Mammalian Mitochondrial Initiation Factor 2 Supports Yeast Mitochondrial Translation Without Formylated Initiator tRNA*

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Short Title: BOVINE MITOCHONDRIAL IF2 IN YEAST

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Abbreviations: fMet-tRNA\textsubscript{Met}, formylated initiator methionyl-tRNA; 5-FOA, 5-
fluoroorotic acid; IF2, initiation factor 2; mIF2, mitochondrial initiation factor 2.
Abstract

Initiation of protein synthesis in mitochondria and chloroplasts is widely believed to require a formylated initiator methionyl-tRNA (fMet-tRNA_{\text{Met}}) in a process involving initiation factor 2 (IF2). However, yeast strains disrupted at the FMT1 locus, encoding mitochondrial methionyl-tRNA formyltransferase, lack detectable fMet-tRNA_{\text{Met}} but exhibit normal mitochondrial function as evidenced by normal growth on non-fermentable carbon sources. Here we show that mitochondrial translation products in *S. cerevisiae* were synthesized in the absence of formylated initiator tRNA. *ifm1* mutants, lacking the mitochondrial initiation factor 2 (mIF2), are unable to respire, indicative of defective mitochondrial protein synthesis, but their respiratory defect could be complemented by plasmid-borne copies of either the yeast *IFM1* gene, or a cDNA encoding bovine mIF2. Moreover, the bovine mIF2 sustained normal respiration in *ifm1 fmt1* double mutants. Bovine mIF2 supported the same pattern of mitochondrial translation products as yeast mIF2, and the pattern did not change in cells lacking formylated Met-tRNA_{\text{Met}}. Mutant yeast lacking any mIF2 retained the ability to synthesize low levels of a subset of mitochondrially-encoded proteins. The *ifm1* null mutant was used to analyze the domain structure of yeast mIF2. Contrary to a previous report, the C-terminus of yeast mIF2 is required for its function *in vivo*, whereas the N-terminal domain could be deleted. Our results indicate that formylation of initiator methionyl-tRNA is not required for mitochondrial protein synthesis. The ability of bovine mIF2 to support mitochondrial translation in the yeast *fmt1* mutant suggests that
this phenomenon may extend to mammalian mitochondria as well.

Introduction

Initiation factor 2 (IF2) plays a critical role in the initiation of translation. IF2 binds the initiator tRNA, then positions it over the initiation codon on the ribosome in a GTP-dependent process (1). In eubacteria, the initiator Met-tRNA\textsubscript{fMet} is formylated by the enzyme methionyl-tRNA formyltransferase. Formylated methionyl-tRNA (fMet-tRNA\textsubscript{fMet}), along with Met-tRNA formyltransferase activity, is also observed in mitochondria of eukaryotes such as \textit{Saccharomyces cerevisiae}, \textit{Neurospora crassa}, and mammals (2-5). These observations, along with the identification of formylmethionine at the N-terminus of several mitochondrially synthesized proteins (6), led to the widely accepted dogma that mitochondrial protein synthesis initiation requires fMet-tRNA\textsubscript{fMet} in a process involving IF2. However, the absolute requirement for formylation of initiator tRNA in mitochondrial protein synthesis has been recently brought into question by genetic and biochemical analyses in \textit{S. cerevisiae}. It was shown that yeast strains unable to synthesize fMet-tRNA\textsubscript{fMet}, due either to mutations in the \textit{FMT1} gene encoding the formyltransferase, or the \textit{MISI} gene encoding mitochondrial C\textsubscript{1}-tetrahydrofolate synthase, exhibit normal mitochondrial function and stability (7). This suggests that protein synthesis in yeast mitochondria can initiate with non-formylated Met-tRNA\textsubscript{fMet}. Furthermore, \textit{in vitro} binding assays have shown that purified yeast
mIF2 supports significant binding of both fMet-tRNA_{fMet} and Met-tRNA_{fMet} to *E. coli* 30S ribosomal subunits, with 6-7 fold higher binding of fMet-tRNA_{fMet} than Met-tRNA_{fMet} (8). In contrast, purified bovine mIF2 showed much greater preference (20- to 50-fold) for fMet-tRNA_{fMet} relative to unformylated Met-tRNA_{fMet} in *in vitro* binding assays (9).

Domain structures of bacterial IF2 (10-12) and bovine mIF2 (13) have been elucidated. The N-terminal domains are highly variable in sequence and length between the different IF2s. A conserved, central G-domain contains primary structure elements typical for GTP/GDP binding and GTPase activity. The C-domain, also with several conserved amino acids, binds the initiator tRNA. More specifically, for *Bacillus stearothermophilus* IF2, the last 90 amino acids have been shown to contain the entire binding site for initiator tRNA (14).

The yeast mIF2 shares 30-40% homology with IF2s from mammalian mitochondria and *E. coli*, with most of the sequence identities found in the G- and C-domains. Yeast mIF2 is nuclear encoded by the *IFM1* gene (15) containing codons for 676 amino acids. Mutations in this gene result in the petite phenotype (15), indicative of impaired mitochondrial protein synthesis (16).

