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Role of the N- and C-terminal extensions on the activity of mammalian mitochondrial translational initiation factor 3

Kalpana Bhargava and Linda L. Spremulli*

Department of Chemistry, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-3290, USA

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ABSTRACT

Mammalian mitochondrial translational initiation factor 3 ($IF3_{mt}$) promotes initiation complex formation on mitochondrial 55S ribosomes in the presence of $IF2_{mt}$, fMet-tRNA and poly(A,U,G). The mature form of $IF3_{mt}$ is predicted to be 247 residues. Alignment of $IF3_{mt}$ with bacterial IF3 indicates that it has a central region with 20–30% identity to the bacterial factors. Both the N- and C-termini of $IF3_{mt}$ have extensions of ~30 residues compared with bacterial IF3. To examine the role of the extensions on $IF3_{mt}$, deletion constructs were prepared in which the N-terminal extension, the C-terminal extension or both extensions were deleted. These truncated derivatives were slightly more active in promoting initiation complex formation than the mature form of $IF3_{mt}$. Mitochondrial 28S subunits have the ability to bind fMet-tRNA in the absence of mRNA. $IF3_{mt}$ promotes the dissociation of the fMet-tRNA bound in the absence of mRNA. This activity of $IF3_{mt}$ requires the C-terminal extension of this factor. Mitochondrial 28S subunits also bind mRNA independently of fMet-tRNA or added initiation factors. $IF3_{mt}$ has no effect on the formation of these complexes and cannot dissociate them once formed. These observations have lead to a new model for the function of $IF3_{mt}$ in mitochondrial translational initiation.

INTRODUCTION

The synthesis and assembly of the oligomeric complexes in mitochondria involved in electron transport and ATP synthesis require genetic information contained in both the nuclear and mitochondrial genomes. Limited information is available on the mechanism by which the mitochondrially-encoded components in these complexes are synthesized and assembled into the oligomeric complexes in the inner membrane of mitochondria. A number of interesting features distinguish the protein synthesizing system of mammalian mitochondria from other translational systems. Of particular interest is the observation that the mRNAs in this organelle have an almost complete lack of 5′- and 3′-untranslated nucleotides. The start codon is generally located within a few nucleotides of the 5′ end of the mRNA (1,2). Thus, a Shine/Dalgarno interaction between the mRNA and the 16S rRNA such as observed in prokaryotes is not used in mammalian mitochondrial protein synthesis. Mammalian mitochondrial ribosomes have low sedimentation coefficients (~55S) and consist of 28S and 39S subunits (3). Animal mitochondrial ribosomes are 31% RNA and 69% protein. In contrast, bacterial ribosomes consists of ~67% RNA and 33% protein (4,5).

In bacteria, three translational initiation factors, initiation factors 1, 2 and 3 (IF1, IF2, and IF3), are required for initiation (6–8). No homolog of IF1 has been detected in mammalian mitochondrial systems. However, mitochondrial initiation factor 2 ($IF2_{mt}$), which promotes the binding of fMet-tRNA to the small subunit of mitochondrial ribosomes has been cloned and characterized (9–15). Recently, the mitochondrial homolog of initiation factor 3 ($IF3_{mt}$) has been cloned and expressed. In bacterial protein synthesis initiation factor 3 has been assigned a number of discrete functions including (i) dissociation of ribosomes (7,16); (ii) increasing the forward rate constant for codon:anticodon interaction at the P-site (17); (iii) dissociation of fMet-tRNA at AUG codons at the 5′ end of leaderless mRNAs (18); (iv) proofreading the selection of the initiator tRNA and an AUG codon at the P-site (19–21) and (v) adjusting the position of the mRNA on the small subunit from a stand-by position to the decoding position (22).

The mature form of $IF3_{mt}$, lacking the predicted mitochondrial import signal, is active in initiation complex

*To whom correspondence should be addressed. Tel: +1 919 966 1567; Fax: +1 919 966 3675; Email: Linda_Spremulli@unc.edu
Present address:
Kalpana Bhargava, Department of Molecular Biology and Biochemistry, School of Biological Sciences, University of Missouri, Kansas City, MO 64110, USA

