Identification of Mammalian Mitochondrial Translational Initiation Factor 3 and Examination of Its Role in Initiation Complex Formation with Natural mRNAs*

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Abstract

Human mitochondrial translational initiation factor 3 (IF3mt) has been identified from the human expressed sequence tag data base. Using consensus sequences derived from conserved regions of the bacterial IF3, several partially sequenced cDNA clones were identified, and the complete sequence was assembled in silico from overlapping clones. IF3mt is 278 amino acid residues in length. MitoProt II predicts a 97% probability that this protein will be localized in mitochondria and further predicts that the mature protein will be 247 residues in length. The cDNA for the predicted mature form of IF3mt was cloned, and the protein was expressed in Escherichia coli in a His-tagged form. The mature form of IF3mt has short extensions on the N and C termini surrounding a region homologous to bacterial IF3. The region of IF3mt homologous to prokaryotic factors ranges between 21–26% identical to the bacterial proteins. Purified IF3mt promotes initiation complex formation on mitochondrial 55 S ribosomes in the presence of mitochondrial initiation factor 2 (IF2mt), [35S]fMet-tRNA, and either poly(A,U,G) or an in vitro transcript of the cytochrome oxidase subunit II gene as mRNA. IF3mt shifts the equilibrium between the 55 S mitochondrial ribosome and its subunits toward subunit dissociation. In addition, the ability of E. coli initiation factor 1 to stimulate initiation complex formation on E. coli 70 S and mitochondrial 55 S ribosomes was investigated in the presence of IF2mt and IF3mt.

Mammalian mitochondria synthesize 13 polypeptides that are essential for oxidative phosphorylation. These 13 proteins are translated from nine monocistronic and two dicistronic mRNAs with overlapping reading frames (1, 2). The protein-synthesizing system of mammalian mitochondria has a number of interesting features not observed in prokaryotes or the cell cytoplasm (3). The mRNAs in this organelle have an almost complete lack of 5′- and 3′-untranslated nucleotides. The start codon is generally located within three nucleotides of the 5′ end of the mRNA (1, 4). Thus, mammalian mitochondrial ribosomes do not recognize the start codon using the Shine/Dalgonaro interaction between the mRNA and the 16 S rRNA as observed in prokaryotes. Further, this system does not use a cap-binding and scanning mechanism such as observed in the eukaryotic cytoplasm.

Three translational initiation factors, IF1, IF2, and IF3, are required for the initiation of protein synthesis in bacteria (5–7). Prior to the present report, the homolog of only one of these factors, IF2mt, had been identified, cloned, and characterized in mammalian mitochondria (8–12). Similar to its prokaryotic counterpart, IF2mt promotes the binding of fMet-tRNA to the small subunit of mitochondrial ribosomes in response to synthetic polynucleotides such as poly(A,U,G).

The current report describes the identification and initial characterization of the mammalian mitochondrial factor equivalent to IF3. In prokaryotes IF3 has a number of roles in the initiation of protein synthesis. IF3 binds to the 30 S subunit and inhibits its association with the 50 S subunit, thus ensuring a supply of 30 S subunits for initiation (13, 14). IF3 also promotes an adjustment of the position of the mRNA on the 30 S subunit facilitating codon-anticodon interactions between the AUG codon and fMet-tRNA in the P site (15–18). IF3 acts to switch the decoding preference of the small ribosomal subunit from elongator tRNAs to the initiator tRNA in the P site, thus playing a proofreading role in initiation (19–21). IF3 is a small protein of 180 amino acids that folds into two distinct domains separated by a long flexible linker. The C-terminal domain is thought to carry out most of the direct functions of this factor, whereas the N-terminal domain stabilizes the interaction of IF3 with the 30 S subunit (22).

No factor equivalent to IF1 has been observed in the mitochondria from any system nor can an EST for this protein be identified in the human EST data bases. A gene for IF1 is, however, apparent in many chloroplast genomes. This small protein (less than 90 residues) binds to the 30 S subunit around helix 44 in the region that will become the A site (23). By binding to this site, IF1 is postulated to prevent accidental initiation from the A site and to promote the correct positioning of the mRNA on the 30 S ribosome. The current report describes the identification and initial characterization of the mammalian mitochondrial factor equivalent to IF3.

