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Mitochondrial Initiation Factor 2 of *Trypanosoma brucei* Binds Imported Formylated Elongator-type tRNA^{Met}*

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The mitochondrion of *Trypanosoma brucei* lacks tRNA genes. Its translation system therefore depends on the import of nucleus-encoded tRNAs. Thus, except for the cytosol-specific initiator tRNA^{Met}, all trypanosomal tRNAs function in both the cytosol and the mitochondrion. The only tRNA^{Met} present in *T. brucei* mitochondria is therefore the one which, in the cytosol, is involved in translation elongation. Mitochondrial translation initiation depends on an initiator tRNA^{Met} carrying a formylated methionine. This tRNA is then recognized by initiation factor 2, which brings it to the ribosome. To guarantee mitochondrial translation initiation, *T. brucei* has an unusual methionyl-tRNA formyltransferase that formylates elongator tRNA^{Met}. In the present study, we have identified initiation factor 2 of *T. brucei* and shown that its carboxyl-terminal domain specifically binds formylated trypanosomal elongator tRNA^{Met}. Furthermore, the protein also recognizes the structurally very different *Escherichia coli* initiator tRNA^{Met}, suggesting that the main determinant recognized is the formylated methionine. *In vivo* studies using stable RNA interference cell lines showed that knockdown of initiation factor 2, depending on which construct was used, causes slow growth or even growth arrest. Moreover, concomitantly with ablation of the protein, a loss of oxidative phosphorylation was observed. Finally, although ablation of the methionyl-tRNA formyltransferase on its own did not impair growth, a complete growth arrest was observed when it was combined with the initiation factor 2 RNA interference cell line showing the slow growth phenotype. Thus, these experiments illustrate the importance of mitochondrial translation initiation for growth of procyclic *T. brucei*.

All organisms have two distinct tRNAs^{Met}, one specialized for decoding the initiation codon (mainly AUG) and another one dedicated for the insertion of methionine into internal peptidic linkages (1, 2). The basic features of translation initiation, namely the binding of the initiator tRNA^{Met} (tRNA$^{Met\text{-}i}$)[1] to initiation factor 2 (IF2) and the subsequent GTP-dependent interaction of the complex with the ribosome are universally conserved (3). Thus, a general feature of IF2 from all organisms is that it must specifically bind tRNA$^{Met\text{-}i}$ and interact with the ribosome. However, a more detailed analysis reveals some interesting differences between the translation initiation process in bacteria and in the eukaryotic cytosol. In the latter, the aminoacylated eukaryotic tRNA$^{Met\text{-}i}$, carrying the diagnostic A1:U72 base pair, directly binds to eukaryotic IF2 and forms a GTP-dependent ternary complex that binds to the 40 S ribosome. In bacteria binding of aminoacylated bacterial-type tRNA$^{Met\text{-}i}$ (recognized by the C1:A72 mismatch) to bacterial IF2 requires prior formylation of the methionine on the charged tRNA$^{Met\text{-}i}$. This reaction is catalyzed by the methionyl-tRNA formyltransferase (MTF), which is not found in the eukaryotic cytosol (1, 3). Furthermore, bacterial ribosomes are smaller and in many ways qualitatively different from their eukaryotic counterparts. However, although bacterial and eukaryotic IF2 both bind tRNAs$^{Met\text{-}i}$, they are not (even though implied by their names) evolutionarily related. Instead, it is eIF5B that is the eukaryotic orthologue of bacterial IF2. eIF5B does not directly bind tRNA but, just as proposed for bacterial IF2, facilitates association of the two ribosomal subunits (4).

Mitochondria are of bacterial evolutionary origin, and their translation system is therefore of the bacterial type. Thus, the two key factors involved in bacterial translation initiation, MTF and IF2, are also found in mitochondria (5). MTF of yeast (6, 7) and bovine mitochondria (8) have been characterized and shown to formylate their respective mitochondria-encoded tRNA substrates. Interestingly, in mammalian mitochondria, only a single tRNA^{Met} is found, which is used as an initiator in the formylated state and as an elongator when carrying a non-derivatized methionine (5). In contrast to bacterial MTF, the yeast enzyme appears to be dispensable for mitochondrial translation, because a MTF disruption strain was still able to grow on non-fermentable carbon sources (6). Mitochondrial IF2 of yeast (9) and bovine (10, 11) have also been characterized. They are similar to the bacterial proteins, the highest homology being found in the GTP-binding domain. Less, but still recognizable, similarity is also detected in the carboxyl-terminal half of the protein, which binds the formylated tRNA$^{Met\text{-}i}$. The domain organization of the amino-terminal part of mitochondrial IF2s, however, is quite variable (5). In line with this, it has been shown in yeast that this domain is dispensible for IF2 function *in vivo* (12).