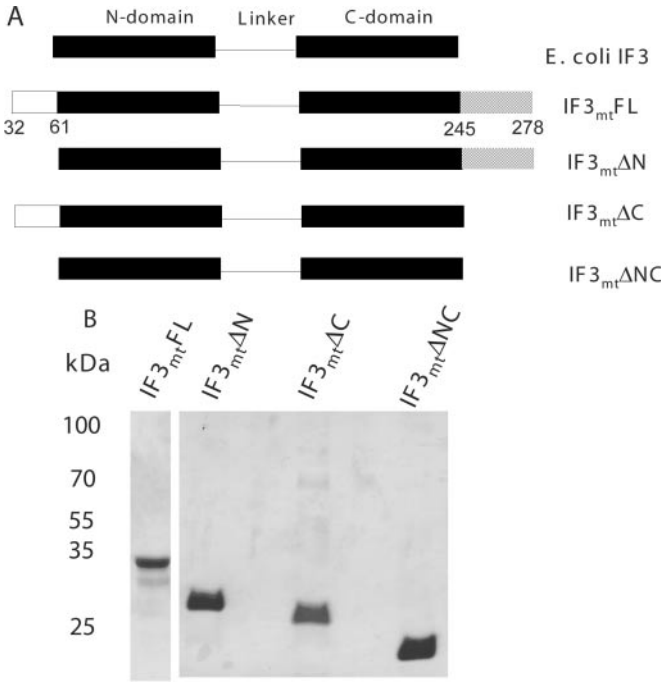

Figure 1. Domain organization of prokaryotic IF3 and mammalian $IF3_{mt}$ and deletion constructs for $IF3_{mt}$. (**A**) Schematic diagram of the organization of *E.coli* IF3 and $IF3_{mt}$ and its deletion derivatives. $IF3_{mt}$ begins with an N-terminal import sequence that is not shown. The full-length version of the mature protein ($IF3_{mt}FL$) encompasses residues 32–278. The region with homology to *E.coli* IF3 begins at residue 61 and goes through residue 245. Deletion of the N-terminal extension ($IF3_{mt}\Delta N$) gives a derivative that includes amino acids 61–278. Deletion of the C-terminal extension ($IF3_{mt}\Delta C$) includes amino acids 32 through and including residue 245. The deletion of both extensions ($IF3_{mt}\Delta NC$) gives a construct that includes residues 61 through 245. (**B**) Analysis of the purity of the full-length and deletion derivatives by SDS–PAGE. Samples (1–2 μg) of $IF3_{mt}FL$ and its deletion derivatives were applied to a 12% SDS–PAGE gel and stained with Coosmassie blue.

formation on both mitochondrial 55S ribosomes and bacterial 70S ribosomes (23). $IF3_{mt}$ has a central region with weak homology (21–26% identity) to bacterial IF3 (Figure 1A). This homology region is divided into two domains (N- and C-domains) separated by a flexible linker as observed for the prokaryotic factors (23). The C-domain is thought to carry out the basic functions of IF3 with the N-domain increasing its affinity for the small subunit (24). In mammalian $IF3_{mt}$, the central region with homology to the bacterial factors is preceded by an N-terminal extension of ~30 residues, which is predicted to form a helical structure. The homology domain is followed by a hydrophilic C-terminal extension of ~30 residues, which is also predicted to have significant helical content. The only organellar IF3 that has been studied at a detailed biochemical level to date is chloroplast IF3 ($IF3_{chl}$) from *Euglena gracilis* (25–28). This factor has a long extension (~150 amino acids) at the N-terminus and an acidic C-terminal extension of 63 residues. In $IF3_{chl}$, sequences near the junction of the N-terminal extension and the homology domain and the C-terminal extension inhibit the activity of this factor (28) and may play a regulatory role in chloroplast protein synthesis. In the present work, we have examined some of the properties of human $IF3_{mt}$ and have tested the effects of the N-terminal and C-terminal extensions on the activity of this factor.

MATERIALS AND METHODS

Materials

Oligonucleotides used for mutagenesis were synthesized at Nucleic Acid Core Facility at the University of North Carolina, Chapel Hill. Bovine mitochondrial 55S ribosomes were prepared as described (29). Mitochondrial 28S and 39S subunits were purified on sucrose gradients (30). *Escherichia coli* ribosomes were prepared from *E.coli* W (31,32) and tight couples were collected from a sucrose gradient in the presence of 5 mM Mg^{2+} (33). Bovine $IF2_{mt}$ and *E.coli* initiation factors were prepared as described (13,23,32). *E.coli* IF2 was also prepared from an expression construct providing a mixture of the α and β forms of IF2 (A. C. Spencer and L. L. Spremulli, unpublished data). Yeast [^{35}S]fMet-tRNA and [^{14}C]Phe-tRNA were prepared and the [^{14}C]Phe-tRNA was acetylated as described (32,34). A transcript encoding subunit 2 of bovine cytochrome oxidase was prepared by *in vitro* transcription (35–37).

Cloning and expression of $IF3_{mt}$ deletion derivatives

The construct carrying the N-terminal deletion was amplified by PCR using the mature $IF3_{mt}$ cDNA as template (23), the forward primer GGGAATTCCATATGACCCAGAATGAAGGAAAAAAGA and the reverse primer CGCGGATCCGCTCGAGCTGATGCAGAACAT. Deletion of the C-terminal extension was carried out using the forward primer CGCGGATCCAATTCATATGACAGCACCAGCACAG and the reverse primer CGCGGATCCGCTCGAGTTTGCTCAAAGCACG. The double deletion of the N- and C-terminal extensions was obtained using the forward primer GGGAATTCCATATGACCCAGAATGAAGGAAAAAAGA and the reverse primer CGCGGATCCGCTCGAGTTTGCTCAAAGCACG. These PCR products were digested with NdeI and XhoI and cloned into pET-21(+) (Novagen). This vector provides a sequence encoding six His residues (His-tag) at the C-terminus. The PCR products were transformed into *E.coli* ER2267 and the nucleotide sequence of the inserted DNA was confirmed. The plasmids were subsequently transformed into *E.coli* BL21(DE3) for expression.

Purification of $IF3_{mt}FL$ and deletion derivatives using Ni-NTA

The full-length mature form of human mitochondrial initiation factor 3 ($IF3_{mt}FL$) was expressed in *E.coli* as described (23). The His-tagged protein was purified on Ni-NTA and on S-Sepharose. This later step separates the $IF3_{mt}FL$ from a 19 kDa degradation product. Ni-NTA preparations of the deletion derivatives did not contain this degradation product and did not require further purification. The N-terminus of the double truncated derivative ($IF3_{mt}\Delta NC$) was sequenced using Edman degradation to ensure that the correct N-terminus was present on the expressed protein. Protein concentrations were determined using the Bradford assay with BSA as a standard (BioRad).