Materials and Methods

Preparation of Ribosomes and Initiation Factors—Bovine mitochondria and 55 S ribosomes were prepared as described previously (26). Escherichia coli ribosomes were prepared as described (27, 28), and tight couples were collected from a sucrose gradient in the presence of 5 mM MgCl2 (29). Bovine IF2mt, yeast [35S]fMet-tRNA, and E. coli initiation factors were prepared as described (12, 28). E. coli IF2 was also prepared from an expression construct providing a mixture of the α and

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The abbreviations used are: IF, initiation factor; IF2mt, mitochondrial IF2; IF3mt, mitochondrial IF3; EST, expressed sequence tag; Ni-NTA, nickel-nitrilotriacetic acid; CoII, cytochrome oxidase subunit II; N domain, N-terminal domain; C domain, C-terminal domain.
Fig. 1. Sequence and domain organization of IF3mt. A, domain organization of prokaryotic and mitochondrial IF3. B, amino acid sequence of human IF3mt and alignment with its prokaryotic homologs from B. stearothermophilus (B.st) and E. coli. The predicted site of cleavage following import into mitochondria is indicated by the arrow (→). The location of the proteolytic cleavage observed in a portion of the factor during expression in E. coli is indicated by the arrow (↑). The asterisks indicate residues implicated in the binding of bacterial IF3 to 30 S subunits. C, alignment of the amino acid sequence of human IF3mt with its homologs from B. taurus (Bovine), M. musculus (Mouse), F. rubripes (Fugu), and D. melanogaster (Drosophila). The alignment was done with the CLUSTALW program in Biology Workbench, and the results are displayed in BOXSHADE. The full sequence of the F. rubripes IF3mt is not shown for convenience. D, alignment of human IF3mt with the putative IF3mt from S. pombe.
β forms of IF2 (kindly provided by Angela Coursey, University of North Carolina). The genes for E. coli IF3 and IF1 (kindly provided by Drs. Roberto Spurio and Claudio Gualerzi, University of Camerino, Italy, and Dr. Rebecca Alexander, Wake Forest University, respectively) were also amplified by PCR and cloned into pET-21(c). The constructs carrying E. coli IF3 and IF1 were transformed into an E. coli BL21(DE3) strain that also carried the plasmid pArgU218 (kindly provided by Dr. Yamada, Mitsubishi Chemical Corp., Yokohama, Japan). The His-tagged forms of the E. coli initiation factors were purified on Ni-NTA resins as described below.

**Cyberprobing for Mitochondrial Translational Initiation Factor 3**—EST and genomic data base searches for human IF3mt were performed using BLAST (National Center for Biotechnology Information) and the sequences of various prokaryotic IF3s as virtual probes (30). Sequence analysis was done using the GCG DNA analysis software package (Wisconsin Package, version 10, Genetics Computer Group, Madison WI), Vector NTI (Informax Inc.), and Biology WorkBench 3.2. The results were displayed using BOXSHADE (written by K. Hofmann and M. Baron). Prediction of the cleavage sites for the mitochondrial signal sequence was carried out using PSort and MitoProt II (31, 32). The results were displayed using BOXSHADE (written by K. Hofmann and M. Baron). Prediction of the cleavage sites for the mitochondrial signal sequence was carried out using PSort and MitoProt II (31, 32). Sequence analysis was done using the GCG DNA analysis software package (Wisconsin Package, version 10, Genetics Computer Group, Madison WI), Vector NTI (Informax Inc.), and Biology WorkBench 3.2. The results were displayed using BOXSHADE (written by K. Hofmann and M. Baron). The genes for IF3 and IF1 were transformed into BL21(DE3) carrying the plasmid pArgU218 (kindly provided by Roberto Spurio and Claudio Gualerzi, University of Camerino, Italy, and Dr. Rebecca Alexander, Wake Forest University, respectively) were also amplified by PCR and cloned into pET-21(c). The constructs carrying bacterial IF3 were transformed into E. coli BL21(DE3) carrying the plasmid pArgU218 (Dr. Yamada, Mitsubishi Chemical Corp., Yokohama, Japan), which provides the gene for the isoacceptor of tRNAArg recognizing poly(A,U,G), or 10 pmol of the CoII mRNA, 3.8 pmol of [35S]fMet-tRNA, and the indicated amounts of various initiation factors. All of the initiation complex formation assays were incubated at 37 °C for 15 min and analyzed as described (8).