Mitochondrial translation in the parasitic protozoa *Trypanosoma brucei* is unusual, because (due to the complete absence of mitochondrial tRNA genes) it depends exclusively on the import of cytosolic, nucleus-encoded tRNAs. Thus, trypanosomal tRNAs all are of the eukaryotic type and function in both the

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[1] The abbreviations used are: tRNA$^{Met\text{-}i}$, initiator transfer RNA^{Met}; IF2, initiation factor 2; MTF, methionyl-tRNA formyltransferase; GST, glutathione *S*-transferase; TLC, thin layer chromatography; RNAi, RNA interference; aa, amino acid(s); MOPS, 4-morpholinepropanesulfonic acid.

cytosol and the mitochondrion (13–15). The only exception is the eukaryotic-type $tRNA^{Met-i}$, which is found in the cytosol only (15). The elongator $tRNA^{Met}$, identical to all other elongator tRNAs, is in part imported into the mitochondrion and represents the only $tRNA^{Met}$ present in this compartment. A fraction of this tRNA becomes formylated after import (16). This results in the surprising situation in that, *T. brucei* mitochondria (a typical elongator $tRNA^{Met}$) functions in translation initiation, provided that it is formylated (17). In agreement with this, it has been shown that the trypanosomal MTF, unlike its counterpart in other organisms, selectively recognizes elongator $tRNA^{Met}$. The unusual nature of trypanosomal MTF is also confirmed by sequence comparisons, which show that the trypanosomal enzyme has twice the size of any of its homologues (16). The aim of the present study was to characterize mitochondrial IF2 of *T. brucei* to see whether, as predicted, it was able to interact with the formylated fraction of the imported elongator $tRNA^{Met}$.

EXPERIMENTAL PROCEDURES

Cells—Procyclic *T. brucei*, stock 427, was grown at 27 °C in SDM-79 medium supplemented with 5% fetal bovine serum. Cells were harvested at 3.5–4.5 × 10^7 cells/ml. Procyclic *T. brucei*, strain 29–13, on which the RNAi knockdown cell lines are based, was grown in SDM-79 supplemented with 15% fetal calf serum, 50 μg/ml hygromycin, and 15 μg/ml G-418 and was harvested at a density of 1–2 × 10^7 cells/ml.

Recombinant IF2—The carboxyl-terminal region of *T. brucei* IF2 (aa 412–721) (Gene DB annotation Tb07.28B13.850), or a variant thereof lacking the last 44 aa, were expressed as glutathione *S*-transferase (GST) fusion proteins. To do so, IF2 gene fragments were prepared by PCR using 5′-TAGGGATCCCCCGGGAAGACTACTTGCAG-3′ as a forward and 5′-TACTCGAGTCATATGGTGGTCTTCAC-3′ or 5′-TACTCGAGTCACACATCACGTGGCTCCTC-3′, for the variant lacking the carboxy terminus, as reverse primers and cloned into the BamHI- and XhoI-digested pGex-5x-3 plasmid (Amersham Biosciences). The resulting constructs were transfected into *Escherichia coli* BL21, and the cells were grown to mid-log phase. Induction was done by adding 0.1 mM of isopropyl-β-D-thiogalactoside for 2 h at 25–27 °C. Induced cells (250 ml) were washed in IF2 binding buffer (50 mM MOPS-NaOH, pH 7.5, 10 mM $MgCl_2$, 20 mM KCl, 1 mM dithiothreitol) and resuspended in ½₀ the volume of the same buffer. Lysis by sonication and batch mode purification of the recombinant protein by glutathione-Sepharose (Amersham Biosciences) were performed as described by the manufacturer, except that IF2 binding buffer was used. Binding was done overnight at 4 °C using 250 μl of a 50% slurry of glutathione-Sepharose and the clarified extract of a 250-ml culture. The yield was ~190 μg of recombinant protein/125 μl of bed volume of glutathione-Sepharose beads. The purity of the recombinant proteins was tested by SDS-PAGE. For the IF-2/tRNA binding assay described below, glutathione-Sepharose-bound recombinant proteins were used. To remove endogenous *E. coli* $tRNA^{Met-i}$, the glutathione-Sepharose-bound recombinant proteins had to be treated with micrococcus nuclease (20 units/250 μl slurry) for 15 min on ice. To do so, 1 mM of $CaCl_2$ had to be added. The reaction was stopped by washing the beads in 500 μl of IF2 binding buffer containing 3 mM EGTA. Subsequently, the beads were washed three times in 500 μl of IF2 binding buffer alone and, finally, resuspended in IF2 binding buffer to reach a 50% slurry.