In this report, the domain structure of yeast mIF2 was investigated by expressing full-length and truncated forms of the gene in an *ifm1* deletion strain. In addition, a cDNA encoding bovine mIF2 was expressed in yeast *ifm1* deletion strains and its activity in yeast mitochondrial protein synthesis was analyzed. Finally, the ability of bovine
mIF2 to initiate protein synthesis in yeast mitochondria without formylation of the initiator tRNA was investigated using fmt1 mutants.

**EXPERIMENTAL PROCEDURES**

*Materials*--The following reagents and materials were used: media (Difco Laboratories,), 5-fluoroorotic acid (5-FOA) (Diagnostic Chemicals Limited, Oxford, CT), *Pfu* DNA Polymerase (Stratagene, La Jolla, CA), restriction enzymes and Geneticin (Gibco BRL, Life Technologies, Rockville, MD), erythromycin, chloramphenicol and cycloheximide (Sigma, St. Louis, MO), [35S]Methionine (Perkin Elmer Life Sciences, Boston, MA), TRI-Reagent (Molecular Research Center, Inc., Cincinnati, OH). All other chemical reagents were from Sigma or Fisher.

*Strains and Growth Conditions*--The *S. cerevisiae* strains used in this work are described in Table 1. All strains were derived from the EUROSCARF deletion set (Frankfurt, Germany), including LOY1, which was obtained by sporulation and tetrad dissection of EUROSCARF diploid Y21714. The Y03039 X Y11714 diploid was constructed by mating the two haploid strains and selecting for diploids nutritionally. Haploid double mutant strains (Δifm1 Δfmt1) were obtained by sporulation and tetrad dissection of the Y03039 X Y11714 diploid and the disruptions were confirmed by PCR.

Rich media contained 1% yeast extract, 2% Bacto-peptone and either 2% glucose (YPD) or 3% glycerol and 2% ethanol (YPEG) as carbon sources. Synthetic minimal medium contained 0.7% yeast nitrogen base without amino acids, 2% glucose or 2% galactose as carbon sources, and was supplemented with the following as necessary for
growth (final concentration): L-leucine (30 mg/liter), L-histidine (20 mg/liter), L-lysine (30 mg/liter), L-methionine (20 mg/liter), and uracil (20 mg/liter). Solid media contained 2% agar. To select for yeast strains with an integrated kanamycin resistance cassette, Geneticin was added at a final concentration of 300 µg/ml. Diploids were sporulated on solid medium at 25°C and tetrads dissected as described previously (17). Plasmid shuffling in ∆ifm1 ∆fmt1 yeast strains required the addition of 5-FOA to agar plates. 5-FOA plates contained 0.7% yeast nitrogen base, 0.1% 5-FOA, 2% glucose, and amino acids at the concentrations used in minimal medium, except 50 mg/liter uracil.

Plasmid Construction--Plasmid constructs are shown in Table 1. Pfu DNA polymerase was used for PCR amplification of DNA when plasmids were constructed. The complete sequence of the yeast IFM1 gene was PCR-amplified from yeast genomic DNA using primers IFM1-5’ and IFM1-3’fl, containing HindIII and BamHI restriction sites, respectively. The resulting PCR product was digested and ligated into yeast expression vector, pVT101U (18) to generate the pVTU-IFM1 construct. Similarly, the nucleotide sequence encoding the N-terminal 421 amino acids of IFM1 was amplified using primers IFM1-5’ and IFM1-3’422, which contained HindIII and XbaI recognition sites, respectively. The IFM1-3’422 primer was designed to replace amino acid 422 with a stop codon. The resulting PCR product was digested with HindIII and XbaI and ligated into pVT101U, to generate the pVTU-IFM1-422 construct. Full length and truncated forms of yeast mIF2 were also cloned into the yeast vector, YEp352 (19). The full-length IFM1 gene was subcloned from pVTU-IFM1 with SphI and BamHI. The truncated version of IFM1 was cleaved from pVTU-IFM1 using SphI and BglII, which cleaves at codon 423. The resulting DNA fragments were ligated into the SphI and
BamHI sites of YEp352 to construct YEp352-IFM1 and YEp352-IFM1-422, respectively. Both constructs include the alcohol dehydrogenase promoter (ADH) from the pVT101U vector to drive expression of the IFM1 insert. The YEp352-IFM1-422 construct does not have a stop codon in the insert sequence and when expressed produces a protein product with an additional 34 vector-encoded amino acids at its C-terminus before an in-frame stop codon is read.