**Dissociation of Mitochondrial 55 S Ribosomes by IF3mt**—The reaction mixtures (100 μl) were prepared containing 25 mM Tris-HCl, pH 7.6, 2 mM MgCl2, 100 mM KCl, 0.5 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 0.2 OD260 units of 55 S ribosomes, and variable amounts of IF3mt (0–1.72 μg). The reactions were incubated for 15 min at 37 °C. The Mg2+ concentration was then adjusted to 7 mM by the addition of 2.5 μl of 0.1 M MgCl2, and the samples were analyzed for 28, 39, and 55 S particles on 10–30% (w/v) linear sucrose gradients prepared in the buffer described above containing 7 mM Mg2+ and analyzed as described previously (38).

**RESULTS AND DISCUSSION**

**Identification of the cDNA for IF3mt and Analysis of the Coding Region**—Although the mammalian mitochondrial ribosome has a low percentage of rRNA and a high protein content compared with bacterial ribosomes, portions of the rRNA where IF3 is thought to bind are present (39). Further, the ribosomal proteins with which IF3 interacts (S7, S11, and S18) have homologs in the 28 S subunit. Hence, it was logical to postulate that mammalian mitochondria contain a homolog of bacterial IF3. Probing the human EST data with the amino acid sequence of E. coli or most other IF3 species fails to provide any convincing evidence for a mammalian mitochondrial homolog of IF3. However, extensive data base searches with the sequences of IF3 from the Mycoplasma and the IF3 homology domain of Euglena gracilis chloroplast IF3 provide a hint in both the human and mouse EST data bases. The sequence detected

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**Preparation of mRNA Transcripts**—The previously described clone carrying the bovine cytochrome oxidase subunit II (CoII) gene (36) was modified to provide a sequence of 30 A residues at the 3′ end. The transcript prepared from this vector mimics mitochondrial mRNAs produced in vivo, which generally have poly(A) stretches up to 70 residues added following transcription and processing (37). In vitro transcripts were prepared as described previously (36).

**Initiation Complex Formation Assays**—The activity of IF3mt in promoting initiation complex formation with E. coli and mitochondrial ribosomes was assayed using conditions basically described previously (8, 9). Reaction mixtures (100 μl) contained 0.5 OD260 units of E. coli 70 S or 0.075–0.15 OD260 units of mitochondrial 55 S tight couples, 12.5 μg of poly(A,U,G), or 10 pmol of the CoII mRNA, 3.8 pmol of [35S]fMet-tRNA, and the indicated amounts of various initiation factors. All of the initiation complex formation assays were incubated at 37 °C for 15 min and analyzed as described (8).

**Identification and Examination of IF3mt**—The full-length cDNA as a template. The portion of the IF3mt cDNA predicted to correspond to the mature protein was cloned between the I sites of pET-21(c) using the forward primer 5′-CGCGGATCC-3′ and the reverse primer 5′-/H11032-3′. The construct carrying the bovine cytochrome oxidase subunit II (CoII) gene (36) was modified to provide a sequence of 30 A residues at the 3′ end. The transcript prepared from this vector mimics mitochondrial mRNAs produced in vivo, which generally have poly(A) stretches up to 70 residues added following transcription and processing (37). In vitro transcripts were prepared as described previously (36).
Identification and Examination of IF3<sub>mt</sub>

The percentage identity between human IF3<sub>mt</sub> and the bacterial factors is reported for the homology region only.

| Organism                             | Identity | Length | Accession number |
|--------------------------------------|----------|--------|------------------|
| *B. stearothermophilus*              | 25.9     | 172    | P03000           |
| Mycoplasma genitalium                | 24.6     | 184    | P47438           |
| Mycoplasma pneumoniae                | 21.5     | 201    | NP_109803        |
| *E. coli*                            | 20.8     | 180    | P02999           |
| *E. gracilis* (chloroplast)          | 23.7     | 538    | L23760           |
| *A. thaliana* (chloroplast)          | 24.4     | 312    | NP_179984        |
| *S. pombe*                           | 20.9     | 233    | T39948           |
| *S. cerevisiae*                      | 16.2     | 370    | NP_011356        |
| *B. taurus* (mitochondrial)          | 69.5     | 273    | cDNAb           |
| Mouse (mitochondrial)                | 66.0     | 276    | BAB28438         |
| *Drosophila* (mitochondrial)         | 22.6     | 226    | AAF5534          |
| *F. rubripes* (mitochondrial)        | 39.9     | 362    | JGI_17846        |