Isolation of Total and Mitochondrial RNA—RNA from total cells and isolated mitochondrial fractions of *T. brucei*, as well as *E. coli* RNA, were purified by the acidic guanidinium isothiocyanate method (18). Isolated RNAs were resuspended in 10 mM Na-acetate, pH 4,0 to avoid deacylation during storage. Mitochondria were purified by the hypotonic lysis method as described previously (19). Mitochondrial fractions from the MTF-RNAi cell line (see Fig. 5) were obtained by digitonin extraction (15, 20).

IF2/tRNA Binding Assay—Mitochondrial or total RNA of *T. brucei* or total *E. coli* RNA (7 μg of each) was dissolved in 50 μl of IF2 binding buffer containing 20 units of SuperRNasin (Ambion). The binding reaction was initiated by the addition of 120 μl of a 50% slurry of IF2 binding buffer and glutathione-Sepharose beads containing ~90 μg each of IF2-GST fusion proteins or of GST alone. After incubation for 10 min at 23 °C, the reaction was spun at 500 × *g*, and 100 μl of the supernatant was removed and kept at 4 °C. The resulting pellet was washed twice in 100 μl of IF2 binding buffer, and the supernatant fractions from the washes were pooled with the first supernatant. The pellet (representing the bound RNAs) and the combined supernatants (representing the unbound RNA) were brought to 400 μl by adding H_2O. After the addition of 0.2 M Na-acetate, pH 4, the samples were extracted with H_2O-equilibrated phenol, and after the addition of 20 μg of glycogen, the samples were precipitated with 100% of ethanol. After a final wash with 75% of ethanol, each RNA pellet was processed for electrophoresis on a denaturing 8 M urea/10% polyacrylamide gel by resuspension in 10 μl of 90% formamide and 20 mM EDTA. Northern blots were done as described previously (14). The following 5′-end-labeled oligonucleotides were used as probes: 5′-GTGAGGCTCGAACTCACG-3′ (for the *T. brucei* elongator $tRNA^{Met}$), 5′-CCCACGCCTACGAATAGA-3′ (for the *T. brucei* $tRNA^{Leu}$), 5′-AGGCTGCTCCACCCCGCG-3′ (for the *E. coli* initiator $tRNA^{Met}$), and 5′-CCGCTCGGGAACCCCACC-3′ (for the *E. coli* $tRNA^{Tyr}$).

The ^{35}S-labeled mitochondrial RNA fraction used for the binding assay (shown in Fig. 2*B*) was prepared by *in organelle* aminoacylation using [^{35}S]methionine, as described previously (15). The labeled methionine species were released by deacylation in 0.1 N NaOH for 30 min at 37 °C. Analysis of the released [^{35}S]methionine species was done by thin layer chromatography (TLC) on a cellulose plate, which was developed in a 13:3:1 mixture of diethyl ether, acetic acid, and water (15).