Initiation complex formation assay for $IF3_{mt}FL$ and its deletion derivatives

The activities of $IF3_{mt}FL$ and its deletion derivatives were determined by measuring their abilities to stimulate the

binding of [^{35}S]fMet-tRNA to either *E.coli* or mitochondrial ribosomes in filter-binding assays essentially as described previously (23). Reactions (100 µl) on *E.coli* ribosomes contained 50 mM Tris–HCl, pH 7.6, 1 mM DTT, 80 mM NH_4Cl, 5 mM $MgCl_2$, 0.25 mM GTP, 12.5 µg poly(A,U,G), 0.06 µM [^{35}S]fMet-tRNA, 0.25 µM $IF2_{mt}$, 0.24 µM *E.coli* 70S tight couples and varying amounts of $IF3_{mt}$ or its deletion derivatives as indicated. For initiation complex formation assays on mitochondrial ribosomes, reaction mixtures (100 µl) contained 50 mM Tris–HCl, pH 7.6, 1 mM DTT, 0.1 mM spermine, 35 mM KCl, 4.5 mM $MgCl_2$, 0.25 mM GTP, 1 mM DTT, 12.5 µg poly(A,U,G), 0.42 µM $IF2_{mt}$, 0.06 µM [^{35}S]fMet-tRNA, 0.05 µM mitochondrial 55S ribosomes and varying amount of $IF3_{mt}$ or its deletion derivatives. Reaction mixtures were incubated for 15 min at 37°C then analyzed using a nitrocellulose filter-binding assay as described (9).

Proofreading assays

This assay has been modified from that described in (38,39) for *E.coli* IF3. A complex carrying [^{14}C]AcPhe-tRNA bound to *E.coli* 30S subunits [AcPhe-tRNA:poly(U):30S] was formed by incubation of activated *E.coli* 30S subunits (0.08 µM), poly(U) (10 µg) and [^{14}C]AcPhe-tRNA (0.3 µM) in a reaction mixture (50 µl) containing 50 mM Tris–HCl, pH 7.6, 0.1 mM spermine, 1 mM DTT, 50 mM NH_4Cl and 15 mM $MgCl_2$. After incubation at 37°C for 30 min, various amounts of $IF3_{mt}$ or buffer (50 µl) were added to the mixture and the incubation was continued for an additional 5 min at 37°C. The mixtures were then diluted 25-fold with pre-warmed dilution buffer (50 mM Tris–HCl, pH 7.6, 1 mM DTT, 50 mM NH_4Cl and 15 mM $MgCl_2$). The diluted reaction mixtures were incubated for 5 min at 37°C. The amount of initiation complex remaining was determined by a nitrocellulose filter-binding assay. A similar assay was carried out using a complex formed with *E.coli* 30S subunits (0.08 µM), poly(A,U,G) (12.5 µg) and [^{35}S]fMet-tRNA (0.2 µM).

A complex containing [^{14}C]AcPhe-tRNA bound to 28S subunits [AcPhe-tRNA:poly(U):28S] was formed by incubation of mitochondrial 28S subunits (0.2 µM) with poly(U) (10 µg) and [^{14}C]AcPhe-tRNA (0.3 µM) in a reaction mixture (50 µl) containing 50 mM HEPES–KOH, pH 7.8, 1 mM DTT, 0.1 mM spermine, 35 mM KCl and 25 mM $MgCl_2$. After incubation at 27°C for 30 min, various amounts of $IF3_{mt}$ or compensating buffer (50 µl) were added to the mixture and incubated for 5 min at 27°C. The mixture was then diluted 50-fold with pre-warmed dilution buffer (50 mM HEPES–KOH, pH 7.8, 1 mM DTT, 0.1 mM spermine 35 mM KCl and 25 mM $MgCl_2$). The reaction mixtures were incubated for an additional 15 min at 27°C. The amount of initiation complex remaining was determined by a nitrocellulose filter-binding assay. Attempts were also made to form a similar complex with mitochondrial 28S subunits (0.2 µM), poly(A,U,G) (12.5 µg) and [^{35}S]fMet-tRNA (0.2 µM) and $MgCl_2$ concentrations ranging from 15 to 50 mM (Results).

Effect of $IF3_{mt}$ on the binding of fMet-tRNA to 28S subunits in the presence or absence of mRNA

Reaction mixtures (100 µl) contained various amounts of $IF3_{mt}$ and 50 mM Tris–HCl, pH 7.6, 35 mM KCl, 0.1 mM spermine, 1 mM DTT, 7.5 mM $MgCl_2$, 0.25 mM GTP, 1.25 mM phosphoenolpyruvate, 0.7 U pyruvate kinase, 0.06 µM [^{35}S]fMet-tRNA, 0.14 µM $IF2_{mt}$, 0.068 µM 28S subunits and, where indicated, 12.5 µg poly(A,U,G). Reaction mixtures were incubated for 20 min at 27°C and the amount of [^{35}S]fMet-tRNA bound to the 28S subunit was determined using a filter-binding assay (9).

Sucrose gradient analysis of initiation complexes

Initiation complexes (200 µl) were assembled as described above containing 0.05 µM 28S subunits or 0.066 µM 39S subunits. Samples were incubated for 20 min at 27°C and then applied to a 5 ml sucrose gradient (10–30% sucrose in 50 mM Tris–HCl, pH 7.6, 40 mM KCl, 7.5 mM $MgCl_2$ and 2 mM DTT). The gradients were subjected to centrifugation for 1 h 45 min at 48 000 r.p.m. in a Beckman SW50.1 rotor. Following centrifugation, gradients were fractionated on an Isco gradient fractionator at a flow rate of 0.8 ml/min. Fractions (0.2 ml) were collected and filtered through nitrocellulose membranes, dried and counted.

