B centrifuge using 1 L swinging buckets in the H-6000A rotor. The cell pellets were resuspended in buffer containing 50 mM Tris-HCl (pH 7.8), 50 mM NH₄Cl and 10 mM MgCl<sub>2</sub> and collected again by centrifugation at 7,000 rpm (5,856 g).
for 30 min in an SS34 rotor. The cell pellets were carefully drained and weighed. At this point, the cells could be stored at -70 °C after being fast-frozen in a dry ice isopropanol bath. The yield of cells was generally around 5 g/L of media. Cells were resuspended in ice cold lysis buffer (10 mL buffer per g of cell pellet) containing 50 mM HEPES-KOH (pH 7.8), 7 mM MgCl₂, 10 % glycerol, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 100 mM KCl, 6

<table>
<thead>
<tr>
<th><strong>IF₃⁻mt:</strong></th>
<th><strong>Forward Primer</strong></th>
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<tbody>
<tr>
<td>1a</td>
<td>GACAAAGGCGAATAAAAACAGCTTTTAGTAACG</td>
</tr>
<tr>
<td>1b</td>
<td>AGACAGGCAGCAATAAAAACAGCTTTTAGTAACG</td>
</tr>
<tr>
<td>1c</td>
<td>CACCCAGAATGAAGGAGCCTTTTAGTAACGCAG</td>
</tr>
<tr>
<td>1d</td>
<td>GAATGAAAGGACGGAGCAGAGCAGCAGCAG</td>
</tr>
<tr>
<td>2</td>
<td>GGTTCAAAGGACGCACGCACACCTGCAAGCTAT</td>
</tr>
<tr>
<td>3a</td>
<td>CAGAGCTGCGATGAGCGCCACCTGCAAGCTAT</td>
</tr>
<tr>
<td>3b</td>
<td>GCTGGATGGGCTGCGCCACCTGCAAGCTAT</td>
</tr>
<tr>
<td>4</td>
<td>GCAGTGGATGCGGCACACCTAGTC</td>
</tr>
<tr>
<td>5</td>
<td>TCTTCAAATTGAGCAAGCTGCTTGGACACAAAGACTAAACAG</td>
</tr>
<tr>
<td>6</td>
<td>GATTTGGACACAGCGACTAAACAGATTCAG</td>
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<tr>
<td>7</td>
<td>CACATATTACCAGCCGAGGAGAAAAATGTAAGCG</td>
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<td>8</td>
<td>GTTCAGAAAATGGAATGCGGCGATATTTCATC</td>
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<td>9</td>
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</tr>
<tr>
<td>10</td>
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</tr>
<tr>
<td>11</td>
<td>GACCCAGAAGGAGCGCTTTGAACAAAGACC</td>
</tr>
<tr>
<td>12</td>
<td>GAGACACTTGGACGCAGCCATGGAAATGATAAGG</td>
</tr>
</tbody>
</table>

Table 2-2. Primers used in preparation of IF₃⁻mt constructs. Mutated nucleotides are highlighted in gray. The reverse primers used were the inverse complements of the forward primers in each case. To make the four amino acid mutations in IF₃⁻mt:1, four sequential mutations were required using primers 1a-1d sequentially. To make the four amino acid mutations in IF₃⁻mt:3, two sequential mutagenic reactions were carried out using primers 3a and 3b as indicated.
mM β-mercaptoethanol (BME), 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 0.8 % Triton X-100 and sonicated for seven pulses (10 s on, 50 s off). Solid NH₄Cl was added to a final concentration of 0.5 M over a period of 15 min while stirring on ice. Cell debris was removed by centrifugation at 15,000 rpm (27,000 g) for 30 min in a Sorvall SS34 rotor at 4 °C. The supernatant was retained. Ni-NTA agarose was equilibrated in Ni-NTA wash buffer containing 50 mM Tris-HCl (pH 7.8), 7 mM MgCl₂, 10 % glycerol, 1M KCl, 10 mM imidazole, 7 mM BME, and a 50 % slurry in this buffer was prepared. This slurry (1 mL/2 L cell culture) was added to the cell lysate and the sample was mixed for 30 min at 4 °C using a test tube rocker. This mixture was then sequentially transferred to a (5 mL) polypropylene column (Qiagen). The column was washed with 100 mL of Ni-NTA wash buffer, after which the protein was eluted with six sequential 1 mL aliquots of Ni-NTA elution buffer containing 50 mM Tris-HCl (pH 7.8), 7 mM MgCl₂, 10 % glycerol, 40 mM KCl, 150 mM imidazole, 7 mM BME. Each aliquot of Ni-NTA elution buffer was allowed to sit on the column for 5 min before being collected. All fractions were combined and dialyzed against one hundred volumes of ice cold dialysis buffer (Buffer I) containing 20 mM HEPES-KOH (pH 7.6), 10 mM MgCl₂, 50 mM KCl, 10 % glycerol, and 6 mM BME for 2 h, with a change of Buffer I after 1 h.

*Purification of IF3 mt by High Performance Liquid Chromatography (HPLC)*

The IF3 mt preparation has one major contaminant following the Ni-NTA step. The contaminant is a 19 kDa C-terminal fragment of IF3 mt (11). This contaminant often represents 30-40 % of the total protein in the sample. Due to the presence of this contaminant, a second step of purification was carried out using HPLC or gravity
chromatography. For the HPLC method, the partially purified IF3\textsubscript{mt} (~3 mg in ~5 mL) prepared from 2 L of cell culture was dialyzed against a 100-fold excess of Buffer I for 1 h. The dialyzed sample was filtered through a 0.45 μm filter and applied at a flow rate of 0.6 mL/min to a TSKgel SP-5PW column (7.5 cm x 7.5 mm, Tosohaas Inc., Japan) that was equilibrated in Buffer I containing 150 mM KCl. The column was washed until the absorbance at 280 nm returned to baseline and was then developed with a linear gradient (40 mL) from 0.15 M to 0.55 M KCl in Buffer I at a flow rate 0.5 mL/min. Fractions (0.5 mL) were collected using a Shimadzo fraction collector into Eppendorf tubes from which the lids had been removed and that were balanced in 100 x 13 mm glass test tubes. Fractions containing full-length IF3\textsubscript{mt} were identified by electrophoresis on 15 % SDS-polyacrylamide gels. These fractions were pooled, fast-frozen in a dry ice isopropanol bath and stored at -70 °C.

Purification of IF3\textsubscript{mt} by Gravity S-Sepharose Column

Since HPLC equipment is not standard in all laboratories, we also developed a gravity chromatographic method for the purification of IF3\textsubscript{mt}. In this procedure, the dialyzed sample (~5 mL, ~3 mg from 2 L cell culture) from the Ni-NTA column was applied to an S-Sepharose column (8 mL, 6.5 cm x 1.75 cm) equilibrated in Buffer I containing 250 mM KCl. The column was developed with a linear gradient (40 mL) from 250 mM to 550 mM KCl in Buffer I. Fractions (0.75 mL) were collected at a flow rate of 1 mL/min while the absorbance at 280 nm was monitored using an Isco UA-6 UV-Visible detector at an absorbance scale setting of 0.5 and a 5 mm optical flow cell. Fractions containing the 29
kDa mature form of the tagged IF3$_{mt}$ were identified by SDS-polyacrylamide gel electrophoresis. The S-Sepharose column could be reused. Before reuse, it was washed with ten volumes of Buffer I containing 550 mM KCl, followed by ten volumes of Buffer I containing 1M KCl. It was then equilibrated in Buffer I containing 250 mM KCl. The column was allowed to sit overnight in this buffer and then washed again briefly with Buffer I containing 250 mM KCl before use.

The IF3$_{mt}$ eluted from the S-Sepharose column described above still contained small amounts of the 19 kDa fragment and other minor contaminants. Fractions containing the 29 kDa band were combined, diluted 1.5-fold with Buffer I and dialyzed against 100 volumes of ice cold Buffer I at 4 °C for two h with a change of buffer after one h. The dialysate was applied to the S-Sepharose column again. The column was developed with a 40 mL linear gradient from 250 to 550 mM KCl in Buffer I and the absorbance at 280 nm was monitored as described above. Aliquots of various fractions were analyzed by SDS-polyacrylamide gel electrophoresis on 15 % gels for the presence of IF3$_{mt}$. The second run through the S-Sepharose column eliminated the 19 kDa fragment and had a negligible effect on activity. Fractions containing full-length IF3$_{mt}$ were then combined, dialyzed against 100 volumes of ice cold Buffer I at 4 °C for two h with a change of buffer after one h, and then stored at -70 °C until further use.

Assay of IF3$_{mt}$ on E. coli Ribosomes

The standard assay (0.1 mL) contained 50 mM Tris-HCl (pH 7.8), 60-80 mM KCl, 5 mM MgCl$_2$, 0.25mM GTP, 1 mM phospho(enol)pyruvate (PEP), 0.9 unit pyruvate kinase, 1
mM dithiothreitol (DTT), 12.5 µg poly(A,U,G), 0.055 µM (5.5 pmol) \([^{35}S]\)fMet-tRNA (60,000-70,000 cpm/pmol), 0.4 µM \(E.\ coli\) 70S ribosomes, a saturating amount (0.1 µM, 10 pmol) of IF2\(_{mt}\), and the desired amount of IF3\(_{mt}\) generally ranging from 0 to 0.1 µM (0 to 10 pmol). Samples were incubated at 37 °C for 15 min. After incubation, each sample was rapidly diluted with 3-4 mL of cold Mg\(_5\) Wash Buffer containing 50 mM Tris-HCl (pH 7.8), 80 mM KCl, and 5 mM MgCl\(_2\), and filtered through a nitrocellulose membrane that was wetted with cold Mg\(_5\) Wash Buffer. Filtration was facilitated by use of a gentle vacuum provided by a pump. The filters were washed with 3 aliquots (3-4 mL each) of cold Mg\(_5\) Wash Buffer with the vacuum on. Background binding (generally 0.2-0.5 pmol) obtained in the absence of IF3\(_{mt}\) was subtracted from each value. One unit is defined as the binding of 1 pmol of \([^{35}S]\)fMet-tRNA to ribosomes under the assay conditions described above. The specific activity of the purified protein was about 1,700 units/mg when tested on 70S ribosomes. The total yield of purified protein was about 2 mg from 2 L cell culture.

**Assay of IF3\(_{mt}\) on Bovine Mitochondrial Ribosomes**

Reaction mixtures (0.1 mL) contained 50 mM Tris-HCl (pH 7.6), 0.1 mM spermine, 35 mM KCl, 4.5 mM MgCl\(_2\), 0.25 mM GTP, 1 mM DTT, 1 mM PEP, 0.9 unit pyruvate kinase, 12.5 µg poly(A,U,G), 0.42 µM IF2\(_{mt}\), 0.06 µM \([^{35}S]\)fMet-tRNA, 0.04-0.06 µM (about 4-6 pmol) mitochondrial 55S tight couples (1 A\(_{260}\) unit is 32 pmol of 55S ribosomes), 0.04 µM \([^{35}S]\)fMet-tRNA, a saturating amount of IF2\(_{mt}\) (about 0.1 µM, 10 pmol) and the desired amount of IF3\(_{mt}\) (generally 0.005 to 0.1 µM). These initiation complex formation assays were incubated at 37 °C for 15 min and processed as described above using Mg\(_5\) Wash
Buffer in which the concentration of KCl was reduced to 40 mM. All of the values reported were corrected for the amount of radioactivity retained on the filters in the absence of IF3\textsubscript{mt} (about 0.1 pmol).

**Preparation of Mutated Derivatives of IF3\textsubscript{mt}**

The IF3\textsubscript{mt} derivatives were purified using the methodology listed above for the purification of wild-type IF3\textsubscript{mt} (IF3\textsubscript{mt}:WT). For use in assays, the derivatives were dialyzed in Buffer 1 for 2 h with a change of buffer after 1 h. Alternatively, the mutated derivatives were dialyzed in 50 mM phosphate buffer (pH 7.6) for 4 h with a change of buffer after 2 h. The mutated derivatives in phosphate buffer were tested for structural integrity by comparing the α-helical content present determined by circular dichroism (CD) to that of IF3\textsubscript{mt}:WT using an Aviv Model 62DS CD Spectrometer. IF3\textsubscript{mt}:1 was excluded from CD measurements due to the fact that it failed to separate from its truncated 19 kDa fragment during HPLC purification.

**Preparation of Polynucleotide Phosphorylase**

Initiation complex assays were carried out using poly(A,U,G) as the mRNA. Since this polymer is no longer commercially available, it was prepared using polynucleotide phosphorylase. The gene encoding *E. coli* polynucleotide phosphorylase was previously cloned into the pET11a plasmid in *E. coli* BL21 (DE3) pLysS cells and was a generous gift from Dr. George H. Jones (Dept. of Biology, Emory University, Atlanta, GA) (15). The enzyme was purified as described (15). To follow the enzyme during purification, activity
assays were carried out. Reaction mixtures (100 µL) contained 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 5 mM [³H]ADP (7 cpm/pmol), and 20 µL of appropriate fractions. The reactions were incubated at 37 °C for 20 min, at which point 500 µg bovine serum albumin (BSA) was added, and the reactions were precipitated with cold 5% trichloroacetic acid (TCA) (16). The precipitate was collected by filtration using impure nitrocellulose filters (Millipore). The amount of polymerized [³H]ADP was quantitated using a scintillation counter. Following final purification of the enzyme, fractions with ADP polymerization activity were pooled and dialyzed against 50% ammonium sulfate overnight without stirring at 4 °C. The enzyme was stored in 1 mL aliquots at -20 °C.

Synthesis and Purification of Poly(A,U,G)

A 10 mL reaction containing 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 5 mM ADP, pH 7.0, 5 mM UDP, pH 7.0, 5 mM GDP, pH 7.0, and 1 mg polynucleotide phosphorylase was incubated at 37 °C overnight. Following synthesis, the reaction mixture was extracted using phenol (pH 8.0)/chloroform, and the RNA was precipitated with ethanol overnight at -20 °C. RNA was collected by centrifugation at 15,000 rpm in the Sorvall SS-34 rotor for 30 min. The pellets were resuspended in 5 mL sterile H₂O and applied to a Sephadex G-50 column (74 cm x 1.1 cm) equilibrated in 3 mM EDTA, pH 8.0, and 25 mM Tris-HCl (pH 7.8). The column was developed with 150 mL of the same buffer, and 1 mL fractions were collected at a flow rate of 1 mL/min. UV absorbance was monitored at 260 nm. Fractions containing poly(A,U,G) were pooled and precipitated using 2.5 volumes 100% ethanol overnight at -20 °C. The poly(A,U,G) was collected by centrifugation at 15,000 rpm for 30 min in the SS-34
Rotor and stored at -20 °C. Before use, the pellets were dissolved in sterile H₂O to a final concentration of 13 µg/µL.

Quantitation of the Binding of IF₃₇ and Its Derivatives to Mitochondrial 28S Subunits Using Microcon Centrifugation

Ribosome binding reactions were carried out as described (10) in Binding Buffer (100 µL) which contained 10 mM Tris-HCl (pH 7.6), 40 mM KCl, 7.5 mM MgCl₂, 1 mM DTT, and 0.1 mM spermine. Reactions also contained 50 nM 28S subunits (5 pmol) and IF₃₇ (0.15-1 pmol, 1.5-10 nM) or its derivatives. The reactions were incubated for at 25°C for 20 min, at which time the reaction mixtures were added to the Microcon spin columns (Millipore) and centrifuged at 12,000 rpm (10,900 g) for 2 min. Binding Buffer (100 µL) was used to wash the Microcon columns, using centrifugation as described above to collect the Binding Buffer. To recover the IF₃₇ bound to 28S subunits (retained on the filter), 100 µl Binding Buffer was added to the column, the column was inverted, and then centrifuged for 1 min as described above. IF₃₇ was released from the ribosome by the addition of EDTA to a final concentration of 20 mM. The sample containing the bound IF₃₇ was applied to a dot blot apparatus (Bio-Rad). The amount of IF₃₇ bound to 28S subunits was determined colorimetrically using a dot blot probed with antibodies against IF₃₇ as described previously (10). A control curve for analysis of the dot blot was prepared by spotting various concentrations of IF₃₇ (0.5-1.2 pmol, 5-12 nM), 28S (5 pmol, 50 nM), and EDTA (20 mM) directly onto the dot blot nitrocellulose membrane to quantify the change in color versus the amount of protein. A blank value corresponding to the color change of the
membrane in the absence of IF3\textsubscript{mt} was subtracted from each value. The calibration curve was linear over this range. The Image Quant software program was used to calculate the number of pixels on the dot blot membrane.

**Dissociation of Mitochondrial 55S Ribosomes by IF3\textsubscript{mt} and its Mutated Derivatives**

Mitochondrial 55S ribosomes (8 pmol, 0.08 µM) were incubated in a buffer (100 µL) containing 50 mM Tris-HCl (pH 7.6), 5 mM MgCl\textsubscript{2}, 40 mM KCl, and 1 mM DTT in the absence or presence of 80 pmol (0.8 µM) IF3\textsubscript{mt} or its mutated derivatives at 37 °C for 15 min. The samples were placed on ice for 10 min and then loaded onto 10-30 % sucrose gradients prepared in the buffer above and analyzed as previously described by monitoring the A\textsubscript{254} during fractionation of the gradients (10). Each 55S ribosome sample contained some free 39S subunits. Therefore, the percentage of 55S particles was determined by comparing the area of the 55S peak on the absorbance profile to the total area of the 28S, 39S, and 55S peaks combined to account for this contamination. Areas under the peaks were determined using the width of the peak at ½ the maximal height multiplied by the height. To determine the percentage dissociation, the amount of 55S particles in the absence of IF3\textsubscript{mt} was set to 100% and compared to the amount of 55S particles observed in the presence of IF3\textsubscript{mt}.