A plasmid containing the cDNA encoding the mature bovine mIF2 was obtained from Dr. Linda Spremulli (Univ. of North Carolina). To express the protein in yeast mitochondria, this cDNA, along with a yeast mitochondrial targeting sequence, was cloned into a yeast expression vector, using overlap extension PCR (20). Specifically, the DNA encoding the bovine mIF2 was PCR-amplified from the plasmid using 5’ and 3’ primers, bIF2C and bIF2D. The 100 nucleotide mitochondrial targeting sequence of the yeast MIS1 gene (21) was PCR-amplified from a plasmid containing the full-length MIS1 ORF using primers Mis1A and Mis1B. The bIF2C primer contained 10 nucleotides of MIS1 mitochondrial targeting sequence on the 5’ end, and the Mis1B primer contained 10 nucleotides of bovine mIF2 cDNA on the 5’ end. The resulting PCR products from these two reactions were purified using the Qiagen PCR purification kit, and combined in a second PCR reaction with primers Mis1A and bIF2D. This second PCR reaction generated a product with the MIS1 targeting sequence fused to the mature bovine mIF2 cDNA, at codon 78. The final PCR product was cleaved with XbaI and HindIII and ligated into pVT101U, to generate the pVT-bmIF2 construct.

A plasmid containing the yeast IFM1 G+C domain fused behind the predicted IFM1 mitochondrial targeting sequence was also constructed using overlap extension PCR
The IFM1 sequence was PCR-amplified from pVTU-IFM1 starting at the nucleotides encoding amino acid 144 of the full-length protein. Oligonucleotide primers IFM1-144-5’ and CS-A, which hybridizes downstream of the pVT101U multiple cloning region, were used for this reaction. A second PCR reaction amplified the first 41 codons of IFM1 with primers IFM1-41-3’ and CS-B, which hybridizes upstream of the pVT101U multiple cloning site. These two PCR products were used in a third reaction, along with primers CS-A and CS-B to generate a product containing the IFM1 G+C domain fused behind the predicted IFM1 presequence. This final PCR product was digested with HindIII and BamHI and ligated into pVT101U to generate the pVTU-IFM1-pre+G+C construct. DNA sequence analysis of the inserts of all plasmid constructs was performed at the DNA facility of the Institute for Cellular and Molecular Biology at the University of Texas at Austin.

For plasmid shuffling experiments, the full-length IFM1 gene and the IFM1-pre+G+C insert were subcloned from the pVT101U vectors into pVT101L plasmids (18). The inserts were removed from pVTU-IFM1 and pVTU-IFM1-pre+G+C using SphI and ligated into the SphI-cleaved pVT101L plasmid to generate pVTL-IFM1 and pVTL-IFM1-pre+G+C constructs.

Complementation Analyses—Plasmids were transformed into various yeast strains as described previously (17). Due to the high rate of irreversible loss of mitochondrial DNA in ifm1 disrupted strains, heterozygous diploid strains (IFM1/ifm1::KanMX4) were typically transformed with a plasmid expressing IF2 prior to sporulation and tetrad dissection when testing the function of the plasmid-borne gene. Following sporulation and tetrad dissection, haploid spores were screened for mutant
ifm1 or fmt1 loci by yeast colony PCR (22).

Plasmid shuffling was used to test the function of pVTL-IFM1 and pVTL-IFM1-pre+G+C constructs in Δifm1 Δfmt1 mutant yeast strains. The double mutant strain carrying the pVTU-bmlIF2 plasmid was transformed with pVTL-IFM1, pVTL-IFM1-pre+G+C or pVT101L, selecting for transformants on minimal medium lacking uracil and leucine. Transformants were grown in +Ura media and plated onto 5-FOA plates to select for strains that evicted the pVTU-bmlIF2 plasmid. The resulting Δifm1 Δfmt1 strains carrying the pVTL-IFM1, the pVTL-IFM1-pre+G+C, or the empty pVT101L vector were tested for growth on YPEG plates.

Isolation of Yeast Mitochondria--Mitochondria were isolated from yeast by differential centrifugation according to Glick and Pon (23), scaled down for 4 liters of yeast culture. The concentration of mitochondrial protein in crude mitochondrial pellets was estimated as described (23). These pellets were resuspended in 0.6 M sorbitol/20 mM HEPES-KOH (pH 7.4) and 10 mg/ml fatty acid-free BSA. Resuspended mitochondria were frozen in liquid nitrogen and stored at –70° C for in organello aminoacylation assays. Actual concentration of mitochondrial protein was 10-12 mg/ml according to BCA (bicinchoninic acid) assays (Pierce).

In Organello Aminoacylation Assays--In organello aminoacylation assays for detection of Met-tRNA formyltransferase activity were performed essentially as described (24), except 20-40 μCi of [35S]methionine were used rather than a labeled methionine/cysteine mixture. Following a 15 minute, 30°C incubation with radioactive methionine, total RNA was isolated from mitochondrial pellets using TRI-Reagent...
according to the manufacturers protocol. Total RNA was resuspended in 15 \( \mu l \) 100 mM Tris-HCl (pH 9.2) and incubated for 1-2 hours at 37\(^0\)C, to deacylate the tRNA. \textit{E. coli} tRNA\textsubscript{f}Met (Sigma) was aminoacylated as described (8) and approximately 1 pmol (0.015 \( \mu \)Ci) of each, \[^{35}\text{S}]\text{Met-tRNA}_{f}\text{Met}\) and \[^{35}\text{S}]\text{f-Met-tRNA}_{f}\text{Met}\), were deacylated along with the mitochondrial tRNA and used as standards. The deacylated tRNA was ethanol precipitated and the supernatant containing the released \[^{35}\text{S}]\text{methionine or formyl}[^{35}\text{S}]\text{methionine was lyophilized and resuspended in 15 } \mu l \text{ of water. 2 } \mu l \text{ of each reaction, along with 1 } \mu l \text{ of each standard, were spotted onto a cellulose 300 thin-layer chromatography plate (Selecto Scientific) which was developed in butanol:acetic acid:H}_2\text{O (2:1:1). The dried plate was exposed to a phosphorimaging screen and scanned with a Molecular Dynamics Phosphorimager 445SI, to detect labeled methionine and formylmethionine.}