<sup>a</sup> Putative mitochondrial initiation factor 3.
<sup>b</sup> cDNA sequences encoding for bovine IF3<sub>mt</sub>; AW658729 (nucleotides 316–582, aa residues 1–89), AW445348 (nucleotides 1–408, aa residues 26–161), BM106922 (nucleotides 270–112, aa residues 149–201), and BM255983 (nucleotides 79–360, aa residues 180–273).

FIG. 2 Three-dimensional models of human IF3<sub>mt</sub> based on the crystal structures of the N- and C-terminal domains of *B. stearothermophilus* IF3. A, N domain of *B. stearothermophilus* IF3 taken from the Protein Data Bank (accession number 1TIF) and displayed using RasMol (51). B, model of the N domain of IF3<sub>mt</sub> generated using Swiss-Model and the coordinates from the N domain of *B. stearothermophilus* IF3. The site of cleavage generating the 19-kDa fragment of IF3<sub>mt</sub> observed in the factor expressed in *B. stearothermophilus* is indicated by the arrow. The linker region could not be modeled because of the low degree of homology observed between the bacterial and mitochondrial proteins in this region of the sequence. C, C domain of *B. stearothermophilus* IF3 taken from the Protein Data Bank (accession number 1TIF). D, partial model of the C-terminal domain of IF3<sub>mt</sub> generated using Swiss-Model and the coordinates of *B. stearothermophilus* IF3. Only a portion of this domain could be modeled because of the low degree of sequence conservation. The helical regions are indicated as H1–H4.

by this search encodes a 278-amino acid protein (Fig. 1A). MitoProt II gives this protein a 97% probability to be localized in mitochondria and predicts that the mature protein will be 247 residues in length. The mature form of IF3<sub>mt</sub> is predicted to have an N-terminal extension of about 30 residues (Fig. 1, A and B) that can form a coiled region followed by an α-helix. An N-terminal extension of about 150 residues has been noted on *E. gracilis* chloroplast IF3 (IF3<sub>chl</sub>) (40). IF3<sub>mt</sub> also has a C-terminal extension just over 30 residues long. Overall, it is quite hydrophilic and highly charged having nine acidic and five basic residues. The C-terminal extension, like the N-terminal extension, is predicted to have significant helical content. A 63-residue acidic C-terminal extension on *E. gracilis* IF3<sub>chl</sub> has been shown to reduce the activity on the chloroplast factor in initiation complex formation and may serve as a potential regulatory region (41).

Alignment of IF3<sub>mt</sub> with prokaryotic and chloroplast IF3 indicates that the mitochondrial factor has diverged considerably from other IF3s (Table I). Overall, it has only 20.8% identity to *E. coli* IF3, which explains the failure of database searches with the sequence of *E. coli* IF3 to locate the corresponding mitochondrial factor. IF3<sub>mt</sub> is 25.9% and 23.7% identical to *Bacillus stearothermophilus* IF3 and to *E. gracilis* chloroplast IF3, respectively (40). Alignment of the sequence of IF3<sub>mt</sub> with the bacterial factors indicates that regions of identity are rather scattered throughout the structure (Fig. 1B). Residues that are responsible for the binding of IF3 to the small subunit are thought to be located primarily in the C-terminal domain. Crystallography experiments place the C-terminal domain of IF3 on the solvent side of the platform on the 30 S subunit (42), whereas cryo electron microscopy and footprinting suggest that it is located on the interface side (39, 43). Important regions of IF3 include residues 99–116, 127–137, 145–155, and 168 (*E. coli* numbering) as indicated by NMR experiments, mutagenesis, and structural studies (39, 42, 44). A number of the residues in these regions are conserved or are conservative replacements in the C-terminal domain of human IF3<sub>mt</sub>. The coordinates from the Protein Data Bank (accession number 1TIF) and displayed using RasMol (51).