**Inhibition of fMet-tRNA Binding to 28S Subunits by IF3\textsubscript{mt} in the Absence of mRNA**

The inhibition of [\textsuperscript{35}S]fMet-tRNA binding to 28S subunits in the absence of mRNA was tested as described (7). Small subunits (6.2 pmol, 0.062 µM) were incubated under the
assay conditions described for mitochondrial ribosomes above with saturating amounts of IF2_{mt} (25 pmol, 0.25 µM) and [^{35}S]fMet-tRNA (5 pmol, 0.05 µM) in the presence or absence of IF3_{mt} (0-12 pmol, 0-0.12 µM) or its mutated derivatives for 15 min at 25 °C in 100 µL reaction mixtures. The amount of [^{35}S]fMet-tRNA remaining bound to the subunits was determined using a nitrocellulose filter binding assay (17).

RESULTS

Design of IF3 Mutations

A model of the region of IF3_{mt} homologous to the bacterial factor was developed using the modeling program Insight II (Figure 2-2). The N-terminal homology domain was modeled after the crystal structure of the Bacillus stearothermophilus IF3 (PDB coordinates 1TIF (18)). This region of IF3 folds into a mixed 4-stranded β-sheet packed against a single helical unit (Helix 1) lying across the sheet and a second α-helix (Helix 2) extending into the linker region. The model of the N-terminal homology domain of IF3_{mt} is quite similar to that of the crystal structure of the B. stearothermophilus factor despite the low percent identity.
(<23 %) between these domains. The C-terminal homology domain was modeled after the NMR structure of the mouse IF3mt (PDB coordinates 2CRQ, to be published) which has been shown to consist of two α-helices (H3 and H4) lying on top of a 4-stranded β-sheet. The sequence of the C-terminal homology domain of the human factor is 72 % identical to that of mouse IF3mt. The predicted structure of this domain of human IF3mt is quite similar to that of the mouse factor, except that one of the β-strands is modeled in two sections due to the presence of an internal proline residue. In the model, the linker region is depicted as being partially α-helical. However, biochemical studies of the linker regions of IF3 from several sources suggest that this region has considerable flexibility (8;19-21). The model does not include the N- and C-terminal extensions, which were predicted to be disordered.

Mitochondrial 55S ribosomes are composed of approximately 2/3 protein and 1/3 RNA. The interface between the 28S and 39S subunits is rich in RNA (5). Significant electrostatic interactions between IF3mt (pI ~10) and the 12S small subunit rRNA are expected to play an important role in the function of this factor. To identify residues in IF3mt potentially involved in binding to the small subunit or in other functions of this factor, clusters of charged amino acids predicted to be exposed on the surface in the model of IF3mt (Figure 2-3) were changed to alanine (Figure 2-4). Twelve different mutated derivatives (IF3mt:1-12) were made in the N- and C-terminal domains, the linker, and the C-terminal extension. The charges of these residues are generally conserved among vertebrate IF3mt, and partially conserved with the mosquito, fruit fly, and bacterial factors (Figure 2-4). No mutations were made in the N-terminal extension, because this region is
poorly conserved and no known function has been attributed to the N-terminal extension alone.

Figure 2-3. Model of IF3\textsubscript{mt} showing IF3\textsubscript{mt}:1-8. The N-terminal domain is shown in red, the linker is in green, and the mutated residues are in blue. IF3\textsubscript{mt}:1 could not be modeled based on the crystal structure of \textit{B. stearothermophilus} because these residues are not present in this IF3 nor are they present in the NMR structure of the N-domain of \textit{E. coli} IF3 (2;3). IF3\textsubscript{mt}:1 has been placed in the model at the N-terminus of the protein for illustrative purposes. The C-terminal homology domain is shown in blue and the mutated residues are shown in orange.

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<th>3</th>
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<td>EKK</td>
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<td>K</td>
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<td>EE</td>
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<td>DA</td>
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Figure 2-4. Conservation of the charged residues mutated in IF3\textsubscript{mt}:1-12. Positively charged residues are shown in dark gray and negatively charged residues are shown in light gray. The numbers above each cluster of residues indicate the IF3\textsubscript{mt} derivative containing mutations of those residues. \textit{B. stearothermophilus} IF3 does not have residues corresponding to IF3\textsubscript{mt}:1. These residues are present in \textit{E. coli} IF3 as KRVQT.
The N-terminal domain of *E. coli* IF3 does not bind independently to 30S subunits (9). In contrast, the N-terminal domain of IF3<sub>mt</sub> binds 28S subunits with a $K_d$ of 390 ± 60 nM (8). Two clusters of residues in the N-terminal domain were mutated (Figure 2-3). The selected residues were fairly well conserved among the mammalian factors (Figure 2-4) but less conserved with the IF3<sub>mt</sub> of other organisms. IF3<sub>mt</sub>:1 is located five residues into the N-terminal domain of IF3<sub>mt</sub> in a region where no structural information is available. Its predicted location is shown in the model (Figure 2-3). This region was selected for mutagenesis since the first six residues of *E. coli* IF3 are important for the function of this factor (22). IF3<sub>mt</sub>:2 is also located in the N-terminal domain in a loop between two $\beta$-strands facing away from the linker region. IF3<sub>mt</sub>:3 is located in a predicted $\alpha$-helical region in the IF3<sub>mt</sub> linker. IF3<sub>mt</sub>:4-6 are located in the C-terminal domain of IF3<sub>mt</sub> in an $\alpha$-helix (Helix 3) predicted previously to be essential for the function of the *E. coli* protein (23). IF3<sub>mt</sub>:7 is also in the C-terminal domain at the tip of a $\beta$-strand (corresponding to $\beta$-strand 7 in the prokaryotic factors) in the center of the domain. IF3<sub>mt</sub>:8 is located behind IF3<sub>mt</sub>:7 on the opposite face of the C-terminal domain (Figure 2-3). IF3<sub>mt</sub>:9-12 are located in the C-terminal extension of IF3<sub>mt</sub>, where no structural data is currently available. A summary of the IF3<sub>mt</sub> mutations is shown in Table 2-1.

Purification of IF3<sub>mt</sub> by Ni-NTA chromatography did not eliminate the 19 kDa contaminant. Thus, subsequent purification by HPLC chromatography was necessary. HPLC purification resulted in a column elution profile characterized by two distinct peaks (Figure 2-5A). The two peaks correspond to the 19 kDa fragment, which elutes from the column first, followed by the 29 kDa full-length protein. Polyacrylamide gel electrophoresis of
fractions eluted from the HPLC column show the separated 19 kDa (fractions 30-34) and 29 kDa (fractions 35-40) forms of IF3_{mt} (Figure 2-5B).

Purification of mutated variants of IF3_{mt} resulted in the same approximate ratio of 19 kDa contaminant to the 29 kDa full-length protein as is shown in Figure 2-5B.

The mutated proteins were purified and tested for structural integrity by measuring the α-helical content using CD. The CD spectrum of IF3_{mt}:WT is shown in Figure 2-6.

IF3_{mt}:WT was determined to have 21 ± 3% α-helical content using the CD Pro analysis software. This agrees with the model if the
linker region is considered to be flexible instead of a rigid α-helix. All mutated proteins had CD profiles similar to that of IF3<sub>mt</sub>:WT, and analysis of the α-helical content showed that all mutated proteins contained the same helical content as IF3<sub>mt</sub>:WT within error. IF3<sub>mt</sub>:1 was excluded from CD measurements because it failed to separate from its truncated fragment using HPLC purification.

*Mutations in the N-terminal Domain and Linker*

IF3<sub>mt</sub> derivatives with mutations in the N-terminal domain and linker were tested for their abilities to promote [<sup>35</sup>S]fMet-tRNA binding to both *E. coli* and mitochondrial ribosomes in the presence of IF2<sub>mt</sub>. This assay primarily measures the ribosomal subunit dissociation activity of this factor (11). A representative graph showing initiation complex formation as a function of increasing amounts IF3<sub>mt</sub> is shown in Figure 2-7. IF3<sub>mt</sub>:1 and IF3<sub>mt</sub>:2 showed activity similar to the unmutated factor (IF3<sub>mt</sub>:WT) on both *E. coli* and

![Figure 2-7](image-url)

**Figure 2-7.** Effect of IF3<sub>mt</sub>:WT, IF3<sub>mt</sub>:7, and IF3<sub>mt</sub>:5 on initiation complex formation using mitochondrial 55S ribosomes. [<sup>35</sup>S]fMet-tRNA binding to mitochondrial 55S particles was tested in the presence of saturating amounts of IF2<sub>mt</sub> using IF3<sub>mt</sub>:WT (closed circles), IF3<sub>mt</sub>:7 (closed squares), or IF3<sub>mt</sub>:5 (closed diamonds). A blank representing the amount of [<sup>35</sup>S]fMet-tRNA bound to ribosomes in the absence of IF3<sub>mt</sub> (~0.1 pmol) was subtracted from each value.
mitochondrial ribosomes (Figure 2-8), indicating that these residues are not important for the activity of this protein. IF3\textsubscript{mt}:3, located in the linker region of the protein, showed a slight reduction in activity in initiation complex formation on both \textit{E. coli} and mitochondrial ribosomes (Figure 2-8). The slight loss of activity for IF3\textsubscript{mt}:3 was further explored in an assay that measures the ability of the protein to dissociate mitochondrial 55S ribosomes using sucrose density gradient centrifugation (Figure 2-9). IF3\textsubscript{mt}:3 showed the same subunit dissociation pattern as IF3\textsubscript{mt}:WT in this assay, which indicates that it did not have any significant defect in this function (Figure 2-10).

Figure 2-8. Summary of the activities of IF3\textsubscript{mt}:1-12 in initiation complex formation. This assay primarily measures ribosome dissociation. The activity of IF3\textsubscript{mt}:WT was set to 100 % and the mutated derivatives were compared to that value using the linear regions of the dose response curves. The activity on 70S ribosomes is shown in black, while activity on mitochondrial ribosomes is in striped gray. Derivatives identical to wild-type IF3\textsubscript{mt} within error are shown as 100 %.
*Mutations in the C-terminal Domain*

The C-terminal domain of IF3\textsubscript{mt} with the linker binds to the small ribosomal subunit with nearly the same affinity as the full-length protein and shows the same activity as the full length protein in stimulating fMet-tRNA binding to mitochondrial ribosomes (8). IF3\textsubscript{mt} derivatives with mutations in the C-terminal domain were tested for activity in initiation complex formation on *E. coli* and mitochondrial ribosomes. IF3\textsubscript{mt}:7 and IF3\textsubscript{mt}:8 had the same activity as the wild-type protein (IF3\textsubscript{mt}:7 Figure 2-7, IF3\textsubscript{mt}:8 Figure 2-8). IF3\textsubscript{mt}:4 showed slightly reduced activity, but still maintained its ability to dissociate 55S ribosomes as nearly as well as the wild-type protein (Figure 2-10). Its slight deficiency in ribosome dissociation agrees with the slight deficiency in initiation complex formation (Figure 2-8). However, IF3\textsubscript{mt}:5 and IF3\textsubscript{mt}:6 had almost no activity in promoting initiation complex formation on mitochondrial 55S ribosomes. Fractionation profiles of mitochondrial 55S ribosomes after centrifugation on a 10-30 % sucrose gradient. Mitochondrial 55S ribosomes (8 pmol) were incubated as described in Materials and Methods in the absence (A) or presence of 80 pmol IF3\textsubscript{mt} (B) or its mutated derivative IF3\textsubscript{mt}:6 (C) and subsequently subjected to centrifugation on a 10-30 % sucrose density gradient. Gradients were fractionated while monitoring the A\textsubscript{254}.

![Diagram](image-url)
formation on *E. coli* ribosomes, and had significantly reduced activity on mitochondrial ribosomes (IF3<sub>mt</sub>:5 Figure 2-7, IF3<sub>mt</sub>:6 Figure 2-8).

Since neither IF3<sub>mt</sub>:5 nor IF3<sub>mt</sub>:6 were active in stimulating initiation complex formation, an assay that basically measures ribosome dissociation, their abilities to promote the dissociation of 55S ribosomes was examined directly by sucrose density centrifugation. Both proteins were deficient in dissociation activity (IF3<sub>mt</sub>:6, Figure 2-9; IF3<sub>mt</sub>:5 Figure 2-10). IF3<sub>mt</sub>:5 dissociated 55S ribosomes by only ~15%, compared to just over 50% dissociation with the same level of IF3<sub>mt</sub>:WT (Figure 2-10). IF3<sub>mt</sub>:6 had essentially no activity in the

![Figure 2-10. Percentage of mitochondrial 55S ribosomes remaining after the addition of IF3<sub>mt</sub>:WT or its mutated derivatives as measured by sucrose density gradient centrifugation.](image)

One possible explanation for the lack of activity observed with IF3<sub>mt</sub>:5 and IF3<sub>mt</sub>:6 is that these mutated proteins are unable to bind 28S subunits, and, therefore, are unable to effectively prevent the interaction of the 28S and 39S ribosomal subunits. To test this
possible explanation directly, the binding of IF3\textsubscript{mt} and its mutated derivatives to 28S subunits was measured using Microcon-100 centrifugation followed by immunological detection of IF3\textsubscript{mt} on a dot blot apparatus (10). This method takes advantage of the observation that, in the absence of 28S subunits, IF3\textsubscript{mt} passes through the membrane while IF3\textsubscript{mt} bound to 28S subunits does not. The binding curve for IF3\textsubscript{mt}:6 is shown in Figure 2-11. IF3\textsubscript{mt}:WT binds to 28S subunits with a K\textsubscript{d} of 35 ± 13 nM (Table 2-3). As indicated in Table 2-

![Figure 2-11](image)

Figure 2-11. Binding assays performed using Microcon spin columns as described (2,2,6). Inset: Calibration curve of IF3\textsubscript{mt}:WT using a colorimetric assay and a dot blot apparatus. This curve was used to determine the amount of IF3\textsubscript{mt} bound to mitochondrial 28S ribosomes in the Microcon centrifugation assay as described in Materials and Methods.

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<td>IF3\textsubscript{mt}:WT</td>
<td>35 ± 13</td>
</tr>
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<td>IF3\textsubscript{mt}:5</td>
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<tr>
<td>IF3\textsubscript{mt}:6</td>
<td>45 ± 17</td>
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Table 2-3. Binding constants of IF3\textsubscript{mt} and its mutated derivatives to 28S mitochondrial subunits. K\textsubscript{d} values of IF3\textsubscript{mt}:WT and its mutated derivatives binding to 28S mitochondrial subunits as determined by Microcon centrifugation.
3, IF3\textsubscript{mt}:6 binds 28S subunits with a K\textsubscript{d} of 45 ± 17 nM and IF3\textsubscript{mt}:5 binds to 28S subunits with a K\textsubscript{d} of 19 ± 11 nM. Since both mutated proteins show the same binding to 28S subunits as IF3\textsubscript{mt}:WT, the lack of activity of the mutated proteins in initiation complex formation and in the dissociation of 55S ribosomes into the 28S and 39S subunits cannot be attributed to a defect in their abilities to bind the SSU.

*Mutations in the C-terminal Extension*

One unusual property of IF3\textsubscript{mt} is that its C-terminal extension is thought to play an important role in decreasing the amount of fMet-tRNA bound to mitochondrial 28S subunits in the absence of mRNA. This observation suggests that mRNA binding should precede fMet-tRNA binding in the mitochondrial system (7). No structural

![Graph](image_url)
data is currently available for the C-terminal extension of IF3\textsubscript{mt}. Modeling of this region indicated that it is probably disordered in solution. Regions of highly charged amino acids (acidic and basic) conserved in vertebrate IF3\textsubscript{mt} lineages were selected for mutation to alanine residues (Figure 2-4). All of these derivatives were as active as IF3\textsubscript{mt}:WT in promoting initiation complex formation on both \textit{E. coli} and mitochondrial ribosomes (Figure 2-8). As indicated in Figure 2-12, IF3\textsubscript{mt}:WT inhibits the binding of fMet-tRNA to 28S subunits in the absence of mRNA. IF3\textsubscript{mt}:5 and IF3\textsubscript{mt}:6 in the C-terminal domain, along with IF3\textsubscript{mt}:10, IF3\textsubscript{mt}:11, and IF3\textsubscript{mt}:12 in the C-terminal extension showed normal activity in this assay (data not shown). However, IF3\textsubscript{mt}:9, also in the C-terminal extension, was unable to reduce the amount of fMet-tRNA bound to ribosomes in the absence of mRNA (Figure 2-12). This observation pinpoints a region of the C-terminal extension that is critical for inhibiting initiation complex formation before the mRNA is bound. Interestingly, the linker region of IF3\textsubscript{mt} also appears to have a role in preventing fMet-tRNA binding in the absence of mRNA (8).