RESULTS

Role of the extensions on $IF3_{mt}$ in initiation complex formation

Mammalian $IF3_{mt}$ has N-terminal and C-terminal extensions just over 30 residues long surrounding the central region homologous to bacterial IF3. To assess the importance of these extensions on the activity of $IF3_{mt}$, three deletion derivatives were constructed (Figure 1A). One derivative lacked the N-terminal extension ($IF3_{mt}\Delta N$). A second lacked the C-terminal extension ($IF3_{mt}\Delta C$) while the third lacked both extensions ($IF3_{mt}\Delta NC$). These derivatives expressed well and were purified from *E.coli* (Figure 1B).

The activities of the full-length mature $IF3_{mt}$ ($IF3_{mt}FL$) and the deletion derivatives were tested in initiation complex formation on bovine mitochondrial 55S ribosomes (Figure 2A). Interestingly, deletion of either the N-terminal or the C-terminal extension in $IF3_{mt}$ increased the activity of the factor slightly in promoting the binding of fMet-tRNA to mitochondrial ribosomes. This observation is reminiscent of the effects of removing the long extensions observed in *E.gracilis* $IF3_{chl}$. However, removal of the extensions on $IF3_{chl}$ has a significantly larger positive effect (3.5- to 4-fold) on the activity of this factor in initiation complex formation. Removal of the extensions on $IF3_{mt}$ also increased the activity of this factor to a small extent in initiation complex formation with the mRNA for subunit 2 of cytochrome oxidase (Figure 2B). Finally, deleting the extensions on $IF3_{mt}$ had a slightly positive effect when this factor was tested in initiation complex formation on *E.coli* 70S ribosomes (Figure 2C) indicating that the effect is not dependent on the interaction of $IF3_{mt}$ with mitochondrial ribosomes. Structural information on bacterial IF3 (Figure 2D) suggests that the extensions in $IF3_{mt}$ could be positioned to interact with the linker region (Figure 1A). Since the linker is believed to play an important role in the binding of $IF3_{mt}$ to the ribosome (27), removal of these extensions may actually increase the binding of $IF3_{mt}$ to the small subunit slightly. In this context, it should be noted that the deletion of both extensions on $IF3_{chl}$ increases the