RNAi—The RNAi constructs were prepared using the same previously described pLew 100-derived stem loop plasmid that carries convenient restriction sites for cloning (21). A 549-bp-long fragment, corresponding to position 156–704 of the IF2 coding sequence, was PCR-amplified using, as a forward primer, 5′-GCGGGATCCAAGCTTGAGTGATGACCCCCGATG-3′ and, as a reverse primer, 5′-GCGCTCGAGTCTAGATTGTGTGCAAGCTCAATC-3′. The resulting fragment was cloned into the plasmid in the sense direction using HindIII/XbaI sites and in the antisense direction using BamHI and XhoI sites. Two versions of the plasmid were prepared. In the first one (TbIF2-Tb), the sense and antisense sequences were separated by a fragment corresponding to 690 nucleotides of the trypanosomal spliced leader sequence (22), whereas in the second one (TbIF2-Mm), a 439-bp fragment, corresponding to positions 341–779 of the mouse Pex11b mRNA, was used as a spacer (23). Using the mouse sequence as a spacer yielded a more efficient down-regulation of the targeted mRNA than if the *T. brucei* sequence was used.[2] The plasmids were linearized with NotI and transfected into the procyclic *T. brucei* strain 29–13, which expresses T7 RNA polymerase and the tetracycline repressor. Selection with phleomycin, cloning, and induction with tetracycline were done as described previously (24). For the IF2/MTF double RNAi cell line (see Fig. 3*D*), we used the same plasmid that was used for the previously described MTF RNAi cell line (16), except that the phleomycin resistance gene was replaced by the puromycin resistance gene.

ATP Production Assay—ATP production assays using digitonin-purified mitochondria were done exactly as described previously (21, 25).

RESULTS

Primary Structure of IF2 of T. brucei—Searching the *T. brucei* genomic data base, we found an open reading frame of 721 aa predicted to encode the orthologue of bacterial IF2. The protein showed an overall identity of 33–35 and 30–32% to bacterial and mitochondrial IF2s, respectively. The predicted domain structure for mitochondrial IF2 (12) was retained (Fig. 1). A highly divergent amino-terminal domain (aa 1–135) was followed by the highly conserved GTP binding domain (aa 136–283). The carboxyl-terminal region (aa 284–721) was less conserved but contained the IF2 signature sequence (aa 628–650) as described in the PROSITE data base (Fig. 1*B*). Bioinformatic analysis did not predict an obvious mitochondrial targeting signal. However, in *T. brucei*, these signals were often difficult to identify, because they can be very short.

It has been shown, for bacterial IF2, that the carboxyl-terminal 110 aa are sufficient for binding of formylated $tRNA^{Met-i}$ (26). Fig. 1*B* shows a multiple sequence alignment of the carboxyl-terminal 310 aa of the *T. brucei* IF2 with the corresponding regions of two bacterial and two mitochondrial orthologues. The best characterized IF2 carboxyl-terminal domain is the one of *Bacillus stearothermophilus*, which has been subjected to

[2] E. Ullu, personal communication.