The C-terminal extension is predicted to emerge from the C-terminal domain near the linker region according to the model (Figure 2-3). The linker, and, thus, the C-terminal extension, may span the region between the platform, where the C-terminal domain is thought to bind, and the head of the small subunit. The linker may be positioned toward the mRNA binding channel as predicted in the model of McCutcheon \textit{et al.} (24). A transient association between of these regions of IF3\textsubscript{mt} with the mRNA channel near the P-site may permit this factor to distinguish prematurely formed initiation complexes.
DISCUSSION

Comparison of Mutational Effects in IF3$_{mt}$ and E. coli IF3

Both NMR (25) and mutational analysis (23;26) indicate that there are a number of contacts between E. coli IF3 and the SSU in the C-terminal domain. These interactions appear to span much of Helix 3, the loop regions, and portions of Helix 4. Key differences between IF3$_{mt}$ and the E. coli factor become apparent when the extensive battery of mutations made in Arg residues in E. coli IF3 are compared to those presented here in the mitochondrial factor (Figure 2-13). In E. coli IF3, the most drastic effects on the activity of

\[
\begin{align*}
>90\% \text{ activity} & \quad \geq 70\% \text{ activity} & \quad \leq 20\% \text{ activity} \\
\text{IF3}_m:5 & \quad \text{IF3}_m:6 & \quad \text{IF3}_m:4 & \quad \text{IF3}_m:8 & \quad \text{IF3}_m:7
\end{align*}
\]

Figure 2-13. A. Mutated residues in the C-terminal domain of E. coli IF3 and their effects on activity in initiation complex formation on E. coli ribosomes. The activity remaining was based on the dissociation of 70S ribosomes, one of several assays carried out with these mutated derivatives of E. coli IF3. The most drastic effects on activity are seen with the mutation in the center of the α-helix (Helix 4) on the back of the protein. B. Mutated residues in the C-terminal domain of IF3$_{mt}$ and the effects of these mutations on activity in initiation complex formation on mitochondrial ribosomes. The most drastic effects are seen with the two mutations located at the base of Helix 3.

the factor in initiation complex formation measured by ribosomal subunit dissociation were
caused by a mutation in the center of Helix 4 in the C-terminal domain (Figure 2-13A) (23). This mutation also caused significant reductions in binding of the mutated protein to \textit{E. coli} 30S ribosomal subunits, suggesting that the defect in initiation arises from a weaker interaction with the SSU. However, IF3\textsubscript{mt}:8, located in Helix 4 in the C-terminal domain of IF3\textsubscript{mt}, was able to stimulate initiation complex formation as well as the wild-type factor. This observation suggests that residues in Helix 4 may play a less important role in IF3\textsubscript{mt} than in \textit{E. coli} IF3.

In the mitochondrial factor, the most drastic effects were seen with two mutations in Helix 3 (IF3\textsubscript{mt}:5 and IF3\textsubscript{mt}:6, Figure 2-13B). A mutation made in the center of this helix in \textit{E. coli} IF3 (R112S) had only a modest effect on initiation complex formation (23), and the mutated derivative still retained >60% of the activity observed with the wild-type factor. In the mitochondrial factor, the mutations in Helix 3 are located closer to the base of the helix rather than in the center. The side chains of these residues are predicted to be shifted approximately 90 degrees towards the front of the protein when compared to the side chains of the mutated residues in \textit{E. coli} IF3. IF3\textsubscript{mt} derivatives containing these mutations do not promote initiation complex formation, but are still able to bind to mitochondrial 28S subunits. Thus, residues near the bottom of Helix 3 are not predicted to be in direct contact with the 28S subunits. Rather, they may define a surface of IF3\textsubscript{mt} that contacts the 39S subunit during the dissociation of the 55S ribosomes into subunits.
Model for IF3<sub>mt</sub>-Mediated Dissociation of 55S Ribosomes

Mutation of residues 170-171 and residue 175 in Helix 3 of the C-terminal domain of IF3<sub>mt</sub> drastically reduced the activity of IF3<sub>mt</sub> in promoting initiation complex formation. This assay basically measures the ability of IF3<sub>mt</sub> to dissociate 55S ribosomes. Sucrose density gradient centrifugation confirmed the idea that these mutations inactivate IF3<sub>mt</sub> in ribosomal subunit dissociation. The most logical explanation for these results is that the mutated proteins fail to bind mitochondrial 28S subunits and, therefore, cannot prevent 39S joining. However, both of these mutated derivatives of IF3<sub>mt</sub> bind to 28S subunits as well as the wild-type factor (Table 2-3). This observation indicates that an alternative problem must underlie the loss of activity in these mutated IF3<sub>mt</sub> derivatives. One possible explanation is that there are two distinct functionally important surfaces of IF3<sub>mt</sub>. The first surface would function as a dissociation interface and may interact with one or both of the ribosomal subunits. The second surface would function as a binding interface between the factor and the 28S subunit.

Recent work has indicated that the position of the C-terminal domain of E. coli IF3 blocks bridge B2b of the 70S ribosome, preventing the association of the large and small subunits when IF3 is present (27). Bridge B2b involves 16S rRNA nucleotide 794 (28), which is in proximity to the proposed IF3 binding site on the 30S subunit (29;30). Cryo-electron microscopy studies of mammalian mitochondrial ribosomes (5) indicates that this intersubunit bridge is one of several conserved contacts between the two subunits. IF3<sub>mt</sub> is expected to bind to 28S subunits in a manner similar to the binding of E. coli IF3 to 30S subunits. It is likely that residues 170-171 and 175 play an essential role in allowing IF3<sub>mt</sub> to...
block the formation of bridge B2b. Mutation of these residues could impede the blocking of this intersubunit bridge by IF3_{mt}, preventing these mutated forms of IF3_{mt} from effectively competing with the 39S subunit for binding to the 28S subunit.

There are two current models to explain the action of IF3_{mt} in ribosomal subunit dissociation (Figure 2-14). In the passive model, IF3_{mt} binds to 28S subunits after the subunits dissociate transiently (Figure 2-14A, passive model). In this model, IF3_{mt} acts more as an anti-association factor than a dissociation factor (31). In the active model, an

![Diagram of two models for the mechanism of IF3_{mt} in the dissociation of mitochondrial ribosomes. A. In the passive model, the subunits are in equilibrium with the 55S monosome (passive step 1). IF3_{mt} binds to free 28S subunits, preventing reassociation with the 39S subunit (passive step 2). B. In the active model, IF3_{mt} interacts with the 55S particle, forming a transient 28S:IF3_{mt}:39S complex (active step 1), which then dissociates into a 28S:IF3_{mt} complex and free 39S subunits (active step 2).]
equilibrium exists between 55S ribosomes and a transient 28S:IF3 mt:39S complex (Figure 2-14B, active step 1). This transient complex is in equilibrium with free 39S subunits and 28S subunits bound to IF3 mt (active step 2). The active model suggests that IF3 mt plays a more direct role in dissociating 55S ribosomes than simply binding to 28S subunits that are present at equilibrium. IF3 mt:5 and IF3 mt:6, though they bind well to 28S subunits (active step 1), are deficient in dissociating 55S ribosomes (active step 2). This observation suggests that a transient 28S:IF3 mt:39S complex formed by these mutated derivatives fails to dissociate into its component subunits. This idea is supported by recent kinetic data, which suggest that in E. coli, IF3 may remain associated with the 30S subunit that is partially bound to the 50S subunit (27), and by recent cryo-electron microscopy data, which tentatively suggests that IF3 may not be released from 70S particles until the hydrolysis of GTP by IF2 has occurred (32). The present results suggest that IF3 mt plays an active role rather than a passive role in ribosomal subunit dissociation in the mitochondrial translational system.
REFERENCES


CHAPTER 3

PREFERENTIAL SELECTION OF THE 5’ TERMINAL START CODON ON LEADERLESS mRNAs BY

BOVINE MITOCHONDRIAL RIBOSOMES

The work from this chapter is in preparation to be submitted for publication.
INTRODUCTION

In contrast to other genomes, the mammalian mitochondrial genome is very compact, containing approximately 16 kilobase pairs of DNA. It codes for 2 rRNAs, 13 proteins, and 22 tRNAs (1). The 13 proteins are components of the electron transport chain and ATP synthase. Very few non-coding regions exist in the mitochondrial genome. A maximum of 3 nucleotides are observed prior to the start codon in human mitochondrial mRNAs (2).

Initiation of protein synthesis in mitochondria has several similarities to that in prokaryotes, but this system is also characterized by a number of unique features. Mitochondrial monosomes sediment at 55S and dissociate to form 28S and 39S subunits. The mitochondrial SSU has a unique mRNA entrance gate composed of one or more mitochondrial-specific proteins and the SSU protein MRPS2. MRPS2 is the homolog of the bacterial ribosomal protein S2 but is larger in the mitochondrial ribosome (3). These proteins come together to form a triangular mRNA entrance site, which may play a role in binding mitochondrial leaderless mRNAs. Protein synthesis in mitochondria occurs with the help of two initiation factors: IF2\textsubscript{mt} promotes the binding of fMet-tRNA to 28S small ribosomal subunits, and IF3\textsubscript{mt} stimulates initiation complex formation by aiding in the dissociation of mitochondrial 55S ribosomes. No factor homologous to prokaryotic IF1 has been found in mitochondria.

An important difference between bacterial and mitochondrial translational initiation is the process by which the start codon of the mRNA is placed in the P-site of the ribosome. In prokaryotes, a SD sequence is present that hydrogen bonds with nucleotides near the 3′
end of the 16S rRNA (ASD) (4). These interactions help to properly position the mRNA on the ribosome. Because mitochondrial mRNAs are largely leaderless, the mechanism of the selection of the translational start site must be different from that in prokaryotes.

Leaderless mRNAs, in addition to being translated in mitochondria, are also translated in bacteria and archaea, although each system has unique mechanisms reported for initiation on these leaderless messages. In the archaeal system, two-thirds of all mRNAs are leaderless, and few of the mRNAs that contain leaders contain SD sequences (5). In certain archaeal systems, introduction of an internal AUG to a leaderless mRNA resulted in translation from both the 5’ AUG and the internal AUG, although it has not been determined whether initiation could occur on both AUGs simultaneously (6).

A recent study on the translation of leaderless mRNA in prokaryotes revealed that ribosomes devoid of a number of proteins (61S particles) were specific for translating only leaderless mRNAs (7). However, of the 14 ribosomal proteins that are conserved between the small subunit of mitochondrial and prokaryotic systems, 11 are lost in the formation of the prokaryotic 61S particles. This observation indicates that the mechanism by which prokaryotic ribosomes translate leaderless mRNAs is likely to be different from the mechanism of translational initiation in mitochondria.

In prokaryotes, it is unclear whether initiation of leaderless mRNAs occurs on intact 70S monosomes or on dissociated 30S subunits (8-10). Bacterial IF3 has been shown to reduce translational efficiency on leaderless mRNAs that begin with a 5’ AUG when present in excess (11). However, at low concentrations, E. coli IF3 increased the number of initiation complexes formed on leaderless mRNAs (8). In agreement with the latter
observation, IF3mt stimulates initiation complex formation on the leaderless mitochondrial mRNAs, suggesting that initiation on these mRNAs does not require an intact monosome (12).

Previous data has indicated that mitochondrial mRNAs will bind nonspecifically to the mitochondrial 28S SSU alone, without the help of initiation factors (13). It was also determined that, in the absence of initiation factors, shorter mRNAs (fewer than 400 nucleotides) bind less well to mitochondrial 28S subunits than longer mRNAs (14). SHAPE analysis of mitochondrial mRNAs indicated that the 5’ ends of these mRNAs contain little or no secondary structure (15). Thus, it is unclear how the start codon of mitochondrial mRNAs is correctly positioned on the ribosome. The focus of this chapter is to investigate the selection of a 5’ AUG start codon on mitochondrial mRNAs.

MATERIALS AND METHODS

Materials

General chemicals were purchased from Sigma-Aldrich or Fisher Scientific. Bovine mitochondrial ribosomes (55S), ribosomal subunits (28S and 39S), and yeast [35S]fMet-tRNA were prepared as described (3;16-18). IF2mt and IF3mt were cloned, expressed, and purified as described previously (19). Both proteins were purified by HPLC following Ni-NTA chromatography as described (19).
Cloning Cytochrome Oxidase Subunit I (Col), NADH Dehydrogenase Subunit 2 (ND2), and Met-tRNA/ND2 fragments

The first 151 nucleotides of Col DNA, the first 150 nucleotides of ND2 DNA, and the entire tRNA\textsuperscript{Met} plus the first 150 nucleotides of ND2 were amplified using PCR from the plasmid p11-1 (20), which contains bovine mitochondrial DNA from nucleotides 15,743 – 8,788. Primers used for amplification incorporated the sequence for the Hind III restriction site, the T7 promoter, and a hammerhead ribozyme (ACATCTGATGAGTCCGTGAGGACGAAA CGGTACCCGGTACCGTC; shaded nucleotides vary and are complementary to the first nucleotides of each transcribed sequence) followed by nucleotides for the sequence of interest. The hammerhead ribozyme system was used to generate leaderless mRNAs via self-cleavage prior to the first nucleotide of each sequence (21). The primers also incorporated an XbaI restriction site on the 3’ end of the DNA sequence. The sequences for all primers and thermocycler conditions used are provided in Table 3-1 and 3-2, respectively. Ten different constructs were prepared using the sequence for Col DNA, including: 5’ AUG mRNA, AUG\textsubscript{68} mRNA, Both AUG mRNA, 0 AUG mRNA, 1mer-AUG mRNA, 2mer-AUG mRNA, 3mer-AUG mRNA, 6mer-AUG mRNA, 9mer-AUG mRNA, and 12mer-AUG mRNA (Figure 3-1). Three different constructs were prepared using the sequence for NADH Dehydrogenase Subunit 2 (ND2), including: ND2 5’ AUG mRNA, ND2 AUG\textsubscript{91} mRNA, and ND2 0 AUG mRNA (Figure 3-1). A hybrid mRNA was prepared using the sequence for tRNA\textsuperscript{Met} attached at the 5’ end to the first 150 nucleotides of ND2 (Figure 3-1).
The first construct prepared was the CoI 5’ AUG mRNA. For this construct, the first 151 nucleotides of CoI were amplified from the p11-1 plasmid with primers that added the hammerhead ribozyme sequence to the 5’ end of the CoI sequence and an XbaI restriction site to the 3’ end of the CoI sequence. A second PCR reaction was then performed using the amplified product from the first reaction and added a portion of the T7 promoter sequence to the 5’ end of the hammerhead ribozyme sequence. Finally, a third amplification was performed using the second amplified product above to incorporate the full T7 promoter sequence and a HindIII restriction site on the 5’ end of the T7 promoter sequence. The resulting construct was then digested with both HindIII and XbaI and ligated into HindIII/XbaI digested pUC19. The University of North Carolina at Chapel Hill Genome Analysis Facility was used to confirm all sequences.

The Both AUG construct was prepared using the QuikChange site-directed mutagenesis kit (Stratagene) to change an internal GTG of the 5’ AUG construct to AUG, resulting in a DNA sequence with two ATGs. The 3’ AUG construct was prepared by mutating the 5’ ATG of the 5’ AUG construct to GCG. To make the 3’ AUG construct cleavable by the hammerhead ribozyme, it was also necessary to change the first four nucleotides of the hammerhead sequence (indicated as variable above) to ACGC. To prepare the 0 AUG construct, the 5’ AUG construct was mutated to change the ATG to GTG, and the variable nucleotides were changed to ACGC to make the hammerhead ribozyme functional. The 5’ extended mRNAs constructs were prepared by adding nucleotides to the 5’ AUG construct using the QuikChange site-directed mutagenesis kit (Stratagene), and the
variable nucleotides were mutated to make the hammerhead ribozyme functional in each case.

The tRNAMet/ND2 and ND2 constructs were prepared using the p11-1 starting plasmid and sequential PCR reactions (similar to those used for the CoI constructs) that resulted in a 5’ AUG construct containing a HindIII restriction enzyme site, the T7 promoter, the hammerhead ribozyme, the first 150 nucleotides of ND2, and an Xbal restriction site (Table 2-1). Since ND2 begins with an ATA in the genome, an additional mutagenesis step was necessary to change the ATA to an ATG.

Table 3-1. Summary of primers used for the preparation of CoI, ND2, and tRNA<sup>Met</sup>/ND2 mRNA constructs. Added nucleotides are shaded in dark grey, and mutated nucleotides are shaded in light grey. The hammerhead ribozyme sequence is underlined, and the first four variable nucleotides are shaded in blue.