One of the major roles of prokaryotic IF3 is the discrimination of the initiation codon (AUG or occasionally GUG or UUG) from other codons. This property can be observed in the isolated C-terminal domain of bacterial IF3 (22) but is strongly affected by conserved residues in the linker region (17, 45, 46). Interestingly, these highly conserved residues, Tyr<sup>70</sup>, Gly<sup>71</sup>, and Tyr<sup>75</sup>, in prokaryotic IF3s are not conserved in human IF3<sub>mt</sub>. In mammalian mitochondria, both AUG and AUA (normally an isolucine codon) serve as initiation codons. Consequently, the proofreading properties of human IF3<sub>mt</sub> could be quite different from those of the bacterial factors.

Analysis of the mouse and bovine EST data bases indicates the presence of mammalian homologs of human IF3<sub>mt</sub> that are 65–70% identical to the human factor (Table I and Fig. 1C). In addition, BLAST searches indicate the presence of IF3<sub>mt</sub> in *Fugu rubripes* (puffer fish) and in *Drosophila melanogaster* (Table I and Fig. 1C). No homolog can be detected in *Caenorhabditis elegans*. It is quite reasonable to assume that this organism will have a corresponding factor. However, IF3<sub>mt</sub> does not appear to be highly conserved throughout the animal kingdom, and it may be difficult to detect using BLAST searches.

The IF3<sub>mt</sub> species detected in animals generally have N- and C-terminal extensions that surround a central section that has homology to the bacterial IF3s. The N-terminal extension on puffer fish IF3<sub>mt</sub> is considerably longer than that observed on...
the mammalian factors (Fig. 1C). D. melanogaster IF3mt has a very short N-terminal extension. The mammalian and puffer fish IF3mtS all have C-terminal extensions of around 30 residues compared with the bacterial IF3s. However, D. melanogaster IF3mt again lacks a significant extension at the C terminus. The linker regions of the mitochondrial factors are charged as observed for the prokaryotic proteins.

Two potential homologs of IF3mt can be found in Arabidopsis thaliana. One of these genes probably encodes the chloroplast factor, whereas the other encodes the mitochondrial factor. These two forms (NP-179984 and NP-174696) differ considerably in length (312 and 574 residues, respectively). Alignments of these two species clearly separate the chloroplast IF3 mt from the other factors and their classification as a mitochondrial factor remains to be clarified.

Development of Three-dimensional Models for the N and C Domains of IF3mt—The coordinates for the crystal structures of N and C domains of B. stearothermophilus IF3 were used to model the N and C domains of human IF3mt using Swiss-Model. The N-terminal domain of IF3 has a globular α/β topology consisting of a single α-helix packed against a four-stranded β-sheet (Fig. 2A). This domain leads into the connecting linker, which is helical in the crystal structure but is quite flexible in solution (48). As indicated in Fig. 2B, the N-terminal domain of IF3mt is predicted to fold into a highly similar structure. The linker region is not shown in this model.

The C-terminal domain of bacterial IF3 also consists of an α/β fold with two helices packed against a four-stranded sheet (Fig. 2C). The C-terminal domain of IF3mt could not be fully modeled because of low sequence conservation and the unclear alignment of portions of the molecules (Fig. 2, C and D). However, the first α-helix and the first two strands of the β-sheet can be modeled to resemble the prokaryotic factors quite well. It is likely that the remainder of the C-terminal domain will have a similar overall fold to that observed in prokaryotic IF3 despite the low sequence conservation.

The linker region separating the N and C domains of IF3 is a rigid helix in the crystal structure of B. stearothermophilus IF3 but is more flexible in the NMR structure of E. coli IF3 (48–52). Structural studies suggest that the linker must be flexible to allow IF3 to interact with distant regions of the small
subunit (39, 42, 43). Secondary structure predictions on the linker region of IF3 mt indicate that it could form a helical conformation, particularly as it exits the N-terminal domain. However, the linker in IF3 mt contains two proline residues near its junction with the C-terminal domain. Proline residues are not observed in the linker regions of prokaryotic IF3s. These residues would be expected to reduce the flexibility of the linker and may help confer a specific orientation between the N- and C-terminal domains in the mitochondrial factor. Both proline residues are conserved in the mammalian factors, whereas the second is also seen in puffer fish IF3 mt.