\textit{Labeling of Mitochondrial Translation Products--In vivo} labeling of mitochondrial translation products was performed as described (25), using 1175 Ci/mmole \[^{35}\text{S}]\text{methionine. Cells were grown in minimal media with 2\% galactose. As a negative control, some strains were grown in minimal galactose medium containing 2 mg/ml erythromycin to completely inhibit mitochondrial protein synthesis (26). Erythromycin was also present in the buffer during the labeling procedures for the cell samples grown with this inhibitor. Following the 30 min labeling incubation, samples were processed as described (25), except that the acetone wash was done twice. Samples were resolved by SDS-PAGE and the dried gel analyzed by phosphorimaging.
RESULTS

Predicted domain structure of yeast mIF2--The primary amino acid sequence of yeast mIF2, encoded by the nuclear IFM1 gene, is 30-40% identical to IF2s from E. coli and bovine mitochondria, with highest homology in the G and C domains of the protein. The predicted domain structure for yeast mIF2, based on E. coli and bovine mIF2 domain structures, is shown in Fig. 1A. A mitochondrial targeting sequence, predicted to be 41 amino acids in length (PSORT II analysis, http://psort.nibb.ac.jp/form2.html), is followed by the N-terminal domain, which is quite divergent among all IF2 sequences elucidated to date. A highly conserved G-domain beginning at amino acid 144 follows the N-terminal domain, and is responsible for the GTPase activity of the protein. The C-domain, extending from amino acids 300-676, also contains several conserved residues and is likely responsible for initiator tRNA binding.

Complementation of an ifm1 Null Mutant--Vambutas et al. reported the isolation of a respiratory-deficient ifm1 mutant strain (15). Mutant cells were capable of growth on glucose, but were unable to grow on the non-fermentable carbon source, glycerol plus ethanol. The resulting petite phenotype was rescued by expression of the full-length IFM1 gene. Furthermore, they reported that expression of the N-terminal 422 amino acids of IFM1 (indicated in Fig. 1A) was sufficient to complement the respiratory-deficiency of their ifm1 mutant strain. This truncated protein lacks most of the C-domain and it is the C-terminal part of the C-domain that binds fMet-tRNAfMet in B. stearothermophilus IF2 (14,27). This result therefore, might indicate significant differences in the domain structure of yeast mIF2, as compared to other IF2s. However,
the nature of the chromosomal ifm1 mutant used by Vambutas et al. (15) was not defined, and it is possible that the rescue by this truncated protein resulted from a gene conversion event or from intragenic complementation (28). The truncated protein might not be functional in an ifm1 null mutant. Therefore, we constructed an ifm1 deletion strain (LOY1) by sporulation and tetrad dissection of the EUROSCARF diploid, Y21714 (IFM1/ ifm1::KanMX4). As expected, LOY1 is respiratory-deficient. Plasmids for expression of the full-length IFM1 gene and the first 421 amino acids of IFM1 were constructed and transformed into the LOY1 mutant strain, which was then tested for the ability to grow non-fermentatively on glycerol plus ethanol (Fig. 1B). The plasmid construct expressing the full-length IFM1 gene pVTU-IFM1, was able to rescue the respiratory deficiency (petite phenotype) of the ifm1 deletion strain (Fig. 1B). However, the plasmid expressing the first 421 amino acids of IFM1 in the pVT101U vector (pVTU-IFM1-422) was not able to rescue growth on glycerol plus ethanol plates (Fig. 1B). Because of the potential for irreversible loss of mitochondrial function in strains defective in mitochondrial protein synthesis (29), these two constructs were also transformed into the heterozygous diploid, Y21714 (IFM1/ ifm1::KanMX4). Following sporulation and tetrad dissection, strains carrying both the ifm1 chromosomal mutation and the IFM1-expressing plasmids were tested for growth on glycerol plus ethanol media. As before, the full-length IFM1 was able to rescue the petite phenotype, but the construct expressing the N-terminal 421 amino acids of IFM1 was not (data not shown).