<table>
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<th>Primer</th>
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<td><strong>5’ AUG mRNA</strong></td>
<td><strong>Starting plasmid: p11-1</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Forward:</strong> ACAT&lt;sup&gt;ACAT&lt;/sup&gt;CTGATGAGTCCGTGAGGACGAAACGGGTACCCGGTACCGTGATG</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse:</strong> CTAGTCTAGACGTCTCCGAGCAGAGTTTC</td>
</tr>
<tr>
<td></td>
<td>Thermocycler condition 1</td>
</tr>
<tr>
<td></td>
<td><strong>Forward:</strong> CTAGAAGCTGGAGA&lt;sup&gt;ACAT&lt;/sup&gt;CTGATGAGTCCGTGAGGACGAAACGGGTACCCGGTACCGTGATG</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse:</strong> Same as above</td>
</tr>
<tr>
<td></td>
<td>Thermocycler condition 2</td>
</tr>
<tr>
<td><strong>Both AUG mRNA</strong></td>
<td><strong>Starting plasmid: 5’ AUG mRNA</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Forward:</strong> CCTTTATCTATTTTTAGCTTGGGCGGATATAGTAGG</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse:</strong> CCTACTATACCGCCCAAGCATCAAATAGTAGATAAAGG</td>
</tr>
<tr>
<td></td>
<td>Thermocycler: Mutagenesis 53, 54, 55, then 16</td>
</tr>
</tbody>
</table>
| AUG<sub>68</sub> mRNA | **Starting plasmid: Both AUG mRNA**  
Forward: `CCCGGTACCGTCCGTACCGTTATTTAACCCTG`  
Reverse: `CAGCGGTAAAATACCTACGCCGATACCGGATACCGGCGG`  
Thermocycler condition 3 |
|----------------------|--------------------------------------------------|
| **HH complementary**  
Forward: `CTACGAATACACCTATCAACGCGACGTAGTCGCTGAGC`  
Reverse: `CTCACGAGACTACCATCATGCATCCCTATAG`  
Thermocycler condition 3 |

| 0 AUG mRNA | **Starting plasmid: 5’ AUG mRNA**  
Forward and Reverse: same as 3’ AUG mRNA  
Thermocycler condition 3 |
|----------------------|--------------------------------------------------|
| **HH complementary**  
Forward and Reverse same as 3’ AUG mRNA  
Thermocycler condition 3 |

| 1mer-AUG mRNA | **Starting plasmid: 5’ AUG mRNA**  
Forward: `CGGTACCGGTACCCTGCATGATCCATTAACCCTG`  
Reverse: `CAGCGGTAAAATACCATCACGGACGTACCGGATACCGGCGG`  
Thermocycler condition 3 |
|----------------------|--------------------------------------------------|
| **HH Complementary**  
Forward: `CTACATAGGGAGACACGTAGTGAGTCCCTGAG`  
Reverse: `CTCACGGAGACTACCATCATGCATCCCTATAG`  
Thermocycler condition 3 |

| 2mer-AUG mRNA | **Starting plasmid: 5’ AUG mRNA**  
Forward: `CGGTACCGGTACCCTGCATGATCCATTAACCCTG`  
Reverse: `CAGCGGTAAAATACCATCACGGACGTACCGGATACCGGCGG`  
Thermocycler condition 3 |
|----------------------|--------------------------------------------------|
| **HH Complementary**  
Forward: `CTACATAGGGAGACACGTAGTGAGTCCCTGAG`  
Reverse: `CTCACGGAGACTACCATCATGCATCCCTATAG`  
Thermocycler condition 3 |

| 3mer-AUG mRNA | **Starting plasmid: 5’ AUG mRNA**  
Forward: `GTACCGGTACCCTGCATGATCCATTAACCCTG`  
Reverse: `CAGCGGTAAAATACCATCACGGACGTACCGGATACCGGCGG`  
Thermocycler condition 3 |
|----------------------|--------------------------------------------------|
| **HH Complementary**  
Forward: `CTACATAGGGAGACACGTAGTGAGTCCCTGAG`  
Reverse: `CTCACGGAGACTACCATCATGCATCCCTATAG`  
Thermocycler condition 3 |

| 6mer-AUG mRNA | **Starting plasmid: 5’ AUG mRNA**  
Forward: `GTACCGGTACCCTGCATGATCCATTAACCCTG`  
Reverse: `CAGCGGTAAAATACCATCACGGACGTACCGGATACCGGCGG`  
Thermocycler condition 3 |
|----------------------|--------------------------------------------------|
| **HH Complementary**  
Forward: `CTACATAGGGAGACACGTAGTGAGTCCCTGAG`  
Reverse: `CTCACGGAGACTACCATCATGCATCCCTATAG`  
Thermocycler condition 3 |
<table>
<thead>
<tr>
<th>ND2 Constructs</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>tRNA&lt;sup&gt;Met&lt;/sup&gt;/ND2</strong></td>
<td></td>
</tr>
<tr>
<td>Starting plasmid: p11-1</td>
<td></td>
</tr>
<tr>
<td>Forward: TACT\textcolor{blue}{CTGATGAGTCCG\textcolor{red}{TAGTA}}AGGT\textcolor{blue}{CCTGAATTAACCG}</td>
<td></td>
</tr>
<tr>
<td>Reverse: CTAGCTCAGTGGTTGAT\textcolor{blue}{TATTTTTATAT\textcolor{red}{GATGGG}}</td>
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</tr>
<tr>
<td>Thermocycler condition 4</td>
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</tr>
<tr>
<td>Forward: CTAGAAGCTTGGGAGA\textcolor{blue}{TACT}CTGATGAGTCCG\textcolor{red}{TAGTA}</td>
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</tr>
<tr>
<td>Reverse: same as above</td>
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</tr>
<tr>
<td>Thermocycler condition 5</td>
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</tr>
<tr>
<td>Change AUA to AUG:</td>
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</tr>
<tr>
<td>Forward: CCTTCCGTACTAAT\textcolor{blue}{AGGGAAGACT\textcolor{red}{TGTA}}CTGATGAGTCCG</td>
<td></td>
</tr>
<tr>
<td>Reverse: same as above</td>
<td></td>
</tr>
<tr>
<td>Thermocycler condition 5</td>
<td></td>
</tr>
</tbody>
</table>

| Starting plasmid: Col 6mer-AUG mRNA |  |
| Forward: CGGTACCGCGTACCGTCCCCG\textcolor{blue}{ACTGATGAGTCCG\textcolor{red}{AGTAGT}}GTTTAATTAACCG |  |
| Reverse: CGGTAAATGAACCATCAGTGGCGTGAGACGGTACCGG\textcolor{red}{GGTAGG} |  |
| Thermocycler condition 3 |  |
| Forward: CTACG\textcolor{blue}{CTGATGAGTCCG\textcolor{red}{TAGTA}}GTTGTGATTTTTATGATG |  |
| Reverse: same as above |  |
| Thermocycler condition 3 |  |

| Starting plasmid: Col 9mer-AUG mRNA |  |
| Forward: CGGTACCGCGTACCGTCCCC\textcolor{blue}{GCTG}\textcolor{red}{AGTAGT}GTTTAATTAACCG |  |
| Reverse: CGGTAAATGAACCATCAGTGGCGTGAGACGGTACCG\textcolor{red}{GGTAGG} |  |
| Thermocycler condition 3 |  |
| Forward: CTACG\textcolor{blue}{CTGATGAGTCCG\textcolor{red}{TAGTA}}GTTGTGATTTTTATGATG |  |
| Reverse: same as above |  |
| Thermocycler condition 3 |  |

<p>| Starting plasmid: Col 12mer-AUG mRNA |  |
| Forward: CGGTACCGCGTACCGTCCCC\textcolor{blue}{GCTG}\textcolor{red}{AGTAGT}GTTTAATTAACCG |  |
| Reverse: CGGTAAATGAACCATCAGTGGCGTGAGACGGTACCG\textcolor{red}{GGTAGG} |  |
| Thermocycler condition 3 |  |
| Forward: CTACG\textcolor{blue}{CTGATGAGTCCG\textcolor{red}{TAGTA}}GTTGTGATTTTTATGATG |  |
| Reverse: same as above |  |
| Thermocycler condition 3 |  |</p>
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<td>Reverse: CTAGTCTAGATGGGTTGTGATTTTTATTATGATGGGG</td>
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<tr>
<td>ND2 5’ AUG mRNA</td>
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<td></td>
<td>Reverse: same as above</td>
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<td>Change AUA to AUG:</td>
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<td>Forward:                                                                 GGTACCCGGTACCGTGACCTATAAACCAAATTATC</td>
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<td></td>
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<td></td>
<td>Thermocycler: condition 3</td>
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<tr>
<td>HH Complementary</td>
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<td>Thermocycler: condition 3</td>
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</tr>
<tr>
<td>ND2 AUG₉₁ mRNA</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Starting plasmid: ND2 0 AUG mRNA</td>
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</tr>
<tr>
<td></td>
<td>Forward:                                                                 CTGACTACTTTGCTGATGGGTTGAAATAAATATAC</td>
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</tr>
<tr>
<td></td>
<td>Reverse: GATAATTGTTGTCATGACCGGTACCGGGTACC</td>
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</tr>
<tr>
<td></td>
<td>Thermocycler: condition 3</td>
<td></td>
</tr>
<tr>
<td>ND2 0 AUG mRNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Starting plasmid: ND2 5’ AUG mRNA</td>
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</tr>
<tr>
<td></td>
<td>Forward:                                                                 GGTACCCGGTACCGTGACCTATAAACCAAATTATC</td>
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</tr>
<tr>
<td>HH complementary</td>
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</tr>
<tr>
<td></td>
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<td>Reverse:                                                                 CTCACTCTAGGACCGATGACCGATGACCGGTACCGGGTACC</td>
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Table 3-2. Thermocycler conditions used for preparation of mRNA constructs listed in Table 3-1.

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<th>Temperature</th>
<th>What for?</th>
<th>Number of cycles</th>
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<td>90°C</td>
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<td>72°C</td>
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<td></td>
<td>30 seconds</td>
<td>90°C</td>
<td>Denaturation</td>
<td>30</td>
</tr>
<tr>
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</tr>
<tr>
<td>---------------</td>
<td>-------------</td>
<td>---------------------------</td>
<td>------------------</td>
<td></td>
</tr>
<tr>
<td>30 seconds</td>
<td>60°C</td>
<td>Annealing Primers</td>
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<tr>
<td>60 seconds</td>
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**Thermocycler conditions 2**

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<td>5</td>
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<tr>
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<tr>
<td>60 seconds</td>
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<tr>
<td>30 seconds</td>
<td>90°C</td>
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<tr>
<td>30 seconds</td>
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**Thermocycler conditions 3**

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**Thermocycler conditions 4 (gradient thermocycler)**

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**Thermocycler conditions 5**

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<td>65°C</td>
<td>Annealing Primers</td>
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**Thermocycler conditions 6**

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<td>60 seconds</td>
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<td>70°C</td>
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Figure 3-1. Summary of cytochrome oxidase subunit I (Col), NADH dehydrogenase subunit II (ND2), and tRNA<sup>Met</sup>/ND2 mRNA constructs. The mRNAs prepared for both Col and ND2 that contained either a 5’ AUG, an internal AUG, or both a 5’ and an internal AUG. A control mRNA was also prepared for each mRNA that did not contain any AUG codons. Col mRNAs with additional nucleotides 5’ to the start codon were also prepared.
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 using 25-50 µg DNA (13). Prior to hammerhead cleavage, transcription reactions were diluted 5X in a cleavage buffer containing 30 mM MgCl₂ and 40 mM Tris-HCl (pH 7.6). Hammerhead cleavage was allowed to proceed for 1 h at 60 °C, at which time 2X RNA load dye (Ambion) was added to the reactions and the transcribed mRNAs were separated from the cleaved hammerhead fragments by gel electrophoresis using an 8% polyacrylamide/7 M urea gel. Full-length mRNA transcripts were excised from the gel and eluted in RNase free H₂O (Ambion) for 48 h at 4 °C. The concentration of each mRNA was determined by measuring the A₂₆₀. The sequences of all mRNA constructs used are summarized in Table 3-3.

Table 3-3. Full sequences of CoI, ND2, and tRNA^{Met}/ND2 mRNA constructs. The AUG codons are shown in red and the tRNA^{Met} sequence is shown in blue.

<table>
<thead>
<tr>
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<th>Sequence</th>
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<tr>
<td>CoI</td>
<td>AUGUUCAUUAACCGCUGACUAAUCUCAACCAGCCAUAAGGAUAUUUGGUACCCU</td>
</tr>
<tr>
<td>S' AUG mRNA</td>
<td>UUAUCUACUAUUUGGUGCUUGGCCGGAUAAGUAAGAACACUGCUAAGCCUU</td>
</tr>
<tr>
<td></td>
<td>UUAUUCGCGCUGAAUUAGCCCAACCGGAACUCUGCUGGAGACG</td>
</tr>
<tr>
<td>ND2</td>
<td>AUGAACCAAUAUCUUAUAUAUUCUACUAACCACAUUUAUCUAGGAACUA</td>
</tr>
<tr>
<td>S' AUG mRNA</td>
<td>UUAUUGCAUAAUCAGUUCUCAGUCACUGACUUGUGAAGGUAUGAAAUAAUACUGGCGUUGGAAAUAAUACUACAGUCAUACCACAAUAAUAAAAUACGCAACCAACCA</td>
</tr>
<tr>
<td>tRNA^{Met}/ND2 mRNA</td>
<td>AGUAAGGUCAGCUAAAUAGCUAUCGGGCCCAUCCCCCGAAAAUGUUUGGUUU</td>
</tr>
<tr>
<td></td>
<td>AUAUUCGUUCCGUGACUAAUGGACCAAUAUCUUAUAUAAUACCUACUACCC</td>
</tr>
<tr>
<td></td>
<td>AUAUACUACUAGGAACUUAUAUUUGCAUAAUCAGUUCUCAGUCACUGACUACUUGGCU</td>
</tr>
<tr>
<td></td>
<td>GAAUCGCGGUUUGAAAUAUACUGGCAUCUACCACCUCAAUAAAUACGCAACCAACCAACCAACCAACCA</td>
</tr>
</tbody>
</table>
**Phosphorylation of mRNAs**

Col 5’AUG and Col AUG$_{68}$ mRNAs were phosphorylated in reactions (500 µL) that contained 100 U T4 polynucleotide kinase (New England Biolabs), 50 µL 10X T4 polynucleotide kinase buffer (New England Biolabs), 50 µg BSA, 1 mM ATP, and 1.25 nmol mRNA. Reactions were incubated at 37 °C for 1 h, extracted using phenol/chloroform and the RNA was precipitated with ethanol. Excess ATP was removed from the phosphorylated mRNAs by using a Mini Quick Spin RNA Column (Roche).

**Initiation Complex Formation on Mitochondrial Ribosomes**

Stimulation of fMet-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 (see Chapter 2) (18;22) and contained the indicated amounts of mRNA, [$^{35}$S]fMet-tRNA (70 nM), 0.25 mM GTP, 1.25 mM PEP, 0.04 U pyruvate kinase, saturating amounts of IF3$_{mt}$ (0.25 µM) and IF2$_{mt}$ (0.15 µM), and 55S ribosomes (80 nM) or 28S ribosomal subunits (80 nM). IF3$_{mt}$ was not present in assays using 28S subunits unless otherwise indicated.

**Competition Assays**

The ability of Col AUG$_{68}$ mRNA to compete with Col 5’ AUG mRNA for mitochondrial 55S ribosome binding was tested in a competition assay. 55S initiation complex assays were performed as described above, except that mixtures of Col 5’ AUG and Col AUG$_{68}$ mRNAs were prepared using 0.1 µM 5’ AUG mRNA in the following ratios to AUG$_{68}$ mRNA:
1:0, 1:1, 1:1.5, 1:2, 1:5, and 1:10. The mRNAs were mixed together prior to being added to the reaction. Reactions were incubated at 37 °C for 10 min and processed as described above.

**Toeprints**

Binding of mitochondrial 55S ribosomes to mRNA was examined using a toeprint assay. Reaction mixtures (20 µL) contained 50 mM Tris-HCl (pH 8.0), 10 mM DTT, 7 mM MgCl₂, 40 mM KCl, 0.1 mM spermine, 0.25 mM GTP, 1.25 mM PEP, 0.04 U pyruvate kinase, 0.5 mM dNTPs, 0.4 µM fMet-tRNA, 0.4 µM IF2ₘ, 0.4 µM IF₃ₘ, 0.3 µM mitochondrial 55S ribosomes, 50 nM [³²P] labeled primer ([³²P] labeled primer with the sequence: CGT CTC CGA GCA GAG was prepared as described previously (15) and gel-purified using a 20% polyacrylamide/7 M urea gel), 50 nM mRNA (CoI 5’ AUG, AUG₆₈, Both AUG, or 0 AUG mRNA). Reactions were incubated at 37 °C for 10 min, at which time 200 U Superscript III reverse transcriptase (Invitrogen) were added, and reverse transcription was allowed to proceed at 37 °C for 10 min. The reactions were stopped by the addition of 2 µL 2 M NaOH and then heated for 5 min at 95 °C. The reactions were neutralized by the addition of 29 µL acid stop mix (4:25 (v/v) mix of 1 M unbuffered Tris-HCl and stop dye: 85 % formamide, ½X Tris-Borate-EDTA (TBE), 50 mM EDTA (pH 8), bromophenol blue, and xylene cyanol). One half of each reaction was then loaded onto a 10% polyacrylamide/7 M urea gel and run at 1550 V for 3 h. At this time, the other half of each reaction was loaded into separate wells and the gel was run at 1550 V for another 3 h. The gel was exposed to a phosphorimager screen overnight and then scanned using a Typhoon Trio + (GE Healthcare) variable mode.
mRNA (pmol)
0 2 4 6 8 10
fMet-tRNA Bound (pmol)
0.0
0.1
0.2
0.3
0.4
Both AUG
5' AUG
AUG
68
0 AUG

Figure 3-2. Initiation complex formation on mitochondrial 55S ribosomes and 28S subunits using Col mRNAs. [35S]fMet-tRNA binding to mitochondrial 55S particles was tested in the presence of increasing amounts of 5’ AUG, AUG68, Both AUG, or 0 AUG mRNA. A blank (~0.08 pmol) corresponding to the amount of fMet-tRNA bound to ribosomes in the absence of mRNA was subtracted from each value.