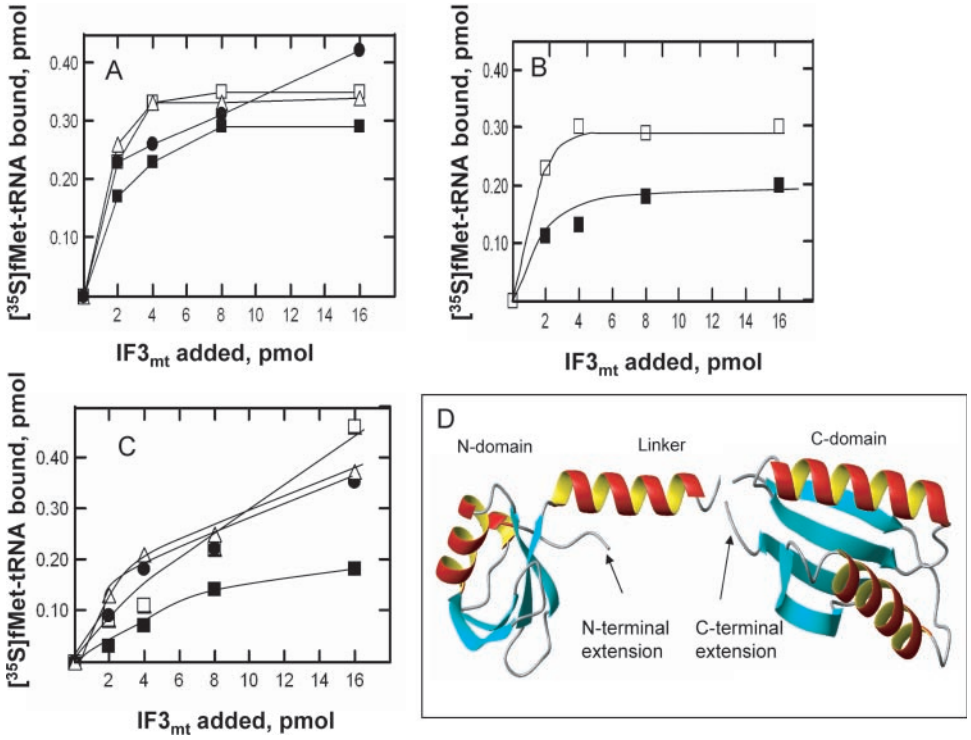

Figure 2. Activity of $IF3_{mt}$ and its deletion derivatives in initiation complex formation. (**A**) The activity of $IF3_{mt}$FL (closed squares), $IF3_{mt}\Delta N$ (closed circles), $IF3_{mt}\Delta C$ (open triangles) and $IF3_{mt}\Delta NC$ (open squares) was tested in initiation complex formation on mitochondrial 55S ribosomes using poly(A,U,G) as the mRNA. A blank containing no $IF3_{mt}$ has been subtracted from each value (0.29 pmol). (**B**) Activities of $IF3_{mt}$FL (closed squares) and $IF3_{mt}\Delta NC$ (open squares) were tested on mitochondrial 55S ribosomes using a transcript of the cytochrome oxidase subunit 2 gene as the mRNA. A blank containing no $IF3_{mt}$ (0.11 pmol) has been subtracted from each value. (**C**) The activities of $IF3_{mt}$FL (closed squares), $IF3_{mt}\Delta N$ (closed circles), $IF3_{mt}\Delta C$ (open triangles) and $IF3_{mt}\Delta NC$ (open squares) were tested on *E.coli* 70S tight couples using poly(A,U,G) as mRNA. A blank containing no $IF3_{mt}$ (0.34 pmol) has been subtracted from each value. (**D**) Model for the N- and C-domains of *Bacillus stearothermophilus* IF3 created from the PDB coordinates (1TIF and 1TIG) using MolMol (55) indicating the location of the N- and C-terminal extensions. Homology modeling suggests that the N-domain of $IF3_{mt}$ has a similar fold to that observed with the *B.stearothermophilus* factor (23). The C-domain of $IF3_{mt}$ is not as highly conserved and cannot be modeled accurately. However, it is probable to have a similar overall fold.

affinity of $IF3_{chl}$ for chloroplast 30S subunits ~100-fold (28). Alternatively, the extensions in $IF3_{mt}$ may be playing a different role in initiation complex formation.

Proofreading activity of $IF3_{mt}$

One of the roles assigned to IF3 in initiation is to proofread the selection of fMet-tRNA and the AUG codon in the P-site (20,38,40). This effect appears to occur through conformational changes in the subunit rather than by a direct interaction of IF3 with the fMet-tRNA bound at the P-site (41). One of the classical methods for measuring the proofreading function of IF3 is to test its ability to promote the dissociation of AcPhe-tRNA bound to the small subunit in response to poly(U). When these experiments are carried out with *E.coli* 30S subunits, the AcPhe-tRNA is bound non-enzymatically (in the absence of IF2) using an elevated concentration of Mg^{2+}. As indicated in Figure 3, *E.coli* IF3 effectively dissociates AcPhe-tRNA bound to the 30S subunit but does not dissociate fMet-tRNA bound to the small subunit. When $IF3_{mt}$ is tested in this assay, it is quite anemic in dissociating the bound AcPhe-tRNA (Figure 3). As expected, it does not promote the release of fMet-tRNA. Deletions of both N- and C-terminal extensions had no effect on the response of $IF3_{mt}$ in this assay (data not shown). In contrast, deletion of these extensions improved that ability of $IF3_{chl}$ to dissociate the 30S:AcPhe-tRNA:poly(U) complex which

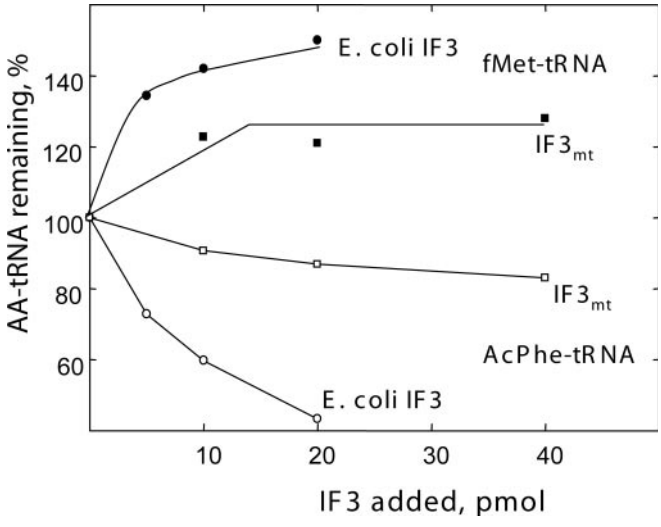

Figure 3. Activity of *E.coli* IF3 and $IF3_{mt}$ in proofreading the initiation complex. The abilities of *E.coli* IF3 (circles) and $IF3_{mt}$ (squares) to promote the dissociation of a pre-formed complex [*E.coli* 30S:poly(U):[^{14}C]AcPhe-tRNA] (open symbols) were measured in the presence of various concentrations of IF3 as described in Materials and Methods. The value for 100% complex remaining is 2.1 pmol. The effects of *E.coli* IF3 and $IF3_{mt}$ on a pre-formed complex [30S:poly(A,U,G):[^{35}S]fMet-tRNA] (closed symbols) were tested under similar conditions. The 100% value for these experiments was 0.14 pmol.

Table 1. Non-enzymatic binding of [^{14}C]AcPhe-tRNA and [^{35}S]fMet-tRNA to mitochondrial 28S subunits in the presence and absence of IF3

Amino acid-tRNA	aminoacyl-tRNA bound to 28S (pmol)	
	No IF3 added	With IF3 added
[^{14}C]AcPhe-tRNA	0.83	0.40
[^{35}S]fMet-tRNA	0.007	0.003

The 28S:poly(U):[^{14}C]AcPhe-tRNA or 28S:poly(A,U,G):[^{35}S]fMet-tRNA complexes were formed non-enzymatically and the effect of adding $IF3_{mt}$ was assessed as described in Materials and Methods. A blank representing the retention of label on the filters in the absence of subunits (~0.16 pmol, 80–100 c.p.m., for [^{14}C]AcPhe-tRNA and ~0.02 pmol for [^{35}S]fMet-tRNA) has been subtracted from each value.

correlated with the improved binding of this factor to chloroplast 30S subunits upon deletion of the extensions (28).