Upon closer inspection, we noted that the truncated IFM1 construct used by Vambutas et al. (15) encoded 34 additional, unrelated amino acids after amino acid 422 before reaching a stop codon in the vector. The C-terminal fusion of these unrelated
amino acids could possibly affect the function of the expressed protein in rescuing the petite phenotype. For this reason, we prepared a second construct (YEp352-IFM1-422) to express an identical protein to the one reported by Vambutas et al. (15), with the additional 34 amino acids at the C-terminus. We also inserted the full-length IFM1 into the same vector (YEp352-IFM1). These constructs are identical to those used by Vambutas et al. (15), except that in our case, expression was driven from the ADH promoter rather than the IFM1 promoter. As shown in Fig. 1B, YEp352-IFM1-422 was unable to rescue the petite phenotype of our ifm1 null mutant. Full-length IFM1 in the same vector, however, did rescue the petite phenotype.

Ma and Spremulli reported that the purified G+C domain of the bovine mIF2 is able to promote GTP-dependent binding of fMet-tRNAf^{Met} to mitochondrial ribosomes in vitro (13). Similarly, binding studies in vitro using yeast mIF2 indicate that the G+C domain alone is capable of binding initiator tRNA (8). To determine if yeast mIF2 is functional in the initiation of protein synthesis in vivo without its N-terminal region, a plasmid expressing just the G+C domain of IFM1 behind its predicted mitochondrial targeting sequence (pVTU-IFM1-pre+G+C) was transformed into the ifm1 mutant strain, LOY1. The resulting transformants grew on glycerol plus ethanol media, showing that the G+C domain of yeast mIF2 is sufficient to sustain mitochondrial protein synthesis in vivo (Fig. 1B).

Upon storage at −70°C, LOY1 lost the ability to be rescued by plasmid-borne IFM1, probably due to loss of mitochondrial DNA. This is consistent with the high frequency of ρ⁰ and ρ⁻ in mutants deficient in mitochondrial protein synthesis (29).
Therefore, subsequent experiments were initiated in heterozygous diploids, to ensure stability of the mitochondrial genome.

*Bovine mIF2 Expression in Yeast*--The cDNA encoding the bovine mIF2, starting with the codon 78 (30), was fused behind the DNA encoding the mitochondrial targeting sequence of the yeast *MIS1* gene (21) and subcloned into a yeast expression vector. This construct, pVTU-bmIF2, was transformed into the diploid Y21714 strain, heterozygous at the *IFM1* locus (*IFM1*/ifm1::KanMX4). The resulting transformants were sporulated and tetrads dissected. Geneticin-resistant spores containing the mutant *ifm1* gene and retaining the pVTU-bmIF2 plasmid were tested for the ability to grow non-fermentatively on YPEG media (Fig. 1C). The growth of the *ifm1* mutant carrying the bovine mIF2 cDNA was compared to those carrying the *IFM1* gene on a plasmid and to wild-type yeast cells. Expression of the bovine mIF2 cDNA rescued the petite phenotype of the *ifm1* mutant yeast strain, demonstrating that bovine mIF2 is capable of replacing yeast mIF2 and supporting yeast mitochondrial function *in vivo* (Fig. 1C).

*Complementation of Δifm1 Δfmt1 Double Mutant Lacking Formylation of Initiator tRNA*--To determine whether the bovine mIF2 is capable, like yeast mIF2, of supporting mitochondrial function without formylated initiator tRNA, it was necessary to express the bovine mIF2 in a yeast strain lacking the ability to produce fMet-tRNA<sub>fMet</sub> in the mitochondria. The nuclear *FMT1* gene encodes the yeast mitochondrial Met-tRNA formyltransferase, responsible for transferring the formyl group from 10-formyl-tetrahydrofolate to the initiator Met-tRNA<sub>fMet</sub> (7). Strains lacking this activity cannot
produce fMet-tRNA\[^{\text{f Met}}\] in the mitochondria, and therefore, should only have the
unformylated initiator Met-tRNA\[^{\text{f Met}}\] present. This was shown to be the case for the
parent \textit{fmt1} mutant strain. An \textit{in organello} aminoacylation assay (24) was used to examine
the formylation status of the initiator tRNA. Intact, isolated yeast mitochondria were
incubated with \[^{35}\text{S}]\text{methionine, and then total RNA was isolated from the mitochondria}
(see Materials and Methods). The total RNA was resuspended in Tris buffer (pH 9) to
decaylate the tRNA. After deacylation and ethanol-precipitation of the RNA, the
released \[^{35}\text{S}]\text{methionine and formyl[^{35}\text{S}]methionine in the supernatant were separated}
by thin layer chromatography and detected using a phosphorimager. The phosphorimage
from the analysis of Y03039 X Y11714 cells, with functional copies of both \textit{IFM1}
and \textit{FMT1} showed both \[^{35}\text{S}]\text{methionine and formyl[^{35}\text{S}]methionine, indicating the presence of}
both Met-tRNA\[^{\text{f Met}}\] and fMet-tRNA\[^{\text{f Met}}\] in the mitochondria (Fig. 2, lane 3). The
phosphorimage from the \textit{fmt1} mutant yeast strain, Y03039, showed only
[^{35}\text{S}]\text{methionine, indicating that no fMet-tRNA\[^{\text{f Met}}\] was produced in the mitochondria, as}
expected when the \textit{fmt1} gene is disrupted (Fig. 2, lane 2).