RESULTS

The Mitochondrial Ribosome Discriminates Between a 5’ AUG and an Internal AUG Codon

Since many mitochondrial mRNAs have the start codon located exactly at the 5’ end, transcription of Col mRNAs was coupled with hammerhead ribozyme cleavage to generate mRNAs with the AUG precisely positioned at the 5’ end. Additional mRNAs were prepared that contained an internal AUG at position 68.
(AUG$_{68}$), two AUGs (Both AUG mRNA, one at the 5' end and one at position 68), or no AUG codon (0 AUG mRNA). Initiation complex formation assays were carried out using each mRNA. Initiation complex formation on the 5' AUG mRNA and the Both AUG mRNA was stimulated as mRNA levels were increased (Figure 3-2). In contrast, no initiation complexes were formed on 55S ribosomes using either the AUG$_{68}$ mRNA or the 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. The same discrimination was seen on 55S ribosomes when IF3$_{mt}$ was excluded from the initiation assay (Table 3-4), although the amount of binding was reduced in the absence of IF3$_{mt}$ due to a limitation in ribosome dissociation. This observation suggests that IF3$_{mt}$ is not necessary for the ribosomal discrimination of a 5' AUG start codon from an internal AUG codon.

Table 3-4. Effect of IF3$_{mt}$ on selection of a 5' AUG by bovine mitochondrial 55S ribosomes. All four Col mRNA constructs were tested for $[^{35}$S]fMet-tRNA binding in the presence and absence of IF3$_{mt}$. No blank corresponding to the amount of $[^{35}$S]fMet-tRNA bound to the ribosome in the absence of mRNA was subtracted, leading to values greater than those reported in Figure 3-2. The estimated error in these experiments is 10%.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>pmol $[^{35}$S]fMet-tRNA bound</th>
<th>+ or − IF3</th>
</tr>
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<tbody>
<tr>
<td>5'AUG</td>
<td>0.17</td>
<td>− IF3</td>
</tr>
<tr>
<td>5'AUG</td>
<td>0.26</td>
<td>+ IF3</td>
</tr>
<tr>
<td>AUG$_{68}$</td>
<td>0.05</td>
<td>− IF3</td>
</tr>
<tr>
<td>AUG$_{68}$</td>
<td>0.08</td>
<td>+ IF3</td>
</tr>
<tr>
<td>Both AUG</td>
<td>0.20</td>
<td>− IF3</td>
</tr>
<tr>
<td>Both AUG</td>
<td>0.31</td>
<td>+ IF3</td>
</tr>
<tr>
<td>0 AUG</td>
<td>0.06</td>
<td>− IF3</td>
</tr>
<tr>
<td>0 AUG</td>
<td>0.08</td>
<td>+ IF3</td>
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It is unclear in the mitochondrial system whether initiation occurs on 55S monosomes or 28S subunits. Hence, initiation assays were also performed using 28S subunits. IF3<sub>mt</sub> was not present in these assays, since it was not shown to affect discrimination by 55S ribosomes and does not stimulate initiation complex formation on 28S SSUs (Figure 3-3) (22). Initiation on 28S subunits was stimulated by increasing amounts of the 5’ AUG and Both AUG mRNAs, as was seen on 55S ribosomes (Figure 3-3). However, no initiation complex formation on 28S subunits was seen using the 0 AUG mRNA, and only a small level of initiation complex formation was observed with the AUG<sub>68</sub> 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 28S subunit. This idea is in agreement with the observation that mRNA binds exclusively to the small subunit (13).
Toeprint Analysis of the Mitochondrial 55S 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 Col mRNAs (Figure 3-4 and Figure 3-5). In the absence of 55S ribosomes, a reverse transcriptase stop is seen at position 20 of the mRNA due to a non-specific stop of the enzyme (Figure 3-4, lanes 1-8).

When initiation complexes were assembled with the 5’ AUG mRNA, a clear and repeatable toeprint of the ribosome was seen 17-19 nucleotides from the 5’ end of the mRNA (Figure 3-4 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 indicates that the prokaryotic ribosome produces a toeprint signal at nucleotide 15 (23). The mitochondrial ribosome is slightly larger in size than its prokaryotic counterpart and is expected to cover a slightly larger section of the mRNA (3). Further, in non-specific complexes between 28S subunits and mitochondrial mRNAs, a region of about 45 nucleotides of the mRNA was protected from degradation by RNase T₁ (13). This value represents the distance between the mRNA entrance tunnel and the point where the mRNA exits the SSU through the platform. Since 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.

In the absence of IF2ₘₜ and fMet-tRNA, the toeprint signal is drastically reduced (Figure 3-4, lane 3), indicating that both are required to stably position the ribosome at the 5’ end of the mRNA. However, a weak signal is observed at position 17. When the 5’ AUG was removed and an AUG was present at position 68, a toeprint signal at nucleotide 17 was present but very weak and was identical in appearance to the toeprint signal in the absence of IF2ₘₜ and fMet-tRNA on the 5’ AUG mRNA (Figure 3-4, lanes 3 and 5). This signal appears to arise from an intrinsic interaction between the mRNA and the ribosome and may represent a signal from a transient complex in which the 5’ end of the mRNA has been fed into the mRNA entrance site and the first codon of the mRNA is transiently positioned at or near the P-site. The strong toeprint signal at nucleotides 18 and 19 was not present in the AUG₆₈ mRNA (Figure 3-4, lane 5). No toeprint signal was observed corresponding to the edge of a ribosome bound at the internal AUG, which would be at nucleotide 85-87 (data...
not shown). The toeprint with the Both AUG mRNA was the same as that observed for the 5’ AUG mRNA. A toeprint at nucleotide 17 only was seen using the 0 AUG mRNA (data not shown).

The importance of fMet-tRNA and IF2_{mt} on the ability of the 55S ribosome to bind specifically to the 5’ end of the mRNA was also examined using the toeprint assay. In the absence of both fMet-tRNA and IF2_{mt}, a weak toeprint signal is seen at the position of nucleotide 17 (Figure 3-5, lane 3). This signal agrees with previous data showing that mRNA can bind to the small subunit of the mitochondrial ribosome in the absence of initiation factors or tRNA and suggests that the mRNA most likely binds to the ribosome prior to the binding of fMet-tRNA or IF2_{mt} (13). This signal is strengthened by the addition of fMet-tRNA in the absence of IF2_{mt} (Figure 3-5, lane 4), but is unaffected by the addition of IF2_{mt} alone (Figure 3-5, lane 5). This data argues that IF2_{mt}, as expected, enhances fMet-tRNA binding to the ribosome and stabilizes the formation of the initiation complex at the 5’ AUG codon,

Figure 3-5. Toeprints showing the position of the 55S ribosome on the 5’ AUG mRNA in the presence and absence of IF2_{mt} and fMet-tRNA. Toeprint assays were performed as described in Materials and Methods. Control reactions were performed in the absence of either fMet-tRNA and IF2_{mt} or 55S ribosomes. Sequencing reactions were carried out using ddCTP and ddATP. Nucleotide G14 is numbered from the 5’ end of the mRNA.
leading to the strong toeprint at positions 17-19. The data also suggests that the additional stabilization of the initiation complex provided by codon/anticodon interactions between the fMet-tRNA and the mRNA are 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 rRNAs, tRNAs, and mRNAs (24). This post-transcriptional mRNA 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 RNA cleavage. Because hammerhead cleavage leaves the mRNAs with a free 5’ hydroxyl group...
instead of a 5’ phosphate group, the Col 5’ AUG and Col AUG$_{68}$ 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 the AUG$_{68}$ mRNAs, regardless of the presence of a 5’-OH or a 5’ phosphate (Figure 3-6), suggesting that the presence of a 5’ phosphate on the mRNAs does not affect the preferential use of the 5’ AUG codon.

**Competition Between the AUG$_{68}$ mRNA and the 5’ AUG mRNA**

It is known that mitochondrial mRNAs bind to the 28S subunit in a sequence independent manner (13). A model of this binding could be that the 28S subunit binds the mRNA at the mRNA entrance gate, and in the absence of a 5’ AUG start codon to trap fMet-tRNA and stabilize initiation complex formation, the mRNA continues to slide through the

![Graph](image)

**Figure 3-7.** Competition of Col AUG$_{68}$ mRNA with 5’ AUG mRNA for initiation complex formation on mitochondrial 55S ribosomes. [$^{35}$S]fMet-tRNA binding to mitochondrial 55S particles was tested in the presence of both Col 5’ AUG and AUG$_{68}$ mRNAs simultaneously. 5’ AUG mRNA and AUG$_{68}$ mRNA were mixed together prior to their additions to the reaction. The molar ratio of the AUG$_{68}$ mRNA divided by the 5’ AUG mRNA is shown on the x-axis.
gate and eventually exits the small subunit. If that model is correct, it is expected that the 
AUG$_{68}$ mRNA would be capable of binding the SSU in a transient fashion and could, thus,
compete with initiation complex formation on the 5’ AUG mRNA. To test this idea,
increasing amounts of AUG$_{68}$ mRNA were added to initiation complex formation assays
using the 5’ AUG mRNA. As indicated in Figure 3-7, addition of the AUG$_{68}$ mRNA led to an
inhibition of $[^{35}\text{S}]$fMet-tRNA binding to 55S ribosomes in response to the 5’ AUG mRNA.
This data suggests that the AUG$_{68}$ mRNA is able to bind ribosomes, but fails to form a stable
initiation complex due to the lack of the 5’ start codon. This model is consistent with the
weak toeprint signal on the AUG$_{68}$ mRNA at nucleotide 17. The high levels of the AUG$_{68}$
mRNA required to compete with the 5’ AUG mRNA for ribosome binding indicate that the
binding of mRNAs that lack a 5’ AUG is not stable, possibly due to a decrease in the $k_{off}$. 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.

Effect of Additional Nucleotides 5’ to the AUG on Initiation Complex Formation

In human mitochondrial mRNAs, the maximum number of nucleotides preceeding a
start codon is three, as observed in Col mRNA (2). In the case of the AUG$_{68}$ mRNA, where
the start codon is located 68 nucleotides into the mRNA, no initiation complexes were
formed on 55S ribosomes. Since 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 were prepared
containing 1, 2, 3, 6, 9, and 12 nucleotides prior to the 5’ AUG (Figure 3-1). The nucleotides
added correspond to the 12 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 Figure 3-8, initiation complex formation was slightly reduced by the addition of a single nucleotide prior to the 5’ AUG. The presence of only 3 nucleotides preceding the AUG codon lead to a steeper decrease 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 80% inhibition of initiation complex formation observed when 12 additional nucleotides were present 5’ to the start codon. This result indicates that the ribosome is less efficient in recognizing the start codon of mRNAs with more than 3 nucleotides 5’ to the AUG.

Figure 3-8. Effect of the addition of nucleotides 5’ to the AUG start codon on initiation complex formation on mitochondrial 55S ribosomes. [35S]fMet-tRNA binding to mitochondrial 55S ribosomes was tested in the presence of increasing amounts of 5’ extended mRNAs. The mRNA was limiting in this assay, giving 0.1 pmol fMet-tRNA bound for the 5’ AUG mRNA. This value was set as 100% initiation complex formation, and the values obtained for the other mRNAs are plotted as a % of this mRNA.
**Preferential Selection of the 5’ AUG is Observed on Additional Mitochondrial mRNAs**

All 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. Since yeast \[^{35}S\]fMet-tRNA was used in the initiation complex formation assays performed here, and AUA is not recognized as a start codon in the yeast translation system, the AUA 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 lacking AUG codons (Figure 3-1) were tested in initiation complex formation. The same preferential use of a 5’ start codon by 55S ribosomes was observed with these mRNAs (Figure 3-9). The ND2 mRNA with the 5’ AUG readily formed initiation complexes. However, no initiation complexes were formed using

![Graph showing the binding of \[^{35}S\]fMet-tRNA to ND2 mRNAs with different AUG codons.](image-url)
the ND2 AUG$_{91}$ mRNA or 0 AUG mRNA. This observation suggests that ribosomal discrimination in favor of a 5’ AUG is a general phenomenon for all mitochondrial mRNAs.

Efficient Initiation Complex Formation Requires Post-Transcriptional mRNA Processing

Both strands of mitochondrial DNA are transcribed into long polycistronic RNAs (24). In many cases, tRNAs exist between protein-coding regions and fold into defined structural elements where they serve as processing signals (25). For example, the ND2 coding region is preceded by the upstream tRNA$^{\text{Met}}$ gene. It is generally assumed that processing to remove the upstream sequence must occur prior to the use of the mRNAs in translation. To test whether the tRNA gene impairs initiation complex formation, we compared initiation complex formation of the ND2 mRNA and a derivative of the mRNA carrying the upstream tRNA$^{\text{Met}}$ (Figure 3-10). In both cases, AUG was used as the start codon in place of AUA. Both mRNAs were prepared by hammerhead

![Figure 3-10. Initiation complex formation on mitochondrial 55S ribosomes using tRNA$^{\text{Met}}$/ND2 mRNAs. [35S]fMet-tRNA binding to mitochondrial 55S particles was tested in the presence of increasing amounts of ND2 5’ AUG and tRNA$^{\text{Met}}$/ND2 mRNAs. A blank (~0.08 pmol) corresponding to the amount of fMet-tRNA bound to 55S ribosomes in the absence of mRNA was subtracted from each value.](image-url)
cleavage to ensure that they were treated identically. As expected, very few initiation complexes were formed on the tRNA$^{\text{Met}}$/ND2 mRNA (Figure 3-10), indicating that translation of ND2 prior to cleavage of tRNA$^{\text{Met}}$ from its 5’ end is inefficient and most likely does not occur.

**DISCUSSION**

Eukaryotic ribosomes bind to mRNA 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 SD/ASD interactions. In this paper, we demonstrated that mitochondrial ribosomes recognize and bind to the start codon at the 5’ end of mitochondrial mRNAs using codon/anticodon interactions between the fMet-tRNA and the mRNA. These interactions are strengthened by the addition of IF2$_{\text{mt}}$, which agrees with the previous proposed role of IF2$_{\text{mt}}$ in promoting the binding of fMet-tRNA to the ribosome.

The sequences of many mitochondrial genomes are known. DNA sequence analysis reveals that few or no nucleotides exist prior to the start codon in animals. Of the 13 proteins coded for by bovine mitochondrial DNA, about half contain noncoding nucleotides prior to the start codon. The highest number of noncoding nucleotides between genes in this organism is four (Table 3-5). No *in vivo* analysis of the 5’ ends of the mRNA has been done in the bovine system. In human mitochondrial DNA, up to 12 nucleotides exist prior to the 5’ start codon of five different genes (the rest lack 5’ untranslated nucleotides), but direct analysis of the mRNAs demonstrated that post-transcriptional processing eliminates the 5’ leader in all but three mRNAs (2). In contrast to these organisms, all noncoding
nucleotides in *Drosophila melanogaster* mitochondrial mRNAs are post-transcriptionally cleaved prior to translation, which indicates that the presence of any nucleotides before the start codon may be inhibitory for translational initiation (27). While mRNAs with few or no nucleotides prior to the 5’ start codon are common in mitochondria, there seems to be no consistency in which genes have noncoding nucleotides and how many nucleotides are present.

Table 3-5. Summary of nucleotides 5’ to the AUG start codon in *Bos taurus, Homo sapiens,* and *Drosophila melanogaster* mitochondrial mRNAs.  

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence 5’ to protein</th>
<th><em>Bos taurus</em> DNA sequence</th>
<th><em>Homo sapiens</em> DNA sequence</th>
<th><em>Homo sapiens</em> mRNA</th>
<th><em>Drosophila melanogaster</em> DNA sequence</th>
<th><em>Drosophila melanogaster</em> mRNA sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND1</td>
<td>tRNA&lt;sub&gt;Leu&lt;/sub&gt;→&lt;sup&gt;3&lt;/sup&gt;</td>
<td>AAATG</td>
<td>ACATA</td>
<td>ACAUA</td>
<td>CTTG</td>
<td>UUG</td>
</tr>
<tr>
<td>ND2</td>
<td>tRNA&lt;sub&gt;Met&lt;/sub&gt;→</td>
<td>ATA</td>
<td>ATT</td>
<td>AUU</td>
<td>ATT</td>
<td>AUU</td>
</tr>
<tr>
<td>ND3</td>
<td>tRNA&lt;sub&gt;Gly&lt;/sub&gt;→</td>
<td>ATA</td>
<td>ATA</td>
<td>AUA</td>
<td>ATT</td>
<td>AUU</td>
</tr>
<tr>
<td>ND4</td>
<td>Overlaps with ND4L</td>
<td>ATG</td>
<td>ATG</td>
<td>AUG</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>ND4L</td>
<td>tRNA&lt;sub&gt;Arg&lt;/sub&gt;→</td>
<td>ATG</td>
<td>ATG</td>
<td>AUG</td>
<td>AATG</td>
<td>AUG</td>
</tr>
<tr>
<td>ND5</td>
<td>tRNA&lt;sub&gt;Leu&lt;/sub&gt;→</td>
<td>ATA</td>
<td>ATA</td>
<td>AUA</td>
<td>ATG</td>
<td>AUG</td>
</tr>
<tr>
<td>ND6</td>
<td>←tRNA&lt;sub&gt;Glu&lt;/sub&gt;</td>
<td>ATG</td>
<td>ATG</td>
<td>AUG</td>
<td>AATT</td>
<td>AUU</td>
</tr>
</tbody>
</table>

1Sequence data obtained from (2;27-29).  
2Between upstream gene and start codon.  
3The forward arrow indicates that the upstream gene is on the same strand as the mRNA coding sequence while the backward arrow indicates that the coding sequence for the upstream gene is on the opposite strand as the mRNA.
The mechanism of post-transcriptional processing in mitochondria has been proposed as a “tRNA punctuation” model. It is known that mitochondrial DNA is transcribed as a long polycistronic mRNA. The tRNA punctuation model predicts that tRNAs are removed from the transcript via post-transcriptional cleavage, and mature mRNAs remain that are ready for translation (25). This model accurately predicts nucleotides 5’ to the start codon for mRNAs with tRNAs immediately preceding them in human mitochondrial DNA. One example where the tRNA punctuation model does not apply is in the case of the gene coding for ColII, which is preceded by the gene coding for ATP6 instead of a tRNA. Thus, cleavage of ColII requires a mechanism distinct from tRNA recognition. In this paper, we clearly demonstrate that post-transcriptional processing in bovine
mitochondria is necessary prior to translation, since initiation of mRNAs containing more than 3-6 nucleotides prior to the 5' AUG is very inefficient.