Initial studies to examine the proofreading activity of $IF3_{mt}$ on mitochondrial 28S ribosomal subunits provided some surprises. Non-enzymatic binding of AcPhe-tRNA could be obtained on 28S subunits at 15–25 mM Mg^{2+} and $IF3_{mt}$ was active in destabilizing these complexes (Table 1). However, the positive control for these experiments should be the stability of complexes formed with fMet-tRNA and the AUG codon in the presence of $IF3_{mt}$. Non-enzymatic binding of fMet-tRNA occurs readily in the *E.coli* system as the concentration of Mg^{2+} is raised. However, essentially no non-enzymatic binding of fMet-tRNA could be detected with 28S subunits even at Mg^{2+} ion concentrations as high as 40 mM (Table 1). The lack of non-enzymatic binding of fMet-tRNA prevented a true assessment of the ability of $IF3_{mt}$ to proofread initiation complex formation in the mitochondrial system.

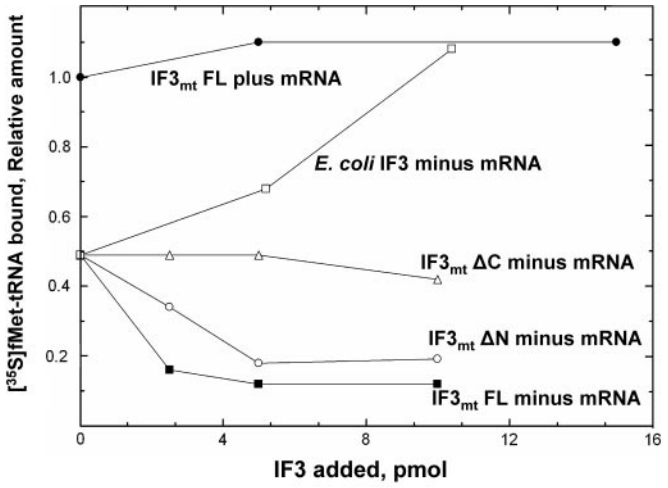

Figure 4. Effect of *E.coli* IF3, $IF3_{mt}$ and its derivatives on the binding of [^{35}S]fMet-tRNA to mitochondrial 28S subunits in the presence and absence of mRNA. Initiation complexes were prepared containing [^{35}S]fMet-tRNA, $IF2_{mt}$ and 28S subunits in the presence and absence of poly(A,U,G) as mRNA. After incubation with the indicated amounts of $IF3_{mt}$ or its derivatives, the amount of complex remaining was determined using a nitrocellulose filter-binding assay. The data are reported as the relative amount of binding obtained since data from different experiments were combined and the absolute numbers obtained depend on the percentage of active 28S subunits in different preparations. For these experiments, the value normalized to 1 for binding in the presence of mRNA represents 0.71 pmol while the value bound in the absence of mRNA was 0.35 pmol. For the experiments testing the effects of deletion of the N- and C-terminal extension, the level of binding obtained in the absence of mRNA and $IF3_{mt}$ was 0.12 pmol. $IF3_{mt}$FL in the presence of mRNA (closed circles), $IF3_{mt}$FL in the absence of mRNA (closed squares), $IF3_{mt}\Delta N$ (open circles) or $IF3_{mt}\Delta C$ (open triangles) or *E.coli* IF3 (open squares) were added as indicated. The amount of fMet-tRNA retained on the small subunit was measured as described in Materials and Methods.

Effect of $IF3_{mt}$ on the binding of fMet-tRNA to 28S subunits in the absence of mRNA

Control experiments used in analyzing the low numbers obtained in the proofreading experiments lead to the realization that a significant amount of fMet-tRNA binds to mitochondrial 28S subunits in the absence of mRNA. This binding is completely dependent on the presence of $IF2_{mt}$ (data not shown). Interestingly, the message-independent binding of fMet-tRNA to 28S subunits is destabilized by $IF3_{mt}$ (Figure 4). In contrast, $IF3_{mt}$ has no effect on the binding of fMet-tRNA to 28S subunits in the presence of mRNA. The destabilization of fMet-tRNA binding to mitochondrial 28S subunits is in contrast to observations made in *E.coli* in which IF3 is reported to stabilize the IF2-dependent binding of fMet-tRNA to 30S subunits in the absence of mRNA (42). We have retested this effect using *E.coli* 30S subunits and have observed that there is some mRNA-independent binding to these small subunits [~10% of the level of binding observed in the presence of poly(A,U,G)]. And, as reported, *E.coli* IF3 stimulated this binding ~2-fold (data not shown). Interestingly, *E.coli* IF3 also stimulates the mRNA-independent binding of fMet-tRNA to mitochondrial 28S subunits ~2-fold. Thus, it behaves quite differently in this assay than does $IF3_{mt}$.

The destabilization of message-independent binding to mitochondrial 28S subunits by $IF3_{mt}$ is quite rapid and is essentially complete within ~20 s (data not shown). $IF3_{mt}$ was able to dissociate fMet-tRNA pre-bound to the 28S subunit in the absence of mRNA indicating that it does not have to be present on the small subunit prior to fMet-tRNA binding to carry out this activity.