Purification of Overexpressed IF3 mt—The portion of the cDNA for human IF3 mt corresponding to the region predicted to be present in the mature form of the protein (amino acids 32–278) was cloned into an expression vector providing a His tag. When the mature form of IF3 mt was expressed, two major bands of protein were observed on SDS-PAGE following purification on Ni-NTA resins (Fig. 3A, lane 2). The highest molecular mass form migrated at 29 kDa, the size expected for the mature form of this factor. A second shorter form of IF3 mt migrated at 19 kDa (Fig. 3A). Both of these bands cross-reacted with the antibody prepared against E. gracilis IF3 chl on Westerns (data not shown). These two forms of IF3 mt were purified by high performance liquid chromatography (Fig. 3). N-terminal analysis of the 29-kDa form showed that it begins with the sequence TAP, indicating that it was expressed from the start site predicted for the mature form of the protein following removal of the initiating Met. N-terminal sequencing of the 19-kDa species gave the sequence GNMHRAN, indicating that it arose from the proteolytic degradation of IF3 mt at amino acid 97 (arrows in Figs. 1B and 2B), which is located in the helical segment in the middle of the N-terminal domain of IF3 mt.

Effect of IF3 mt on the Equilibrium between the 55 S Ribosome and Its Subunits—Bacterial IF3 acts as a ribosome dissociation factor. The ability of IF3 mt to affect the equilibrium between the mitochondrial 55 S ribosome and its 28 and 39 S subunits was examined in a two-step assay. In the first step, IF3 mt was incubated with mitochondrial ribosomes at 2 mM Mg²⁺/H⁺. At this concentration of Mg²⁺, a significant fraction of the 55 S ribosomes dissociates into subunits giving IF3 mt access to 28 S subunits (38). In the second stage, the Mg²⁺ concentration was raised to 7 mM, promoting the reassociation of the subunits.
Binding of IF3mt to the 28 S subunits in the first step would be expected to result in an increased amount of ribosomal subunits following the increase in the Mg$^{2+}$ concentration. The distribution of ribosomal particles was monitored by sucrose density gradient centrifugation. The significant fraction of the mitochondrial ribosomes were present as 55 S particles following the increase in the Mg$^{2+}$ concentration to 7 mM (Fig. 4A). However, in the presence of IF3mt, a substantial increase in the presence of 28 and 39 S subunits was observed (Fig. 4B). This observation demonstrates that IF3mt acts as a subunit anti-association factor in mammalian mitochondria as it does in bacteria.

Activity of IF3mt in Initiation Complex Formation and Ribosome Specificity—The ability of IF3mt to promote initiation complex formation on mitochondrial ribosomes was examined by testing its effect on fMet-tRNA binding to ribosomes in the presence of IF2mt and poly(A,U,G). As indicated in Fig. 5A, the presence of IF3mt increased the amount of fMet-tRNA binding observed. This result is expected based on the ability of IF3mt to increase the availability of 28 S subunits required for the activity of IF2mt. IF3mt did not stimulate binding of fMet-tRNA to 28 S subunits, suggesting that its effect in the initiation...
assay arises primarily from its ability to promote the dissociation of ribosomes.

Previously, it has been shown that E. coli IF3 promotes the dissociation of mitochondrial ribosomes into 28 and 39 S subunits (38, 53). This observation suggests that it might be active in promoting initiation complex formation on mitochondrial ribosomes. As indicated in Fig. 5A, E. coli IF3 also promotes initiation complex formation on mitochondrial 55 S ribosomes as expected from its activity as a ribosome dissociation factor. In contrast to the activity on E. coli IF3 on mitochondrial ribosomes, E. coli IF2 is not active on 55 S ribosomes (9).

The activity of IF3mt on E. coli 70 S ribosomes was tested by examining its ability to promote the binding of fMet-tRNA to these ribosomes in the presence of E. coli IF2 (Fig. 5B). Somewhat surprisingly, IF3mt showed no activity on the bacterial ribosomes under these conditions. This observation is in contrast to the activity of IF2mt, which is quite active on 70 S ribosomes (8). Two possible explanations can be put forward to explain this observation. First, it is possible that IF3mt cannot bind to bacterial ribosomes. Second, IF3mt might bind to bacterial small subunits but not permit the binding of E. coli IF2, which would require for fMet-tRNA binding. To distinguish between these two possibilities, fMet-tRNA binding assays to 70 S ribosomes were carried out using bovine IF2mt, which is active on bacterial ribosomes. As indicated in Fig. 5C, under these conditions, IF3mt is quite active in promoting fMet-tRNA binding to E. coli ribosomes. This observation indicates that IF3mt can bind bacterial ribosomes but that its presence is incompatible with the activity of E. coli IF2.