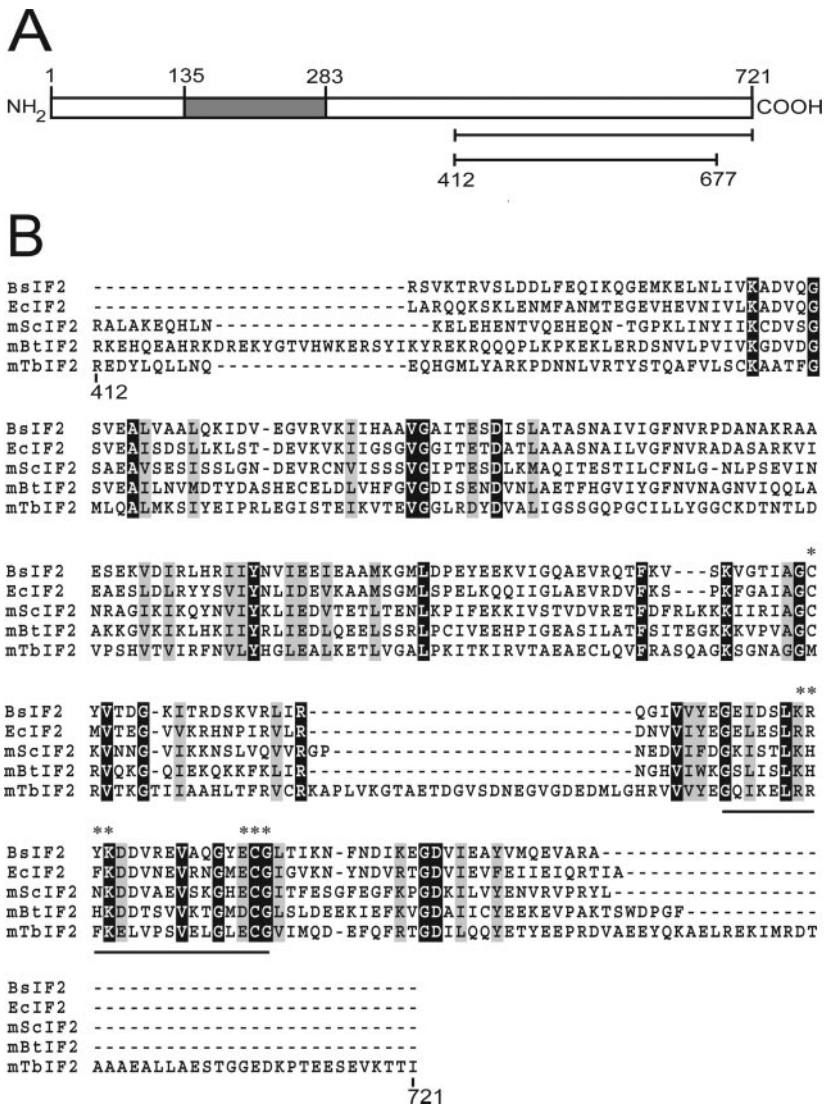

FIG. 1. **The *T. brucei* IF2 orthologue.** *A*, predicted domain structure of *T. brucei* IF2. The highly conserved GTP binding domain is shown in a *gray box*. The two regions of IF2 that were expressed as GST fusion proteins (Fig. 2) are indicated. *B*, multiple sequence alignment by ClustalW of the carboxyl-terminal 310 aa of the *T. brucei* IF2 (*Tb*) with the corresponding regions of two bacterial (*Bs*, *B. stearothermophilus*; *Ec*, *E. coli*) and two mitochondrial (*mSc*, *Saccharomyces cerevisiae*; *mBt*, *Bos taurus*) orthologues. Residues in the *B. stearothermophilus* protein identified as the main determinants for $tRNA^{Met-i}$ binding are indicated by *asterisks* (28). IF2 signature sequences (aa 628–650), as described in the PROSITE data base, are *underlined*.

extensive mutational analysis and which structure has recently been solved by NMR (26–28). These studies identified the Cys-668 and two short peptides, KRYK (aa 699–702) and ECG (aa 713–715), as the main determinants for $tRNA^{Met-i}$ binding (numbers refer to the position of amino acids in the *B. stearothermophilus* protein, Fig. 1*B*). Interestingly, except for three conservative replacements (C668M, K699R, Y701F), identical aa are found in the same relative position in the *T. brucei* protein (Fig. 1*B*, *asterisks*). Although bacterial IF2 interacts with $tRNA^{Met-i}$, the trypanosomal protein is predicted to bind an elongator-type $tRNA^{Met}$. Nevertheless, the elements, which in the bacterial IF2 are required for binding of the $tRNA^{Met-i}$, have been conserved in the trypanosomal protein.

However, there are also sequence elements that are unique for the trypanosomal IF2. These are an insertion of 27 aa and a carboxyl-terminal extension of 44 aa (Fig. 1*B*). Modeling of the trypanosomal sequence on the *B. stearothermophilus* structure (27) shows that the insertion localizes to a loop connecting two β sheets and thus will most likely not significantly disturb the structure. Interestingly, insertions at the same relative position are found in the IF2 orthologues of two other trypanosomatids (*Leishmania major* and *Trypanosoma cruzi*). However, although the length of these insertions (32 aa in *L. major* and 26 aa in *T. cruzi*) are very similar to the 27 aa observed in *T. brucei*, their sequences are not conserved. A similar situation is found for the carboxyl-terminal extensions. They are also present in *L. major* (length, 50 aa) and *T. cruzi* (length, 39); however, in contrast to the trypanosomatid-specific insertion, their sequences are highly similar.