In prokaryotes, it is known that transcription and translation can occur simultaneously. Therefore, it was of interest to determine whether the mitochondrial system, proposed to be derived from a prokaryotic ancestor, operates in the same manner. It is obvious that, since mitochondrial mRNAs do not contain SD sequences in their mRNAs, they must initiate protein synthesis using a mechanism distinct from the prokaryotic system. This paper demonstrated that mitochondrial 55S ribosomes were unable to initiate on an ND2 mRNA that was still attached to the tRNA\textsubscript{Met} on its 5' end, indicating that post-transcriptional processing to remove tRNAs must occur prior to translation in mammalian mitochondria.

Our current model for initiation in mammalian mitochondria is illustrated in Figure 3-11. IF3\textsubscript{mt} first interacts with the 55S ribosome, forming a transient 28S:IF3\textsubscript{mt}:39S intermediate complex (step 1) (30). This transient complex dissociates into free 28S subunits bound to IF3\textsubscript{mt} and free 39S subunits (Figure 3-11, step 2). Following the ribosome dissociation step, the mRNA feeds into the mRNA entrance tunnel on the 28S subunit (Figure 3-11, step 3). It is unclear whether IF2\textsubscript{mt}:GTP binds at this point or later. It is believed that mRNA binding precedes fMet-tRNA binding, because IF3\textsubscript{mt} has been shown to destabilize the fMet-tRNA bound to 28S subunits in the absence of mRNA (12). 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 at position 17 relative to the 5’ end regardless of the presence or absence of a 5’ AUG codon. During this pause,
IF2
mt:GTP promotes the binding of fMet-tRNA to the ribosome (Figure 3-11, step 4) and codon:anticodon interactions between the fMet-tRNA and the AUG start codon promote the formation of a stable initiation complex (Figure 3-11, step 4). The formation of this complex leads 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 AUG
68 mRNA. If fMet-tRNA binds to the 5’ start codon, the large subunit binds, IF2
mt hydrolyzes GTP to GDP, and the initiation factors leave, resulting in a full 55S initiation complex that is then free to move to the elongation phase of protein synthesis (Figure 3-11, 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. This pause creates a kinetic opportunity for the selection of a 5’ start codon by the binding of the initiator tRNA to the P-site.
REFERENCES


CHAPTER 4

ANALYSIS OF THE FUNCTIONAL CONSEQUENCES OF LETHAL MUTATIONS IN
MITOCHONDRIAL TRANSLATIONAL ELONGATION FACTORS

The work from this chapter was done in collaboration with Kenta Akama, Christie Jones, and
Nono Takeuchi, and is in preparation to be submitted for publication.
**INTRODUCTION**

During protein biosynthesis, elongation factor Tu (EF-Tu) forms a ternary complex with aa-tRNA and GTP and promotes the binding of the aa-tRNA to the A-site of the ribosome (1). Following codon:anticodon interactions, an EF-Tu:GDP complex is released from the ribosome. Elongation factor Ts (EF-Ts) binds to the EF-Tu:GDP complex and promotes the exchange of GDP for GTP, regenerating the active EF-Tu:GTP complex (2).

Mammalian mitochondria have a specific protein biosynthetic system responsible for the synthesis of thirteen polypeptides of the respiratory chain complexes. Protein synthesis in this organelle requires elongation factors that correspond to EF-Tu and EF-Ts (EF-Tu\textsubscript{mt} and EF-Ts\textsubscript{mt}). Both of these proteins are encoded in the nuclear genome, synthesized in the cell cytoplasm, and subsequently imported into the mitochondrion. While mutations in mitochondrial DNA are well known to cause disease, few mutations have been identified in nuclear genes that encode mitochondrial proteins required for protein biosynthesis in this organelle. Recently, a mutation in the gene coding for EF-Ts\textsubscript{mt} (Arg312 to Trp)\(^1\) was reported leading to encephalomyopathy in one patient and hypertrophic cardiomyopathy in another (3). Both patients died at 7 weeks of age. This mutation is predicted to disrupt important interactions between EF-Tu\textsubscript{mt} and EF-Ts\textsubscript{mt}.

EF-Ts\textsubscript{mt} is a 30.7 kDa protein that consists of an N-terminal domain and a core domain divided into N- and C-terminal subdomains (Figure 4-1) (4). EF-Tu\textsubscript{mt} and EF-Ts\textsubscript{mt} are over 92% identical.

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\(^1\)In the original paper (3), the position identified as mutated is listed as Arg333. This position corresponds to the residue in isoform 2 of human EF-Ts\textsubscript{mt} (P-43897-2). However, there is no direct experimental evidence for the expression of this isoform. Rather, isoform 1 (P-43897-1) has been chosen as the canonical sequence. In humans, the mutation in isoform 1 is in position 312 (R312W). There is direct experimental evidence for isoform 1 in the bovine system. The mutation created here is in isoform 1 of *Bos taurus* EF-Ts\textsubscript{mt} and corresponds to residue 325 (R325W). The mature forms of human and *Bos taurus* EF-Ts\textsubscript{mt} are over 92% identical.
interact through extensive surface contacts. The N-terminal domain and the subdomain N of the core of EF-Tsmt interact directly with the G-domain (domain I) of EF-Tu mt, while the subdomain C of the core contacts domain III of EF-Tu mt.

Arg325 of EF-Tsmt is highly conserved and is found in most bacterial and mitochondrial factors (3). This residue is located in a β-strand in subdomain C of the core and is shown in blue in Figure 4-1 and Figure 4-2. While it does not make direct contact with EF-Tu mt, other nearby residues in this region do. Molecular modeling has predicted that the R325W mutation could disrupt the tertiary structure of the subdomain C of the core, altering its interactions with domain III of EF-Tu mt (3). Mutations in this region have been shown to reduce the affinity of EF-Tsmt for EF-Tu mt about six to seven-fold (5). For example,
mutation of His231 (shown in purple in Figure 4-2) to Ala caused a 6-fold decrease in the binding constant governing the interaction of EF-Ts_{mt} with EF-Tu_{mt}. Mutation of L302 and L303 (shown in orange) caused a 7-fold decrease in the EF-Tu_{mt}:EF-Ts_{mt} binding constant.

We have directly examined the effect of the R325W mutation by testing the ability of this mutated protein to bind directly to EF-Tu_{mt} and to stimulate its activity in poly(U)-directed polymerization.

EF-Tu_{mt} is a 45.1 kDa protein composed of three domains. Domain I contains the guanine nucleotide binding site. All three domains are involved in binding the aa-tRNA (6), while domains I and III interact with EF-Ts_{mt}. The stable binding of guanine nucleotides and EF-Ts_{mt} to EF-Tu_{mt} are mutually exclusive, allowing EF-Ts_{mt} to serve as a nucleotide exchange factor for EF-Tu_{mt} (7;8).

A mutation in EF-Tu_{mt}, (Arg336 to Gln), was discovered in an infant with lactic acidosis and fetal encephalopathy (9). The infant later died at an age of 14 months. Arg336 of EF-Tu_{mt} is located in domain II of the protein, in a region known to interact with the...
acceptor stem of the aa-tRNAs (Figure 4-1). EF-Tu$_{mt}$ R336Q was previously found to be unable to form a ternary complex with mitochondrial Ser-tRNA (10). The mutated factor was also deficient in poly(U)-directed poly(Phe) synthesis using mitochondrial Phe-tRNA and E. coli 70S ribosomes. However, no studies were carried out with mitochondrial ribosomes and mitochondrial elongation factor G (EF-G$_{1mt}$) (10). In this study, the activity of the mutated EF-Tu$_{mt}$ has been examined on mitochondrial ribosomes, and the effect of the mutation on its ability to form ternary complexes with several additional mitochondrial aa-tRNAs has been examined.

**MATERIALS AND METHODS**

**Materials**

$[^{14}\text{C}]$phenylalanine and $[^{35}\text{S}]$methionine were purchased from Perkin Elmer Life Sciences, Inc. E. coli tRNA was obtained from Boehringer Mannheim. E. coli $[^{14}\text{C}]$Phe-tRNA and the human mitochondrial $[^{35}\text{S}]$Met-tRNA transcript were prepared as described (11;12). Mitochondrial tRNA was purified from bovine mitoplasts using the Qiagen RNA/DNA maxi kit. The tRNA$_{\text{Phe}}$ was aminoacylated using $[^{14}\text{C}]$Phe as described (13). E. coli ribosomes were purified from E. coli W cells; mitochondrial 55S ribosomes, and EF-G$_{1mt}$ were purified as described (14;15).

**Cloning, Expression, and Purification of Proteins**

BMtu/pET24c and BMts/pET24c, the E. coli expression vectors for C-terminal histidine-tagged bovine EF-Tu$_{mt}$ and EF-Ts$_{mt}$, are described in (16;17) and the proteins
expressed from them are referred to as the wild-type (WT) proteins. Expression vectors for the mutant EF-Tu_{mt} (R336Q) and mutant EF-Ts_{mt} (R325W) were constructed by using the Quik Change site-directed mutagenesis kit (Stratagene). EF-Tu_{mt} and EF-Ts_{mt} were purified using described protocols (4;16;18).

Circular Dichroism of EF-Ts_{mt} and EF-Ts_{mt} R325W

Purified EF-Ts_{mt} and EF-Ts_{mt} R325W were dialyzed in 1000 volumes CD buffer containing: 10 mM MgSO_{4}, 50 mM potassium phosphate buffer (pH 7.6), and 5% glycerol for 4 h with a change of buffer after 2 h. CD spectra of EF-Ts_{mt} and EF-Ts_{mt} R325W were obtained using an Aviv Model 62DS CD Spectrometer. Data points were collected in 1 nm steps, and data was analyzed using the CD Pro software.

Poly(Phe) Polymerization Assays

To test the ability of EF-Ts_{mt} to stimulate the activity of EF-Tu_{mt} in poly(Phe) directed polymerization, reaction mixtures (25 µL) contained 50 mM Tris-HCl (pH 7.8), 1 mM DTT, 0.1 mM spermine, 6 mM MgCl_{2}, 80 mM KCl, 0.5 mM GTP, 1.25mM PEP, 0.4 U pyruvate kinase, 3 µg poly(U) mRNA, 8.3 pmol (0.33 µM) E. coli [^{14}C]Phe-tRNA, 5 pmol (0.2 µM) EF-G1_{mt}, 0.25 µM E. coli 70S ribosomes, 0.5 pmol (20 nM) EF-Tu_{mt}, and 0.025-0.075 pmol (1-3 nM) EF-Ts_{mt}. The reaction mixtures were incubated at 37 °C for 30 min and analyzed as described (19). A blank representing the amount of label retained on the filter in the absence of EF-Ts_{mt} was not subtracted.
To test the poly(Phe) polymerization activity of WT and mutated EF-Tu\textsubscript{mt} in the \textit{E. coli} system, reaction mixtures (25 µL) contained 50 mM Tris-HCl (pH 7.8), 1 mM DTT, 0.1 mM spermine, 6 mM MgCl\textsubscript{2}, 80 mM KCl, 0.5 mM GTP, 1.25 mM PEP, 0.4 U pyruvate kinase, 3 µg poly(U), 8.3 pmol (0.3 µM) \textit{E. coli} [\textsuperscript{14}C]Phe-tRNA, 5 pmol (0.2 µM) EF-G1\textsubscript{mt}, 16 µg (0.25 µM) \textit{E. coli} 70S ribosomes, and 0.2-0.8 pmol (8-32 nM) EF-Tu\textsubscript{mt} (WT or R336Q) in a 1:1 mix with EF-Ts\textsubscript{mt}. Reaction mixtures were incubated at 37 °C for 30 min, at which time they were quenched by the addition of 5% TCA and processed as described (19). A blank representing the amount of label retained on the filter in the absence of EF-Tu\textsubscript{mt} (~0.3 pmol) was subtracted from each value.

For assays performed in the mitochondrial system, the reaction mixtures (25 µL) contained 50 mM Tris-HCl (pH 7.8), 1 mM DTT, 0.1 mM spermine, 7.5 mM MgCl\textsubscript{2}, 40 mM KCl, 0.5 mM GTP, 1.25 mM PEP, 0.4 U pyruvate kinase, 3 µg poly(U), 3 pmol (0.12 µM) mitochondrial [\textsuperscript{14}C]Phe-tRNA, 5 pmol (0.2 µM) EF-G1\textsubscript{mt}, 3 pmol (0.12 µM) mitochondrial 55S ribosomes, and 0.2-0.8 pmol EF-Tu\textsubscript{mt} (WT or the R336Q mutated protein) in a 1:1 mixture with EF-Ts\textsubscript{mt}. Reactions were incubated at 37 °C for 30 min, quenched by the addition of 5% TCA, and processed as described (19). A blank representing the amount of label retained on the filter in the absence of EF-Tu\textsubscript{mt} (~0.08 pmol) was subtracted from each value.

\textit{Physical Interaction of EF-Tu\textsubscript{mt} and EF-Ts\textsubscript{mt} Measured Using a Gel-Shift Assay}

The binding of EF-Ts\textsubscript{mt} to EF-Tu\textsubscript{mt} was studied using a gel-shift assay according to (20). Briefly, EF-Ts\textsubscript{mt}:GDP (40 pmol) and EF-Tu\textsubscript{mt} (0-40 pmol) were incubated on ice for 5 min in a reaction mixture (10 µL) containing 50 mM Tris-HCl (pH 7.5), 70 mM KCl, 1.5 mM EDTA,
10% glycerol, 1 mM DTT, 0.004% bromophenol blue. The samples were resolved by native polyacrylamide gel electrophoresis (PAGE). The electrophoresis was performed at 4°C (450 V, 2 h) on a 1 mm x 20 cm x 10 cm 9% PAGE gel (acrylamide:bisacrylamide 29:1) containing 8 mM Tricine-NaOH (pH 8.2), 1 mM EDTA and 5% glycerol. The running buffer contained 8 mM Tricine-NaOH (pH 8.2) and 1 mM EDTA. The proteins were visualized using SYPRO Ruby (Molecular Probes).

*Ternary Complex Formation Measured Using Hydrolysis Protection*

The ability of EF-Tu<sub>mt</sub> and EF-Tu<sub>mt</sub> R336Q to protect aa-tRNA from spontaneous deacylation was monitored as described (20), with slight modifications. Ternary complexes were formed in reaction mixtures (100 µL) containing 75 mM Tris-HCl (pH 7.5), 75 mM NH₄Cl, 15 mM MgCl₂, 7.5 mM DTT, 60 µg/mL BSA, 1 mM GTP, 2.4 mM PEP, 0.1 mU pyruvate kinase, 12.5 pmol (0.125 µM) *E. coli* [¹⁴C]Phe-tRNA, and 0-330 pmol (0-3.3 µM) EF-Tu<sub>mt</sub> (WT or R336Q). The samples were incubated for the indicated times at 30 °C, and precipitated in cold 5% TCA. The remaining aa-tRNA was collected on filter papers (3MM, Whatman) and the amount of EF-Tu:GTP:[¹⁴C]Phe-tRNA was quantified using a liquid scintillation counter.

*Ternary Complex Formation Measured Using RNase Protection*

Ternary complex formation assays were carried out in reaction mixtures (50 µL) containing 20 mM HEPES-KOH (pH 7.0), 1 mM DTT, 6.7 mM MgCl₂, 68 mM KCl, 0.5 mM GTP, 1.25 mM PEP, 0.8 U pyruvate kinase, 7.2 pmol (0.14 µM) *E. coli* [¹⁴C]Phe-tRNA, and 0-20 pmol (0.1-0.4 µM) EF-Tu<sub>mt</sub> (WT or R336Q). For experiments using mitochondrial tRNAs, 5.3
pmol (0.11 µM) native bovine mitochondrial [14C]Phe-tRNA or 9.6 pmol (0.19 µM) of the human mitochondrial [35S]Met-tRNA transcript and 0-60 pmol (0.3-1.2 µM) EF-Tu_{mt} (WT or R336Q) were used. The reactions were incubated for 15 min at 0 °C, at which time 10 µg RNase A was allowed to digest the sample for 30 s. Ice cold 5% TCA was added, and the remaining aa-tRNA was precipitated at 0 °C for 10 min and analyzed as described (12;21). A blank representing the amount of label retained on the filters in the absence of EF-Tu_{mt} (~0.3 pmol using E. coli [14C]Phe-tRNA, ~0.1 pmol using mitochondrial [14C]Phe-tRNA and ~0.2 pmol using [35S]Met-tRNA) was subtracted from each value.