Examination of the structures of E. coli and mitochondrial IF2 indicates that the mitochondrial factor is significantly shorter than E. coli IF2. Indeed, IF2mt lacks both domains I and II in the six-domain model of E. coli IF2 (12, 54). Domain II has been implicated in the binding of E. coli IF2 to 30 S subunits (55). The absence of this domain in IF2mt may allow both IF3mt and IF2mt to bind to the small subunit at the same time.

**Stimulation of Initiation Complex Formation in the Presence of Natural mRNAs by IF3mt**—It has not yet been possible to assemble an initiation complex using a “natural” mRNA in the mammalian mitochondrial system. To further test the effect of IF3mt on fMet-tRNA binding in initiation, its activity was examined in the presence of an in vitro transcript of the cytochrome oxidase subunit II gene (ColI mRNA). As indicated in Fig. 6, IF3mt stimulated fMet-tRNA binding with ColI mRNA on 55 S ribosomes. As mentioned earlier, mitochondrial mRNAs have an almost complete lack of 5’- and 3’-untranslated nucleotides. The translational start codon is generally located within three nucleotides of the 5’ end of the mRNA (1, 4), a situation very similar to the rare leaderless mRNAs found in several prokaryotic systems. Recent studies performed with leaderless mRNAs in bacteria have suggested that IF3 antagonizes translation initiation on these mRNAs, at least with ribosomes containing ribosomal protein S1 and in the presence of a competing mRNA carrying a Shine/Dalgalno sequence (56–59). Because mitochondrial ribosomes do not have a protein equivalent to S1 (60) and have no mRNAs with Shine/Dalgalno sequences, different constraints may be operating in the mitochondrial system permitting initiation on mRNAs with essentially no 5’ leader.

**Requirement for Other Initiation Factor(s) in Mammalian Mitochondrial Initiation**—Prokaryotic translational initiation requires IF1 in addition to IF2 and IF3. With the current work, two of these factors, IF2mt and IF3mt, have been identified in mammalian mitochondria. Extensive searches of the human and mouse EST data bases and of the genomes of S. cerevisiae, D. melanogaster, and C. elegans have failed to provide evidence for the presence of a factor equivalent to IF1 in mitochondria. This small protein presents a considerable challenge to identify in such searches because of its small size (about 70 amino acids) and low degree of sequence conservation. Biochemical tests have also failed to date to identify a factor equivalent to IF1 in mammalian mitochondrial extracts. However, such a protein would only be present in trace amounts making its detection a challenge.

To help assess the possible need for a factor equivalent to IF1 in the mitochondrial system, the effect of E. coli IF1 on initiation complex formation was examined using both E. coli and mitochondrial 55 S ribosomes as well as with E. coli and mitochondrial IF2 and IF3. As indicated in Fig. 7A, the presence of E. coli IF1 has essentially no effect on initiation complex formation on 55 S ribosomes in the presence of IF3mt and IF2mt. The observation suggests that the mitochondrial system may not require a factor directly equivalent to IF1.

Further insight into the question of an interaction between E. coli IF1 and the mitochondrial initiation factors was obtained by examining the effects of this factor on initiation complex formation on 70 S ribosomes. As a control, the previously reported stimulation of E. coli IF2 by IF1 was examined, and a substantial stimulation of fMet-tRNA binding was observed (Fig. 7B). In contrast to the stimulation of E. coli IF2 by IF1, no stimulation of the activity of IF2mt on 70 S ribosomes was observed under identical conditions (Fig. 7C). IF2mt alone actually stimulates initiation complex formation on 70 S ribosomes as effectively as E. coli IF2 in the presence of IF1.

The activity of E. coli IF3, like that of IF2, is stimulated by IF1 (data not shown). However, E. coli IF1 again fails to stimulate the activity of IF3mt on 70 S ribosomes in the presence of saturating levels of IF2mt (Fig. 7D). Taken together, these results suggest that IF2mt and IF3mt function efficiently in initiation complex formation in the absence of IF1. These results suggest that the conformational change caused by the binding of IF1 to 30 S subunits (23) can also be generated by the binding of these mitochondrial initiation factors to 30 S subunits.

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