The pVTU-bmIF2 construct was transformed into Y03039 X Y11714 cells
(\textit{IFM1}/ifm1::\textit{KanMX4}, \textit{FMT1}/fmt1::\textit{KanMX4}). Following sporulation and tetrad dissection,
spores were screened by colony PCR for the disrupted \textit{ifm1} and \textit{fmt1} loci. Spores
containing deletions of both \textit{ifm1} and \textit{fmt1} and carrying the pVTU-bmIF2 construct
were isolated. These transformants grew on YPEG medium, indicating that the bovine
mIF2 can replace yeast mIF2 even in the absence of formylated initiator tRNA (Fig. 3). The plasmids expressing full-length yeast mIF2 and the G+C domain of yeast mIF2 were also tested in ∆ifm1 ∆fmt1 strains. Plasmid shuffling was used to introduce these plasmids into the double mutant strain. The pVTL-IFM1 and pVTL-IFM1-pre+G+C constructs were transformed into ∆ifm1 ∆fmt1/pVTU-bmIF2 selecting for Leu⁺ transformants. Following eviction of the plasmid encoding the bovine cDNA construct by growth on 5-FOA, the resulting strains carrying IFM1 plasmids were streaked onto YPEG plates to observe growth on a non-fermentable carbon source (Fig. 3). Since single fmt1 mutants show no growth defect on YPEG media at 30°C (7), the ∆ifm1 ∆fmt1/pVTL-IFM1 yeast grew on the non-fermentable carbon source as expected. The expression of just the G+C domain of yeast mIF2 (pVTL-IFM1-pre+G+C), however, did not rescue the petite phenotype of the ∆ifm1 ∆fmt1 mutant on YPEG plates. Similar results were seen when these strains were grown in liquid YPEG medium. Double mutant (∆ifm1 ∆fmt1) strains expressing plasmid-borne, full-length yeast mIF2 or bovine mIF2 grew at wild-type rates, while the pVTL-IFM1-pre+G+C and empty pVT101L constructs did not support growth in liquid YPEG medium (data not shown). Thus, the G+C domain only supported respiration in strains that produce fMet-tRNAf.

Mitochondrial Translation in Strains Lacking Formylated Initiator tRNA--

Radioactive labeling of mitochondrial translation products was used to directly demonstrate the activity of bovine mIF2 in yeast mitochondrial protein synthesis. Whole cells were incubated with [³⁵S]methionine and unlabeled amino acids in the presence of cycloheximide to inhibit cytoplasmic protein synthesis. After precipitation of proteins
with trichloroacetic acid, total cellular proteins were separated by SDS-PAGE and labeled mitochondrial translation products were visualized by phosphorimaging. The wild-type strain, Y03039 × Y11714, showed incorporation of [35S]methionine into all the major mitochondrial translation products (Fig. 4A, lane 2). Similar patterns of protein labeling were observed in ∆ifm1 ∆fmt1 mutant yeast expressing either yeast mIF2 or bovine mIF2 from the pVTL-IFM1 or pVTU-bmIF2 plasmids, respectively (Fig. 4A, lanes 3 and 4). This clearly demonstrates that both the yeast and the bovine mIF2s were able to support the initiation of protein synthesis and translation of mitochondrial proteins without formylated initiator tRNA. Surprisingly, labeled proteins were also observed in the ∆ifm1 ∆fmt1 strain carrying the empty pVT101L vector, lacking any mIF2 (Fig. 4A, lane 1). To verify that these protein products were indeed translated in the mitochondria, a control experiment using erythromycin as a mitochondrial protein synthesis inhibitor (26) was performed (Fig. 4B). Two ∆ifm1 ∆fmt1 yeast strains, one carrying the pVTL-IFM1 construct and the other carrying the empty pVT101L vector, were grown with or without erythromycin and used for mitochondrial protein labeling. As expected, in the presence of erythromycin, cells were unable to synthesize mitochondrial proteins, and no radiolabeled protein bands were observed on the phosphorimage (Fig. 4B, lanes 1 and 3). The same strains grown without erythromycin produced labeled translation products as seen before, indicating that these proteins are indeed products of the mitochondrial translation machinery (Fig. 4B, lanes 2 and 4). Some differences in the protein labeling pattern of strains lacking a mIF2 were observed (Fig. 4A, compare lanes 1 and 2). The most noticeable difference is the absence of visible CoxI and CoxII protein bands when
mitochondrial labeling is performed in strains lacking mIF2. Surprisingly, however, other major mitochondrially synthesized proteins can be observed in the Δifm1 ∆fmt1/pVT101L strain.