RESULTS AND DISCUSSION

Biochemical Consequences of the R325W Mutation in EF-Ts_{mt}

A mutated derivative of EF-Ts_{mt}, corresponding to the lethal R325W mutation in humans, was prepared using site-directed mutagenesis (3). To determine whether the mutation adversely affected the structure of EF-Ts_{mt}, the CD spectra of the mutated derivative was compared to that of the

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Figure 4-3. Circular dichroism spectra of EF-Ts_{mt} and EF-Ts_{mt} R325W. CD spectra of EF-Ts_{mt} (WT and R325W) obtained as described in Materials and Methods.
WT factor. As indicated in Figure 4-3, the α-helical content of the mutated protein did not change significantly with respect to the wild-type factor, suggesting that the protein retained its normal secondary structure. A thermal melt monitored by CD indicated that both EF-Ts_{mt} and EF-Ts_{mt} R325W unfold at the same temperature (data not shown), indicating that the mutation has not greatly destabilized the protein.

To investigate the biochemical effect of the R325W mutation, the ability of EF-Ts_{mt} and its R325W variant to stimulate the activity of EF-Tu_{mt} in poly(Phe) polymerization was examined. EF-Tu_{mt} alone has measurable activity in polymerization (Figure 4-4). However, its activity is enhanced by the presence of EF-Ts_{mt} due to an increase in the guanine nucleotide exchange rate, allowing EF-Tu_{mt} to operate with a higher turnover number. Wild-type EF-Ts_{mt} stimulated the activity of EF-Tu_{mt} more than 2-fold under the reaction conditions.

<table>
<thead>
<tr>
<th>EF-Ts (pmol)</th>
<th>[14C]Phe polymerized (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>0.0</td>
</tr>
<tr>
<td>0.03</td>
<td>0.2</td>
</tr>
<tr>
<td>0.05</td>
<td>0.4</td>
</tr>
<tr>
<td>0.07</td>
<td>0.6</td>
</tr>
</tbody>
</table>

![Figure 4-4](image)

Figure 4-4. Activity of EF-Ts_{mt} and EF-Ts_{mt} R325W in poly(Phe) polymerization. The abilities of EF-Ts_{mt} and EF-Ts_{mt} R325W to function in a poly(U)-directed peptide polymerization assay were tested using EF-Tu_{mt} and *E. coli* [14C]Phe-tRNA. EF-Ts_{mt} is shown in closed squares and EF-Ts_{mt} R325W is shown in closed circles. The assays were performed multiple times and data were normalized with respect to the point containing 0.05 pmol of EF-Ts_{mt}. 


conditions used (Figure 4-4). This value is consistent with previous observations (22). However, the stimulation of EF-Tu\textsubscript{mt} by the R325W variant was reduced substantially, and only a 1.2-fold stimulation was observed (Figure 4-4). This reduction could arise from the failure of the mutated EF-Ts\textsubscript{mt} to interact with EF-Tu\textsubscript{mt}:GDP effectively, reducing GDP release and formation of an EF-Tu\textsubscript{mt}:EF-Ts\textsubscript{mt} complex. The reduction could also reflect a failure of the EF-Tu\textsubscript{mt}:EF-Ts\textsubscript{mt} complex to bind GTP, which is necessary to release EF-Tu\textsubscript{mt} for another round of polymerization.

To investigate the interaction between EF-Tu\textsubscript{mt} and EF-Ts\textsubscript{mt} R325W directly, the binding of the two proteins was studied using a gel-shift assay (Figure 4-5). EF-Ts\textsubscript{mt} or EF-Ts\textsubscript{mt} R325W was incubated with increasing amounts of EF-Tu\textsubscript{mt}. After complex formation, the proteins were separated on native-PAGE. This gel system separates EF-Ts\textsubscript{mt} from the EF-Tu\textsubscript{mt}:EF-Ts\textsubscript{mt} complex.

However, the mobility of EF-Tu\textsubscript{mt}:EF-Ts\textsubscript{mt} complex was quite similar to that for EF-Tu\textsubscript{mt}, and the resolution of the complex from free EF-Tu\textsubscript{mt} was R325W

![Figure 4-5. Physical interaction of EF-Ts\textsubscript{mt} and EF-Ts\textsubscript{mt} R325W with EF-Tu\textsubscript{mt}. The abilities of EF-Ts\textsubscript{mt} and EF-Ts\textsubscript{mt} R325W to bind to EF-Tu\textsubscript{mt} were measured using a gel shift assay on native PAGE. WT EF-Ts\textsubscript{mt} (upper panel) or EF-Ts\textsubscript{mt} R325W (lower panel) were incubated with the indicated amount of EF-Tu\textsubscript{mt}. The proteins were separated on native-PAGE and visualized by SYPRO Ruby. The position of EF-Ts\textsubscript{mt}, EF-Tu\textsubscript{mt}:EF-Ts\textsubscript{mt} complex and EF-Tu\textsubscript{mt} are indicated with arrows. Data and figure provided by K. Akama and N. Takeuchi.](image-url)
difficult. Thus, the efficiency of complex formation was assessed by the quantity of free EF-Ts_{mt} remaining. Wild-type EF-Ts_{mt} was effective in forming a complex with EF-Tu_{mt} in a dose dependent manner. Approximately 60% of EF-Ts_{mt} bound EF-Tu_{mt} when incubation was carried out using equal molar amounts of the two factors (Figure 4-5, upper and Figure 4-6, closed circles). In contrast, EF-Ts_{mt} R325W was ineffective in binding EF-Tu_{mt} and remained free, even when incubated with an equal amount of EF-Tu_{mt} (Figure 4-5, lower and Figure 4-6, closed squares). These results indicate that the reduction in poly(U)-dependent poly(Phe) synthesis with the mutated EF-Ts_{mt} arises to a large extent from a failure of the mutated EF-Ts_{mt} to interact with EF-Tu_{mt}.

This effect observed with the R325W mutation is in agreement with previous mutational data as described in the introduction (9). The data provided here and the mutational studies carried out previously indicate that the interactions of residues in the β-sheet of subdomain C and other nearby residues with EF-Tu_{mt} are essential for the stable binding of EF-Tu_{mt} with EF-Ts_{mt}.

![Figure 4-6. Efficiency of the complex formation in Figure 4-5 assessed by quantitating the amount of free EF-Ts_{mt} remaining. The relative amount of free EF-Ts_{mt} in each panel was normalized to a value of 1 for EF-Ts_{mt}. EF-Ts_{mt} is shown in closed circles, and EF-Ts_{mt} R325W is shown in closed squares. Data for figure provided by K. Akama and N. Takeuchi.](image-url)
Biochemical Consequences of the R336Q Mutation in EF-Tu\textsubscript{mt}

Previous work on the lethal R336Q mutation in EF-Tu\textsubscript{mt} indicated that this mutation did not affect the structural integrity of EF-Tu\textsubscript{mt}, as demonstrated by CD spectroscopy (10). However, the mutation drastically reduced the ability of EF-Tu\textsubscript{mt} to promote polymerization using mitochondrial $[^{14}\text{C}]$Phe-tRNA on \textit{E. coli} ribosomes (10). The R336Q mutation was not predicted to influence the interaction of EF-Tu\textsubscript{mt} with EF-Ts\textsubscript{mt}, because the primary interaction with EF-Ts\textsubscript{mt} occurs via interactions involving domains I and III (4). The mutation was also not expected to interfere with GTP binding, which is localized to domain I. Further, previous data indicated that Glu287 in \textit{E. coli} (corresponding to Arg335 in EF-Tu\textsubscript{mt}), the residue adjacent to Arg336, did not affect GTP binding (21). Mutation of Arg335 to glutamic acid did result in reduced activity of EF-Tu\textsubscript{mt} in both ternary complex formation and A-site binding with mitochondrial Phe-tRNA$^{\text{Phe}}$.

The effects of the R336Q mutation on the activity of EF-Tu\textsubscript{mt} were examined further using \textit{E. coli} and mitochondrial ribosomes and aa-tRNAs. Surprisingly, when EF-Tu\textsubscript{mt} R336Q was tested in poly(U)-directed polymerization using \textit{E. coli} $[^{14}\text{C}]$Phe-tRNA and \textit{E. coli} 70S ribosomes, it was as active as the wild-type factor in this assay (Figure 4-7). However, in the homologous system using mitochondrial $[^{14}\text{C}]$Phe-tRNA and ribosomes, EF-Tu\textsubscript{mt} R336Q was almost inactive in polypeptide chain elongation (Figure 4-8). Previous work (10) indicated that EF-Tu\textsubscript{mt} R336Q lacked activity with mitochondrial Phe-tRNA when tested on \textit{E. coli} ribosomes, suggesting that the lack of activity in the mitochondrial system was not a function of the ribosomes used but, rather, due to a failure of the mutated factor to interact with mitochondrial Phe-tRNA. Mitochondrial tRNAs are less structurally stable than \textit{E. coli}
tRNAs (12), and additional stabilizing interactions between EF-Tu\textsubscript{mt} and the mitochondrial aa-tRNA may be important in forming the ternary complex.

The poly(Phe) polymerization assay does not directly measure the interaction between EF-Tu\textsubscript{mt} and aa-tRNA. This interaction can be measured directly using one of two ternary complex formation assays: the hydrolysis protection assay and the RNase protection assay. The hydrolysis protection assay measures the ability of EF-Tu\textsubscript{mt} to protect the aa-tRNA bond from spontaneous hydrolysis. Since the mutated

Figure 4-7. Poly(Phe) polymerization with EF-Tu\textsubscript{mt} and EF-Tu\textsubscript{mt} R336Q using *E. coli* 70S ribosomes and *E. coli* \[^{14}\text{C}]\text{Phe}\text{-tRNA}. The abilities of EF-Tu\textsubscript{mt} and its R336Q derivative to function in a poly(Phe) polymerization assay was tested as described in Materials and Methods. EF-Tu\textsubscript{mt} is shown in closed circles and EF-Tu\textsubscript{mt} R336Q in closed squares.

Figure 4-8. Poly(Phe) polymerization with EF-Tu\textsubscript{mt} and EF-Tu\textsubscript{mt} R336Q using mitochondrial 55S ribosomes and native bovine mitochondrial \[^{14}\text{C}]\text{Phe}\text{-tRNA}. The abilities of EF-Tu\textsubscript{mt} and its R336Q derivative to function in a poly(Phe) polymerization assay was tested as described in Materials and Methods. EF-Tu\textsubscript{mt} is shown in closed circles and EF-Tu\textsubscript{mt} R336Q is shown in closed squares.
EF-Tu<sub>mt</sub> was active using E. coli Phe-tRNA in polymerization, its interaction with bacterial Phe-tRNA was tested directly. The spontaneous hydrolysis of E. coli Phe-tRNA was apparent (Figure 4-9, closed circles). When bound to EF-Tu<sub>mt</sub>, the Phe-tRNA bond was clearly protected from hydrolysis (Figure 4-9, solid lines). However, when the R336Q derivative of EF-Tu<sub>mt</sub> was used in place of the normal factor, substantially less protection of the Phe-tRNA bond was observed (Figure 4-9, dashed lines). At sufficiently high concentrations of EF-Tu<sub>mt</sub>,

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**Figure 4-9.** Activities of EF-Tu<sub>mt</sub> and EF-Tu<sub>mt</sub> R336Q in ternary complex formation measured using a non-enzymatic hydrolysis protection assay. Amount of [<sup>14</sup>C]Phe-tRNA protected from hydrolysis by increasing concentrations of EF-Tu<sub>mt</sub> (solid lines) and EF-Tu<sub>mt</sub> R336Q (dashed lines) as a function of time. Data for figure provided by K. Akama and N. Takeuchi.

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**Figure 4-10.** Relative amount of [<sup>14</sup>C]Phe-tRNA at time 60 min in Figure 4-9 plotted against the EF-Tu<sub>mt</sub> concentration. Data for figure provided by K. Akama and N. Takeuchi.
R336Q, considerable ternary complex is formed (Figure 4-10, closed squares), indicating that R336Q has some ability to bind *E. coli* Phe-tRNA. This data suggests that, although the mutated protein is deficient in binding to *E. coli* Phe-tRNA, the reduced affinity is not sufficient to prevent its efficient use in polymerization (Figure 4-7).

To verify the results obtained from the hydrolysis protection experiments and to examine the interaction with mitochondrial aa-tRNAs, the RNase protection assay was used. This assay measures the ability of EF-Tu<sub>mt</sub> to protect the acceptor stem of the aa-tRNA from degradation by RNase A. Because the RNase A digestion of the tRNA is done in 30 s, it provides a snapshot of the interaction between the two molecules. When the binding of EF-Tu<sub>mt</sub> to *E. coli* [<sup>14</sup>C]Phe-tRNA was tested, the R336Q variant showed a decrease in binding of just over 50% (Figure 4-11), indicating that the mutation reduces but does not abolish the ability of EF-Tu<sub>mt</sub> to form a ternary complex with bacterial aa-tRNAs. These results agree with the observation above, that the reduction in ternary complex formation is not sufficient to compromise the activity of this factor in polymerization (Figure 4-7). It is important to note that the binding of the aa-tRNA to EF-Tu<sub>mt</sub> is probably not the
rate limiting step in polypeptide chain elongation. Rather, interactions with the ribosome, including the release of inorganic phosphate from EF-Tu\textsubscript{mt} following GTP hydrolysis, appear to be rate limiting (23).

Previous work indicated that EF-Tu\textsubscript{mt} R336Q did not bind mitochondrial Ser-tRNA effectively in the ternary complex (10). This observation is in sharp contrast to the significant level of ternary complex formation observed with the \textit{E. coli} Phe-tRNA (Figure 4-11) but in agreement with the lack of polymerization observed with mitochondrial Phe-tRNA (Figure 4-8). To explore whether the failure of EF-Tu\textsubscript{mt} to use mitochondrial Phe-tRNA in polymerization arises from a failure of ternary complex formation, the RNase protection assay was carried out with mitochondrial [\textsuperscript{14}C]Phe-tRNA (Figure 4-12). EF-Tu\textsubscript{mt} was quite active in forming a ternary complex with native bovine mitochondrial Phe-tRNA. However, the R336Q variant did not bind mitochondrial Phe-tRNA detectably (Figure 4-12). This observation agrees with previous work and explains the lack of activity of the variant protein in poly(Phe)-directed polymerization with mitochondrial Phe-tRNA on mitochondrial 55S ribosomes (Figure 4-8).
To address the question of whether other mitochondrial aa-tRNAs also fail to bind the R336Q variant, ternary complex formation assays were carried out using human mitochondrial [\(^{35}\)S]Met-tRNA (Figure 4-13). This tRNA was transcribed in vitro and has been shown to properly fold and to bind EF-Tu\textsubscript{mt} (12). In this assay, EF-Tu\textsubscript{mt} readily formed a ternary complex with mitochondrial [\(^{35}\)S]Met-tRNA. However, once again EF-Tu\textsubscript{mt} R336Q was unable to bind mitochondrial [\(^{35}\)S]Met-tRNA, indicating that the variant protein is unable to form ternary complexes with most or all mitochondrial aa-tRNAs.

It is perplexing that the variant EF-Tu\textsubscript{mt} can bind \textit{E. coli} Phe-tRNA but not mitochondrial aa-tRNAs. This discrepancy must stem from innate differences between the mitochondrial and bacterial tRNAs. Canonical tRNAs fold into a distinct L-shape stabilized by strong G-C base pairs in the stems of the tRNA and tertiary interactions involving many invariant or semi-invariant residues (24). In contrast, mitochondrial tRNAs are characterized by weaker stems with numerous A-U base pairs or mismatches. They often lack the conserved residues that create important tertiary interactions in other tRNAs (25-27).
Although mitochondrial tRNAs can fold into an L-shape, the interactions that stabilize the elbow region are weaker (28). Mitochondrial tRNAs observed on 55S ribosomes have an L-shape but show a “caved in” feature at the corner of the L (28). These unusual structural features of mitochondrial tRNAs may lead to a requirement for interactions with EF-Tu\textsubscript{mt} that are not essential for the binding of canonical aa-tRNAs.

Previous data indicate that mitochondrial aa-tRNAs must be phosphorylated at their 5’ ends in order to bind effectively to EF-Tu\textsubscript{mt} (12). The crystal structure of the \textit{T. thermophilus} ternary complex indicates that Arg300 (corresponding to Arg336 in EF-Tu\textsubscript{mt}) forms a salt bridge with the phosphate group at the 5’ end of the aa-tRNA (Figure 4-14) (6). Replacement of the Arg with Gln in EF-Tu\textsubscript{mt} could disrupt this interaction, since the side chain of Gln is shorter and may not be within bridging distance of the phosphate backbone. This interaction is not critical for the formation of the ternary complex with \textit{E. coli} aa-tRNAs, in agreement with data showing that a 5’-phosphate is not important for ternary complex formation in prokaryotes (29). However, the data presented here indicate that the interaction between Arg336 and the 5’ phosphate of mitochondrial aa-tRNAs is critical for ternary complex formation in the mitochondrial translational system. The interaction between Arg336 and the 5’ phosphate of aa-tRNAs could be also important following the ternary complex formation, since the aa-tRNA in the ternary complex is substantially distorted upon ribosome binding, allowing it to interact simultaneously with the mRNA codon and EF-Tu\textsubscript{mt} (30).
CONCLUSION

Oxidative phosphorylation requires the assembly of the electron transport chain and ATP synthase, which contain protein components synthesized by the mitochondrial translational machinery. A defect in mitochondrial translation caused by the inability of EF-Ts_{mt} R325Q to bind to EF-Tu_{mt} likely leads to the defects in oxidative phosphorylation seen in the clinical manifestation of the EF-Ts_{mt} mutation.