The Carboxyl-terminal Domain of Trypanosomal IF2 Binds Formylated Elongator-type $tRNA^{Met}$—We wanted to test whether trypanosomal IF2, as predicted, binds imported formylated elongator $tRNA^{Met}$. Thus, we expressed the carboxyl-terminal 310 aa of the *T. brucei* IF2 (and a variant thereof lacking the carboxyl-terminal 44 aa) in *E. coli*. To simplify purification, the IF2 peptides were expressed as GST fusions. Affinity chromatography on glutathione-Sepharose beads yielded eluates consisting of single proteins of the expected molecular weights (data not shown). For the tRNA binding assay, the final elution step was omitted, and the beads containing the bound protein were directly incubated with isolated total or mitochondrial RNA fractions from *T. brucei*. Thus, our binding assay, comparable with the situation *in vivo*, was performed in a complex mixture of RNAs. It was important to isolate the RNAs under acidic conditions to keep the tRNAs in an aminoacylated state. After the binding step, the beads were recovered by centrifugation, and RNAs were isolated from the supernatant and, after a washing step, from the pellet. Both RNA fractions were separated by polyacrylamide gel electrophoresis and analyzed by Northern hybridizations. The results in Fig. 2*A* show that, when incubated with isolated mitochondrial RNAs, ~25% of the elongator $tRNA^{Met}$ present in this

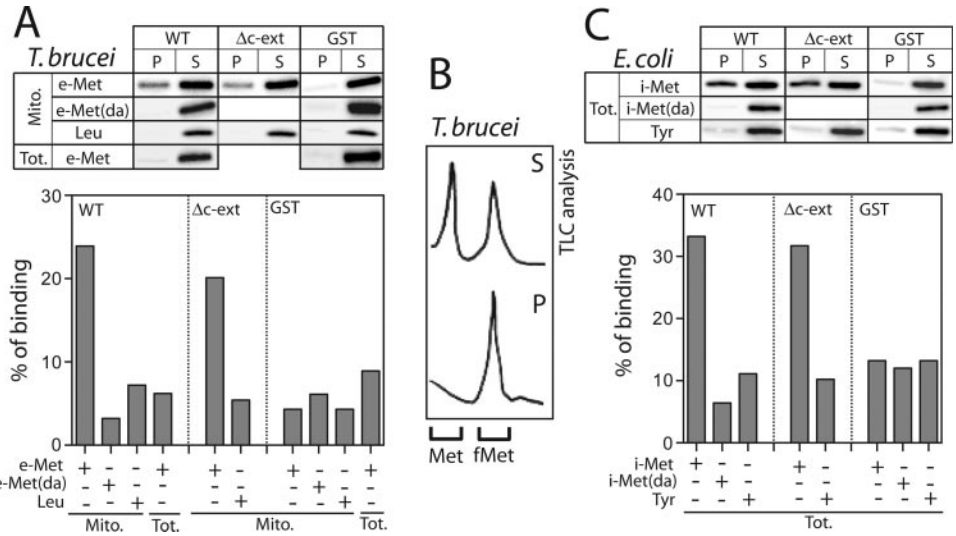

FIG. 2. **The carboxyl-terminal domain of *T. brucei* IF2 binds formylated tRNAs^{Met}.** *A*, IF2 binding assays using *T. brucei* RNA fractions as substrate. The carboxyl-terminal domain of wild-type (*WT*) trypanosomal IF2 (aa 412–721) or a variant thereof lacking the 44 last aa (Δ*c-ext*) were expressed as GST fusions. Glutathione-Sepharose beads saturated with the recombinant proteins or with GST alone were incubated with aminoacylated or deacylated (*da*) isolated mitochondrial RNA (*Mito.*), respectively. Identical experiments were also performed using isolated total RNA (*Tot.*). Northern blots containing bound (*P*) and unbound (*S*) RNA fractions were probed for elongator tRNA^{Met} (*e-Met*) or tRNA^{Leu} (*Leu*). The graph shows a quantification of the Northern blots signals as analyzed by a phosphorimaging device. The combined signals of the P and S fractions were set to 100%. *B*, IF2 binding assay using the *T. brucei* [^{35}S]methionine-labeled mitochondrial RNA fraction as substrate. Both supernatant (*S*) and pellet (*P*) fractions were deacylated, and the released [^{35}S]methionine species were analyzed by TLC. The graph shows the *tracings* of the [^{35}S]methionine signals from TLC as detected on the monitor of a multichannel analyzer of a gas flow Geiger Müller Counter (15). The positions of unlabeled methionine (*Met*) and formyl-methionine (*fMet*) separated under the same conditions as established in a parallel TLC lane are indicated. *C*, IF2 binding assays using total RNA isolated from *E. coli* as substrate. The same recombinant proteins as in *A* were tested for binding of aminoacylated or deacylated (*da*) *E. coli* tRNA^{Met-i} (*i-Met*) and tRNA^{Tyr}, respectively.