**DISCUSSION**

We have used the complementation of a *S. cerevisiae* ifm1 null mutant to show that the domain structure of yeast mIF2 is similar to that of prokaryotic and other mitochondrial IF2s. First, expression of DNA encoding the N-terminal 421 amino acids of yeast mIF2 did not rescue the respiratory deficiency of an ifm1 null mutant strain. Despite an earlier report to the contrary (15), this indicates that the C-terminal part of yeast mIF2 is critical to its function. Furthermore, expression of just the G+C domain of yeast mIF2 behind its predicted mitochondrial targeting sequence was sufficient for the complementation of the petite phenotype of the ifm1 mutant strain. This experiment clearly demonstrates the ability of the yeast mIF2 G+C domain to replace full-length yeast mIF2 *in vivo*, as long as fMet-tRNA<sub>Met</sub> is available, which is in agreement with *in vitro* binding studies showing initiator tRNA binding with just the yeast mIF2 G+C domain (8).

Similar complementation experiments were used to analyze the ability of bovine mIF2 to function in the initiation of yeast mitochondrial protein synthesis. Expression of the cDNA encoding bovine mIF2 and targeting of the resulting protein to yeast mitochondria rescued the respiratory deficiency of the ifm1 deletion strain. This indicates that the basic function and mechanism of mIF2 is conserved between yeast and...
mammals. Hence, *S. cerevisiae* represents a promising model organism for future studies of mammalian mitochondrial protein synthesis.

Formylation of initiator tRNA (Met-tRNA$_\text{Met}$) in prokaryotes and mitochondria has generally been believed to promote its recognition and binding by IF2. However, our previous work demonstrated that *in vivo*, yeast mitochondria can respire without formylation of their initiator Met-tRNA$_\text{Met}$ (7). Two different mutant strains unable to formylate their mitochondrial Met-tRNA$_\text{Met}$ were shown to be respiratory competent. The fmt1 mutant, lacking Met-tRNA formyltransferase activity, grew at wild-type rates on both rich and minimal glycerol plus ethanol media (7). Given the conservation of initiator Met-tRNA$_\text{Met}$ formylation in mitochondria from fungi to mammals, this result was quite surprising. The physiological significance of the ability to use unformylated initiator tRNA in mitochondrial protein synthesis is still being determined. It is likely that the presence of formylated Met-tRNA$_\text{Met}$ does provide some advantage for the cell. Indeed, studies published during the completion of this manuscript show that the lack of initiator tRNA formylation in some *S. cerevisiae* strains impaired the growth of these strains under severe conditions (31). Specifically, growth at high temperature (37°C) in rich medium on glycerol or growth in minimal medium on glycerol at 30°C, was slower in some, but not all, Δfmt1 strains.

We investigated the ability of the yeast mIF2 G+C domain to support mitochondrial function in yeast lacking mitochondrial initiator tRNA formylation (Δifm1 Δfmt1) (Fig. 3). As expected, the expression of full-length *IFM1* rescued the respiratory
deficiency of these strains. However, the G+C domain was not able to replace full-length yeast mIF2 when formylated Met-tRNA_{f^{Met}} was unavailable for translation initiation. *In vitro* experiments demonstrated that both the full-length yeast mIF2 and the yeast mIF2 G+C domain have 6-7-fold lower binding affinity for Met-tRNA_{f^{Met}} as compared to fMet-tRNA_{f^{Met}} (8). Although capable of fMet-tRNA_{f^{Met}} binding in *ifm1* \textit{FMT1}^{+} strains, we suspect that the yeast mIF2 G+C domain is less stable than its full-length counterpart. The potential instability of the G+C domain, combined with lower affinity for non-formylated initiator tRNA, likely reduced IF2 activity below a threshold necessary to sustain growth of the \Delta{ifm1}{\Delta}fmt1 mutants on YPEG medium. An alternative explanation is that the N-domain of mIF2 is involved in the binding of initiator tRNA.

The petite phenotype of \textit{ifm1} null mutants reflects the essential role of mIF2 in mitochondrial respiration. It was therefore surprising that yeast lacking mIF2 carried out a low level of mitochondrial protein synthesis (Fig. 4). However, only a subset of the 8 essential mitochondrial proteins could be detected in these cells. Loss of just one of these mitochondrially-synthesized proteins is known to lead to the petite phenotype.

Because bovine mIF2 was able to replace yeast mIF2 \textit{in vivo}, and because yeast strains lacking Met-tRNA formylation could be constructed, we were able to test whether bovine mIF2 is capable of utilizing unformylated Met-tRNA_{f^{Met}} to initiate yeast mitochondrial protein synthesis. Expression of bovine mIF2 rescued the respiratory deficiency of \Delta{ifm1}{\Delta}fmt1 yeast as efficiently as the yeast mIF2. Furthermore, labeling
of mitochondrial translation products in an Δifm1 Δfmt1 mutant was identical between strains expressing plasmid-borne bovine mIF2 cDNA and those expressing yeast mIF2. Therefore, bovine mIF2 is fully capable of initiating mitochondrial protein synthesis in a yeast strain lacking formylated Met-tRNAf^Met. This result was a surprise given that purified bovine mIF2 has a much greater preference (20- to 50-fold) for fMet-tRNAf^Met relative to unformylated Met-tRNAf^Met in \textit{in vitro} binding assays (9). Perhaps there exist accessory factors \textit{in vivo} that facilitate utilization of non-formylated Met-tRNAf^Met under conditions where fMet-tRNAf^Met is low or unavailable. In any event, this result brings into question whether formylation of the initiator tRNA is required in mammalian mitochondrial protein synthesis. Unlike the mitochondria of \textit{S. cerevisiae}, mammalian mitochondria contain only one tRNA^Met that is used for both translation initiation and elongation (32). It has been assumed that the rate of formylation of this tRNA determines its function in either initiation or elongation (33). But little is known about the mechanism of initiator tRNA recognition by mIF2, and how mammalian mitochondria control the relative levels of formylated versus unformylated Met-tRNA^Met is unclear. Because bovine mIF2 is capable of initiating \textit{S. cerevisiae} mitochondrial protein synthesis, we now have a model system in which to conduct \textit{in vivo} studies of mammalian mIF2 function. The expression of chimeric and mutant forms of mIF2 in yeast should provide more insight into the mechanism of initiator tRNA binding by IF2.