Sucrose gradient analysis was used to assess whether the fMet-tRNA bound to mitochondrial 28S subunits in the absence of mRNA could be incorporated into 55S monosomes. As indicated in Figure 5A, fMet-tRNA could be observed bound to 28S subunits when reaction mixtures were incubated in the absence of $IF3_{mt}$. The presence of $IF3_{mt}$ resulted in a substantial decrease in the amount of fMet-tRNA bound as observed in the filter-binding assay. As expected, no fMet-tRNA binding was observed to mitochondrial 39S subunits in either the presence or absence of $IF3_{mt}$ (data not shown). About 25% of the fMet-tRNA bound in the absence of mRNA was observed in the 55S region of the gradient after 39S subunits were added indicating that at least a portion of this material could be chased into 55S complexes (Figure 5B). No fMet-tRNA was observed remaining in the 28S region of the gradient. Nitrocellulose filter-binding assays suggest that there is no loss of the fMet-tRNA bound in the absence of mRNA when 39S subunits are added suggesting that the lower yield of 55S complexes observed in the sucrose gradients arises from the reduced stability of these complexes compared with fMet-tRNA bound to 28S subunits directly. The loss of a portion of the fMet-tRNA bound to the subunit upon formation of 55S complexes probably reflects the release of $IF2_{mt}$ upon subunit joining.

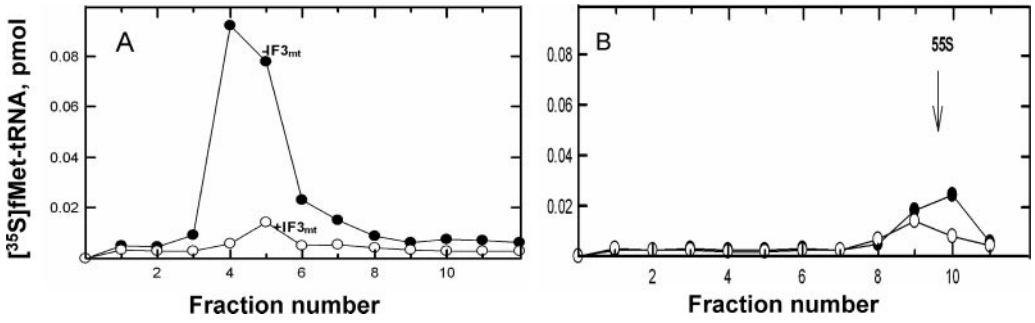

Figure 5. Effect of the addition of 39S subunits on the mRNA-independent binding of [^{35}S]fMet-tRNA formed in the presence and absence of IF3$_{mt}$. (**A**) The 28S subunits were incubated with [^{35}S]fMet-tRNA and IF2$_{mt}$ in the absence (closed circles) or presence (open circles) of IF3$_{mt}$. Reaction mixtures were analyzed by sucrose density gradient centrifugation and the position of the [^{35}S]fMet-tRNA was located by filtering appropriate fractions as described in Materials and Methods. (**B**) [^{35}S]fMet-tRNA binding was initially carried out with 28S subunits in the presence of IF2$_{mt}$ but in the absence of mRNA. Reactions mixtures were prepared in the absence (closed circles) or presence (open circles) of IF3$_{mt}$. Following assembly of these complexes, 39S subunits (0.066 μM) were added and the incubation was continued for an additional 5 min at 27°C. The resulting complexes were then analyzed on sucrose gradients.

Since destabilization of mRNA-independent binding of fMet-tRNA to the small subunit is observed in the mammalian mitochondrial system but not in the prokaryotic system, the effects of the N- and C-terminal extensions on IF3$_{mt}$ on this activity were examined. As indicated in Figure 4, both the full-length factor and the derivative lacking the N-terminal extension were active in promoting the release of fMet-tRNA bound to the small subunit in the absence of mRNA. However, when the C-terminal extension was deleted, no destabilization of the fMet-tRNA bound to the small subunit in the absence of mRNA was observed (Figure 4). Deletion of both extensions gave a result identical to that observed when the C-terminal extension alone was deleted (data not shown). This observation suggests that this unusual activity of IF3$_{mt}$ requires the C-terminal extension and suggests that this extension developed on the mammalian mitochondrial factor in order to promote the dissociation of fMet-tRNA bound to the 28S subunit prior to mRNA binding.

The mitochondrial 28S subunit has the ability to bind mRNAs in a sequence independent manner in the absence of any added factors (35,43). The effect of IF3$_{mt}$ on this interaction was tested by monitoring the formation of the complex between 28S subunits and labeled mRNA for subunit 2 of cytochrome oxidase using a nitrocellulose filter-binding assay. IF3$_{mt}$ had no effect on the direct binding of mRNA to 28S subunits (data not shown) indicating that this complex can form in the presence of IF3$_{mt}$ and remains stable in its presence.

DISCUSSION

One of the classical features of bacterial IF3 is the ability to proofread the selection of fMet-tRNA and an AUG (or GUG) codon at the P-site during initiation. Detailed studies of the discrimination of IF3 against non-canonical initiation codons using tRNAs with the characteristic features of the initiator tRNA indicate that this factor recognizes primarily codon–anticodon interactions, at least at the second and third positions of the codon (20). The results reported here leave open the question of whether IF3$_{mt}$ has a proofreading function comparable with that observed with *E.coli* IF3. One might even question whether proofreading is important in the animal mitochondrial translational system with its limited repertoire of mRNAs to translate. Certain differences must apply to the mitochondrial system since both AUG and AUA serve as methionine codons in this organelle. In humans, 3 of the 13 translational start sites use AUA as the start codon. The basis for the ability of the mammalian fMet-tRNA^{Met} to read the AUA codon is unclear although it has been postulated that the minor base 5-formyl cytidine has a critical role to play in decoding the AUA triplet (44). In humans, mice and presumably several other mammals, AUU also serves as a start codon for at least one mitochondrial mRNA (45,46). In addition, mutation of the AUG start codon to GUG in the ATPase 6 mRNA allows efficient initiation indicating that this codon can also serve as a start codon (47). These observations indicate that there is considerable tolerance for the start codon used in mammalian mitochondria with variations accepted in both position 1 and position 3 of the codon.