fraction is recovered in the pellet and thus is bound to the carboxyl-terminal 310 aa of *T. brucei* IF2. The observed binding was specific for elongator tRNA^{Met}, as another elongator-type tRNA, the tRNA^{Leu}, could not bind. Furthermore, interaction with IF2 required methionylated tRNA^{Met}, because it was prevented by prior deacylation of the RNA fraction. Interestingly, when incubated with isolated total RNA, consisting essentially of cytosolic RNAs, no binding of aminoacylated elongator tRNA^{Met} was observed. The only difference between cytosolic and imported mitochondrial elongator tRNA^{Met} is that 40–50% of the imported fraction is formylated (16), indicating that formylation is required for binding. As expected, GST alone did not significantly bind tRNA. In contrast, the variant of the IF2 peptide, which lacks the conserved trypanosomatid-specific carboxyl-terminal 44 aa, showed a binding activity that was comparable with the wild-type peptide.

To provide direct evidence that formylation is required for binding of the elongator tRNA^{Met}, we performed an IF2/tRNA binding assay using [^{35}S]methionine-labeled mitochondrial RNA as a substrate. The radioactive mitochondrial RNA fraction was prepared by *in organelle* aminoacylation. This procedure labels both the unformylated and formylated form of the imported elongator tRNAs^{Met} (15). After the binding reaction, the bound and unbound RNA fractions were deacylated, and the liberated [^{35}S]methionine and ^{35}S-formyl-methionine were analyzed by TLC. Fig. 2*B* shows that, as expected, it was mainly the ^{35}S-formyl-methionine that was recovered in the pellet fraction representing the bound RNA.

The same binding assays were also performed using isolated *E. coli* RNA as a substrate (Fig. 2*C*). Interestingly, ~35% of the *E. coli* tRNA^{Met-i} was able to bind to the trypanosomal IF2 peptide, provided that it was aminoacylated. Binding was specific, as an elongator tRNA^{Tyr} did not bind. Furthermore, as for the trypanosomal tRNA^{Met}, the very carboxyl terminus of IF2 (aa 678–721) was dispensable for binding.

In summary, these results show that the carboxyl-terminal domain of trypanosomal IF2 (aa 412–677) specifically binds elongator-type tRNA^{Met} of *T. brucei*, provided that the molecule is charged and formylated. Furthermore, when the IF2 peptide was assayed with *E. coli* RNAs, it specifically recognized the bacterial tRNA^{Met-i}.

In Vivo Depletion of IF2 by RNAi—Due to the availability of a tightly controlled tetracycline-inducible expression system (29), RNAi has become the method of choice to disrupt gene function in *T. brucei* (22, 23). Thus, to investigate the function of IF2 by RNAi, we prepared a construct that contained a defined sequence of the IF2 gene in the sense, as well as the antisense, direction. Both sequences were inserted downstream of a tetracycline-inducible promoter and were separated by a stuffer to simplify cloning. In the presence of tetracycline, the RNA was expressed and formed a stem loop that eventually led to the specific degradation of the IF2 mRNA. There have been two types of stuffer sequences used in stem loop constructs, one of trypanosomal origin as described previously (22) and another one of mouse origin (23). Surprisingly, it was shown that the efficiency of RNAi was generally higher when the mouse sequence was used.[2] The reason for this might be that the trypanosomal sequence still contains transcription signals that potentially interfere with the expression of the double-stranded RNA. We decided to take advantage of this situation and prepared RNAi strains that could be used for different purposes. Preparing the RNAi cell line using the construct containing the mouse stuffer sequence (TbIF2-Mm) resulted in the growth curve shown in Fig. 3*A*, which indicated, as expected, that IF2 is essential for growth of procyclic *T. brucei*. With the construct containing the trypanosomal stuffer (TbIF2-Tb), however, an RNAi cell line having a more moderate growth phenotype was obtained (Fig. 3*B*). Analysis of different clones from the two transfections show that the growth curves for the two plasmids, which are depicted in Fig. 3, *A* and *B*, are typical for the whole population. Thus, we think that the different growth phenotypes are caused by different efficiencies of RNAi due to the two stuffer sequences. However, because no antibodies against trypanosomal IF2 were avail-