\textit{Acknowledgments}--We thank Dr. Linda Spremulli for providing the bovine
mitochondrial IF2 cDNA.

REFERENCES


Figure legends:

Fig. 1. Complementation of respiratory defect of ifm1 null mutant. (A) Predicted domain structure for IFM1-encoded yeast mIF2 based on sequence similarity to eubacterial and bovine mIF2. Numbers represent amino acid residues. M, mitochondrial targeting sequence (from PSORT II prediction); N, N-terminal domain; G, G-domain; C, C-terminal domain. Arrow at 422 indicates site of truncation in some constructs. (B) Expression of full-length and domain constructs of yeast mIF2 in ifm1 null mutant. ifm1 yeast (LOY1) harboring the various IFM1 constructs, as described in the text, were streaked onto a YPEG plate and incubated at 30°C for 5 days. Transformation with empty plasmid (either YEp352 or pVT101U) served as negative controls. (C) Expression of bovine mIF2 in ifm1 null mutant. ifm1 yeast harboring either the full-length yeast mIF2 (IFM1) or bovine mIF2 (bmIF2) in the plasmid pVT101U, were streaked onto a YPEG plate and incubated at 30°C for 7 days. Wild-type (IFM1) is an untransformed positive control strain in which the chromosomal IFM1 gene is intact.

Fig. 2. Phosphorimage of thin-layer chromatograph of [35S]methionine and [35S]formylmethionine from in organello aminoacylation assays. Lane 1, formyl-[35S]methionine standard; lane 2, deacylation products from ∆fmt1 mitochondria; lane 3, deacylation products from wild-type (FMT1) mitochondria; lane 4, [35S]methionine standard.
Fig. 3. Complementation of respiratory defect of *ifm1 fmt1* deletion mutant on YPEG.

*ifm1 fmt1* double mutants harboring either the full-length yeast mIF2 (IFM1), full-length bovine mIF2 (bmIF2), or G+C domain of yeast *IFM1* (IFM1-pre+G+C) in either plasmid pVT101U (pVTU-) or pVT101L (pVTL-), were streaked onto a YPEG plate and incubated at 30°C for 5 days. Empty plasmids (pVT101L or pVT101U) served as negative controls. The various plasmids were introduced by plasmid shuffling as described in the text.

Fig. 4. *In vivo* labeling of mitochondrial translation products in cells expressing yeast *IFM1* or bovine mIF2. Cells were labeled with [35S]methionine in the presence of cycloheximide, extracted, and analyzed by SDS-PAGE and phosphorimaging as described in the text. Equal amounts of total protein (~50 µg) were loaded on each lane. Var1, CoxI, CoxII, Cyt b, CoxIII, and Atp6 are abbreviations for products of yeast mitochondrial protein synthesis (25). The two smallest mitochondrially-synthesized proteins, Atp8 and Atp9, migrate with the dye front on these gels. (A) lane 1, ∆ifm1∆fmt1/pVT101L; lane 2, wild-type (Y03039 X Y11714); lane 3, ∆ifm1∆fmt1/pVT101L; lane 4, ∆ifm1∆fmt1/pVTU-bmIF2. (B) Cells were grown and labeling reactions performed in the presence (+) or absence (-) of erythromycin. Lanes 1 and 2, ∆ifm1∆fmt1/pVT101L; lanes 3 and 4, ∆ifm1∆fmt1/pVT101L. Plasmid constructs are as described in the legend to Fig. 3.
## Table I. Yeast strains and plasmid constructs

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<td>pVTU-bmIF2</td>
<td>pVT101U with bovine mIF2 behind MIS1</td>
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pVT101L with mitochondrial targeting sequence and G+C domain of IFM1

Table II. Sequences of oligonucleotide primers

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All primers were synthesized by Integrated DNA Technologies (Coralville, IA) and are listed 5’-3’. Restriction enzyme recognition sequences described in the text are underlined.
Mammalian mitochondrial initiation factor 2 supports yeast mitochondrial translation
without formylated initiator tRNA
Anne S. Tibbetts, Lena Oesterlin, Sherwin Y. Chan, Gisela Kramer, Boyd Hardesty and
Dean R. Appling

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