The same tRNA^{Met} is used for both initiation and elongation in mammalian mitochondria. This tRNA has retained the classical set of three G:C base pairs at the bottom of the anticodon stem, a characteristic of initiator tRNAs (48,49). The three consecutive G:C pairs are critical for the binding of the initiator tRNA to the P-site during initiation and the anticodon stem is examined by IF3 during translational initiation (19,50). Interestingly, tRNA^{Met} is the only mammalian mitochondrial tRNA characterized by three consecutive G:C base pairs at the bottom of the anticodon stem suggesting that this feature remains important for the selection of this tRNA for binding to the P-site during initiation (51).

One of the most unusual observations emerging from these studies is that IF3$_{mt}$ has the ability to dissociate the IF2$_{mt}$-dependent binding of fMet-tRNA to the 28S subunit in the absence of mRNA. Our current working hypothesis to account for this activity is illustrated by the model in Figure 6. In this model, premature binding of fMet-tRNA in the presence of IF2$_{mt}$ would lead to an unproductive complex. IF3$_{mt}$ dissociates this unproductive complex or prevents its formation. In the productive pathway, mRNA binds to the 28S subunit but the subunit is positioned randomly on the message (Step 1). The basis for this idea emerges from the observation that 28S ribosomal subunits bind mRNAs quite tightly (K_d of 25 nM at 50 mM KCl). This binding occurs randomly on the mRNA (35,43). IF3$_{mt}$ is postulated to alter the position of the mRNA, promoting the positioning of the 5′ start codon into the P-site.

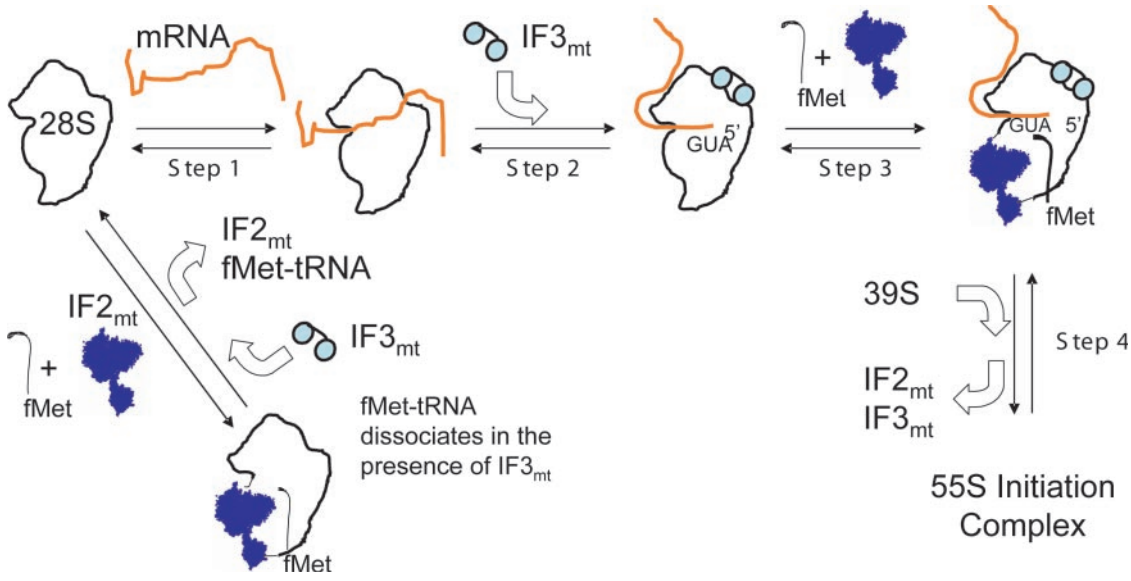

Figure 6. Proposed model for the role of $IF3_{mt}$ in initiation complex formation in mammalian mitochondria. For a description of the steps see text.

This idea has precedents in bacterial initiation in which the 5′-untranslated region near the Shine/Dalgarno sequence lies at the junction of the platform and the head of the small subunit (52) (Step 2). IF3 binding near the platform promotes the rearrangement of the mRNA facilitating the correct placement of the AUG start codon in the P-site (22,53). Following the correct positioning of the mRNA, $IF2_{mt}$ promotes the binding of fMet-tRNA (Step 3). Finally, the 39S subunit joins this complex leading to the release of the initiation factors and the formation of the 55S initiation complex (Step 4).

The data presented here indicate that the N-terminal and C-terminal extensions on $IF3_{mt}$ are not essential for the activity of this factor in promoting initiation complex formation on mitochondrial 55S ribosomes. However, the C-terminal extension appears to be essential for allowing $IF3_{mt}$ to dissociate fMet-tRNA bound in the absence of mRNA. In the bacterial system, the order of binding of mRNA and fMet-tRNA to the small subunit appears to be random. Either will also bind to 30S subunits in the absence of the other although with less stability (7,54). We believe that the evolution of the C-terminal extension on mammalian $IF2_{mt}$ arose as a means to create an ordered pathway for the binding of mRNA prior to the binding of fMet-tRNA during initiation.

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