The R336Q variant of EF-Tu_{mt} is inactive in mitochondrial polypeptide chain elongation as a result of its inability to bind mitochondrial aa-tRNAs. Failure of ternary complex formation leads to defective mitochondrial protein synthesis, causing a decrease in
oxidative phosphorylation. One direct clinical phenotype of the EF-Tu\textsubscript{mt} R336Q mutation is lactic acidosis (9), which most likely resulted from the buildup of lactic acid from pyruvate due to a failure in the synthesis of the respiratory chain.


CHAPTER 5

THE EFFECT OF SPERMINE ON THE INITIATION OF MITOCHONDRIAL PROTEIN SYNTHESIS

The majority of the work reported in this chapter has been published (1) and reprinted with permission.
INTRODUCTION

Spermine and spermidine are aliphatic polyamines that have a number of roles in both prokaryotic and eukaryotic cells. Both polyamines are cationic at physiological pH due to pK values >8 (2). In prokaryotic cells, polyamines are known to affect protein synthesis in several ways. The structure of the SD region on the mRNA is rearranged in the presence of polyamines, which helps promote the formation of the prokaryotic initiation complex (3).

In addition to this structural effect, polyamines lower the optimal concentration of Mg$^{2+}$ required for protein synthesis. Translation initiation on the minor start codon UUG, used, for example, in the translation of adenylate cyclase, is stimulated by polyamines (4). The tertiary polyamine spermidine (Figure 5-1) reduces the dissociation of 70S ribosomes, partially counteracting the action of IF3 in the initiation of translation (5). Spermine, a quaternary polyamine (Figure 5-2), has binding sites on both 16S and 23S rRNA, indicating that it could be important for ribosomal structure (6;7). Spermine crosslinks near the decoding center of the small subunit, suggesting that it could also be involved in aa-tRNA binding (6).

In wheat germ, spermidine has been shown to affect translation by increasing the fidelity of protein synthesis (8). Spermine exists in the cytosol of eukaryotic cells with concentrations in the µM range (9) and is known to be present in mitochondria although it...
is not synthesized there (10). In fact, spermine plays a metabolic role in mitochondria by stimulating the activity of the pyruvate dehydrogenase complex and by activating citrate synthase in the citric acid cycle (10). Because spermine binds to the mitochondrial membrane, the concentration of spermine inside the mitochondrion is difficult to measure accurately and is, therefore, not known (11).

Spermine affects translation in mitochondria by lowering the Mg$^{2+}$ requirement for the association of mitochondrial ribosomal subunits into 55S monosomes by about 1 mM (12). Little is known about other effects that spermine may have on protein synthesis in mitochondria. Because mitochondrial mRNAs are largely leaderless, the effects of spermine on mitochondrial translation may be different from those observed with prokaryotic mRNAs, most of which contain SD sequences.

**MATERIALS AND METHODS**

*Materials*

General chemicals were purchased from Sigma-Aldrich or Fisher Scientific. Bovine mitochondrial ribosomes (55S), ribosomal subunits (28S and 39S), and yeast [$^{35}$S]Met-tRNA were prepared as described (13-16). Poly(A,U,G) was synthesized and purified as described (17). The AUG triplet was purchased from Dharmaco. A 29-mer RNA oligonucleotide
containing the 5' AUG start site (underlined) for subunit I of bovine mitochondrial cytochrome oxidase, AUGUUAUAAACCGCUGACUAUUCUCAAC, was also purchased from Dharmacon.

**Cloning, Expression and Purification**

Bovine IF2<sub>mt</sub> and human IF3<sub>mt</sub> were cloned, expressed, and purified as described previously (18). Both proteins required further purification following the Ni-NTA column. IF2<sub>mt</sub> and IF3<sub>mt</sub> were purified on DEAE-5PW and SP-5PW HPLC columns, respectively, as described (18).

**Dissociation of Mitochondrial 55S Ribosomes by IF3<sub>mt</sub> in the Presence and Absence of Spermine**

Mitochondrial ribosomes (8 pmol, 80 nM) were incubated in the presence or absence of IF3<sub>mt</sub> (41 pmol, 410 nM) and in the presence and absence of spermine (0.1 mM) in 100 µL of gradient buffer containing 25 mM Tris-HCl (pH 7.6), 5 mM MgCl<sub>2</sub>, 40 mM KCl and 1 mM DTT for 15 min at 37 °C. After incubation, reaction mixtures were placed on ice for 10 min and then layered onto cold 4.8 mL 10-30% linear sucrose gradients prepared in gradient buffer with and without spermine. Gradients were centrifuged for 1 h and 45 min at 220,000 g ave in the Beckman SW55 Ti rotor and fractionated as described (19).
Initiation Complex Formation on Mitochondrial Ribosomes

Stimulation of initiation complex formation by spermine was examined by measuring the increase of $[^{35}\text{S}]\text{fMet-tRNA}$ binding to 55S ribosomes or 28S subunits in a filter binding assay. Reaction mixtures (100 μL) were prepared as described previously (16;20) and contained the indicated amounts of IF2$_{mt}$ and/or IF3$_{mt}$, $[^{35}\text{S}]\text{fMet-tRNA}$ (60 nM), 0.25 mM GTP, 1.25 mM PEP, 0.9 U pyruvate kinase, 55S ribosomes (60 nM) and, unless otherwise indicated, 10 μg poly(A,U,G). Mixtures were incubated for 10 min at 37 °C. The amount of $[^{35}\text{S}]\text{fMet-tRNA}$ bound to ribosomes was measured using a nitrocellulose filter binding assay (20;21).

RESULTS AND DISCUSSION

Effect of Spermine on Initiation Complex Formation on Mitochondrial 55S Ribosomes

In the absence of mRNA, fMet-tRNA is able to bind mitochondrial ribosomes to a limited extent (20). Thus, the effect of spermine on the binding of fMet-tRNA to mitochondrial 55S ribosomes was tested in the presence and absence of mRNA, IF2$_{mt}$, and IF3$_{mt}$ (Figure 5-3). In the presence of both initiation factors and poly(A,U,G), a two-fold increase in fMet-tRNA binding correlated with increasing concentrations of spermine up to 0.05 mM (Figure 5-3). This result indicates that spermine has a positive effect on initiation. This effect could occur at one or more or several steps, including: fMet-tRNA binding, mRNA binding, the action of IF2$_{mt}$, or the action of IF3$_{mt}$.

If spermine primarily affects fMet-tRNA binding by promoting the interaction of the mRNA with the ribosome, one might expect a smaller effect of spermine on fMet-tRNA
binding in the absence of mRNA. Binding of fMet-tRNA to ribosomes was significantly lower in the absence of mRNA (Figure 5-3); however, spermine stimulated this binding two-fold, as observed in the presence of mRNA, although higher concentrations of spermine were required to observe this effect. This observation suggests that spermine is affecting a step in initiation distinct from the mRNA binding step.

To further explore this conclusion, we have more fully examined the effects of spermine on initiation complex formation with three different mRNAs (Figure 5-4). These mRNAs included the AUG triplet, a 29-nucleotide RNA derived from the initiation site of bovine mitochondrial subunit I of cytochrome oxidase, and poly(A,U,G). The assays shown in Figure 5-3 were carried out using poly(A,U,G), a long RNA polymer that is not representative of natural mitochondrial mRNAs. Native mitochondrial messages contain few or no nucleotides 5′ to the initiation codon, so the AUG triplet and the leaderless CoI mRNA were used.
In the absence of polyamines, substantial binding of fMet-tRNA to the ribosome was observed with poly(A,U,G). Spermine stimulated this binding nearly two-fold. When the AUG triplet was used in initiation complex formation (Figure 5-4), less fMet-tRNA binding was observed in the absence of spermine, and this polylamine stimulated binding about three-fold, bringing it close to the level observed with poly(A,U,G).

Natural mitochondrial mRNAs are generally leaderless. To examine the effect of spermine on a natural initiation site, we used a 29-mer synthetic oligonucleotide based on the initiation site of subunit I of the bovine cytochrome oxidase mRNA. When this oligonucleotide was tested for initiation complex formation in the absence of spermine, it showed about the same level of binding as observed with the AUG triplet (Figure 5-4). Again, spermine stimulated binding about three-fold.

The more substantial binding obtained in the absence of spermine with poly(A,U,G) probably reflects the ability of this long polymer to trap fMet-tRNA more effectively on the ribosome. Long mRNAs are known to bind more rapidly to the small subunit and to
dissociate more slowly than shorter mRNAs (22). Since stable initiation complex formation requires the simultaneous presence of both fMet-tRNA and mRNA on the ribosome, the more stable binding of poly(A,U,G) to the ribosome allows more initiation complex formation to be detected in the absence of spermine. Regardless, spermine stimulates fMet-tRNA binding in the absence of any mRNA (Figure 5-3) and in the presence of all the mRNAs tested (Figure 5-4), indicating that the effect of this polyamine on initiation is not directly on mRNA binding to the ribosome.

Effect of Spermine on IF3\textsubscript{mt}-Mediated Stimulation of Initiation Complex Formation

Another potential target for the effect of spermine is on the action of IF3\textsubscript{mt}. The major effect of IF3\textsubscript{mt} on initiation complex formation is to loosen the interactions between the ribosomal subunits, making the small subunit available for fMet-tRNA binding. Since spermine is expected to tighten subunit interactions, one might predict that spermine would increase the amount of IF3\textsubscript{mt} required for initiation. To test this theory, fMet-tRNA binding to mitochondrial ribosomes was examined in the presence of IF2\textsubscript{mt} with
increasing amounts of IF3\textsubscript{mt} (Figure 5-5). In the presence of IF2\textsubscript{mt} and mRNA, the addition of IF3\textsubscript{mt} caused a 2-fold increase in fMet-tRNA binding in the absence of spermine (Figure 5-5). This stimulation is believed to arise because IF3\textsubscript{mt} promotes the dissociation of ribosomal subunits and, therefore, enhances initiation complex formation. Surprisingly, the addition of spermine did not result in a need for a higher concentration of IF3\textsubscript{mt} to promote initiation complex formation. In the presence of spermine, IF3\textsubscript{mt} increased the amount of fMet-tRNA bound to 55S ribosomes with basically the same dose response curve as observed in the absence of spermine (Figure 5-5). Spermine caused a 1.4-fold stimulation of fMet-tRNA binding independent of the amount of IF3\textsubscript{mt} added. This observation suggests that the effect of spermine is not directly on IF3\textsubscript{mt}.

In prokaryotes, spermine has been shown to change the conformation of the 30S ribosomal subunit to favor subunit association (6). It also reduces the electrostatic repulsion between the subunits by masking the charges on the rRNA (23). In both prokaryotes and mitochondria, spermine has been shown to tighten the interaction between the ribosomal subunits (12;24). The effect of spermine on the ability of IF3\textsubscript{mt} to promote subunit dissociation was tested directly using sucrose density gradient centrifugation (Figure 5-6). In the absence of both IF3\textsubscript{mt} and spermine, mitochondrial ribosomes existed primarily as 55S particles at 5 mM Mg\textsuperscript{2+}, although a small amount of dissociation was observed (Figure 5-6). The addition of spermine led to a stronger interaction between the ribosomal subunits, and very few subunits were observed in its presence. In the absence of spermine, IF3\textsubscript{mt} was able to promote the dissociation of ribosomal subunits. However, in the presence of spermine, IF3\textsubscript{mt} had almost no effect on
the dissociation of 55S ribosomes. Thus, spermine appears to play a role in tightening of the interactions between the mitochondrial 28S and 39S subunits. It is possible that, in the presence of spermine, IF3\textsubscript{mt} loosens mitochondrial subunit contacts enough to allow initiation complex formation but not enough to stably dissociate the ribosomal subunits. This observation argues that the effect of spermine is not on the action of IF3\textsubscript{mt} in promoting ribosomal subunit dissociation.

**Effect of Spermine on the Action of IF2\textsubscript{mt} in fMet-tRNA Binding to 55S Ribosomes**

IF2\textsubscript{mt} is known to stabilize the binding of fMet-tRNA to the P-site of the ribosome and, as expected, the amount of fMet-tRNA bound to the ribosome was quite low in the absence of IF2\textsubscript{mt} (Figure 5-7). However, even in the absence of IF2\textsubscript{mt}, spermine enhanced the binding of fMet-tRNA to the ribosome. This observation is in agreement with the data above, suggesting that the effect of spermine is directly on fMet-tRNA binding to the P-site. The presence of spermine did not significantly alter the stimulatory effect of IF2\textsubscript{mt}. As the

![Figure 5-6. Effect of spermine on IF3\textsubscript{mt}-induced 55S dissociation measured using sucrose density gradient centrifugation. Fractionation profiles of mitochondrial 55S ribosomes after centrifugation on 10-30% sucrose gradients as described in Materials and Methods.](image)
IF2<sub>mt</sub> concentration was increased, considerable stimulation of fMet-tRNA binding was observed as expected. However, the degree of stimulation of fMet-tRNA binding to the ribosome caused by spermine (1.6-fold) was constant at all concentrations of IF2<sub>mt</sub>. This observation suggests that spermine does not directly affect the action of IF2<sub>mt</sub>.

**Effect of Spermine on fMet-tRNA Binding to 28S Subunits**

The data above demonstrate that the effect of spermine is on fMet-tRNA binding to the ribosome; however, the question arises as to whether spermine affects fMet-tRNA interactions on the small or large ribosomal subunit. If fMet-tRNA binding to the small subunit is being affected, the same increase in fMet-tRNA binding should be observed on 28S subunits as was seen on 55S ribosomes. In the presence of increasing amounts of spermine, an increase in fMet-tRNA binding was observed in the presence of IF2<sub>mt</sub> (Figure 5-8). IF3<sub>mt</sub> was not needed in this assay, since no dissociation of ribosomal subunits was needed. The stimulatory effect of spermine was absolutely dependent on IF2<sub>mt</sub>, reflecting
the lower stability of fMet-tRNA bound to the small subunit alone. However, the same two-fold stimulation of fMet-tRNA binding by IF2\textsubscript{mt} was observed on both 28S subunits and 55S ribosomes. Thus, the full effect of spermine was seen on 28S subunits and can be attributed to a direct stimulation of fMet-tRNA binding to the P-site of the small ribosomal subunit.

**Effect of Polyamines on fMet-tRNA Binding to Prokaryotic and Mitochondrial Ribosomes**

Polyamines have been shown to be important for prokaryotic translational initiation, and the current work demonstrates that spermine is also important in the mitochondrial translational system. Because spermine was shown to crosslink to the decoding center of the prokaryotic ribosome, it was assumed that the effect of spermine could be on fMet-tRNA binding to the SSU. However, it was unclear whether the effect of spermine was primarily on the interaction of the tRNA with the rRNA or the proteins of the SSU. The crystal structure of the *T. thermophilus* ribosome demonstrates that the P-site bound tRNA

![Graph showing the effect of spermine on initiation complex formation using 28S subunits in the presence and absence of initiation factors. [\textsuperscript{35}S]fMet-tRNA binding to mitochondrial 28S subunits was tested using poly(A,U,G) as the mRNA in the presence and absence of IF2\textsubscript{mt} at different concentrations of spermine as described in Materials and Methods.]
has several important contacts with the SSU of the ribosome that could be affected by the presence of spermine (25). Ten of those contacts are with rRNA nucleotides on the SSU, and those ten residues are present in the truncated mitochondrial 12S rRNA. Spermine could enhance these interactions by shielding the repulsion between the backbones of the tRNA and the rRNA. The tRNA in the P-site of the prokaryotic ribosome only interacts with one SSU protein (S13), which is not present in mitochondrial ribosomes. However, additional protein contacts are made between the mitochondrial ribosome and the P-site tRNA (15). An interaction occurs between the T loop of the tRNA bound at the P-site and the P-site finger protein, a structure unique to the mitochondrial system (15). A further interaction is observed between the CCA-arm of the tRNA and an unknown small subunit protein. These protein interactions, unique to the mitochondrial system, could also be enhanced by spermine.

**CONCLUSION**

Initiation of translation in mitochondria is stimulated by the presence of spermine, a polyamine that occurs naturally in mitochondria. The stimulatory effect of spermine does not appear to depend on the mRNA used, the action of IF3_{mt}, or the action of IF2_{mt}. The data suggest that the binding of fMet-tRNA to the P-site of mitochondrial 55S ribosomes is directly stabilized by spermine. This effect could be due to the alteration of the conformations of the ribosomal subunits by spermine or to a direct stabilization of the contacts between fMet-tRNA and the ribosome. The observation that the full effect of spermine is observed on 28S subunits indicates that this polyamine is primarily enhancing
the interaction of the fMet-tRNA with the small subunit. Future work regarding translation in mitochondria should carefully consider the spermine concentration used.
REFERENCES