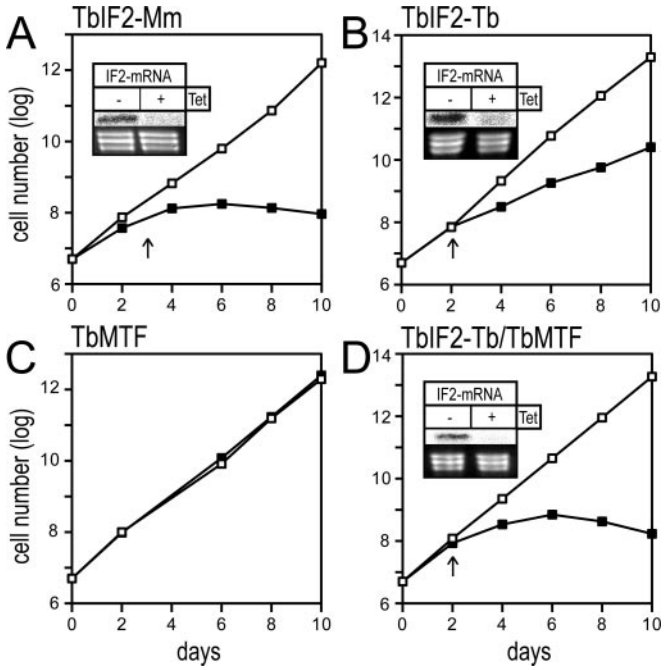

FIG. 3. **Mitochondrial translation initiation is essential for growth of procyclic *T. brucei*.** *A*, growth curve of a representative clonal *T. brucei* RNAi cell line ablated for IF2. The cell line was obtained using the stem loop construct containing the mouse stuffer sequence (TbIF2-Mm). *Open* and *filled squares* represent growth in the absence or presence of tetracycline (*Tet*), respectively. The *inset* shows a Northern blot for IF2 mRNA; time of sampling is indicated by the *arrow*. The rRNAs in the *lower panel* serve as loading controls. *B*, same as *A*, but the RNAi cell line was obtained using the stem loop construct containing the trypanosomal stuffer sequence (*TbIF2-Tb*). *C*, growth curve of a representative clonal *T. brucei* RNAi cell line ablated for MTF (*TbMTF*). Same results were obtained for the mouse and the trypanosomal stuffer sequence. MTF activity was measured by the assay described previously (16) and shown to be reduced to ~10–20% after three days of induction. *D*, growth curve for a representative clonal double RNAi cell line ablated for IF2 and MTF simultaneously. The cell line was obtained by transfecting TbIF2-Tb cells with the plasmid used to create the original MTF cell line (16), except that the resistance marker was changed. Down-regulation of IF2 mRNA and MTF activity was shown to be the same as in the corresponding single RNAi cell lines shown in *B* and *C*.

able, we could not verify this on the protein level.

Previous work from our laboratory has shown that, even though ablation of MTF resulted in an ~80–90% reduction of formylation activity (16), no growth phenotype was observed (Fig. 3*C*). Thus, the residual activity of MTF might still be sufficient to support growth, or MTF (as in yeast) might indeed not be required for normal growth (6, 7). The absence of a growth phenotype in MTF-ablated cells is surprising, because the enzyme is required for formylation of mitochondrial $tRNA^{Met}$, and the $tRNA^{Met}$ can only bind to IF2 in its formylated state (see Figs. 2 and 5). Both MTF and IF2 are therefore expected to be essential for the mitochondrial translation initiation pathway in *T. brucei*. Thus, to demonstrate the *in vivo* importance of trypanosomal MTF, we have used the clonal IF2 RNAi cell line (TbIF2-Tb) showing the moderate growth phenotype (Fig. 3*B*) and transfected it with the construct used to produce the original MTF-RNAi cell line (Fig. 3*C*) (16). Control experiments showed that, in induced cells of the resulting double RNAi knockdown cell line (TbIF2-Tb/TbMTF), IF2 mRNA is degraded, and MTF activity is reduced to the same level as in the two original cell lines (Fig. 3, *B* and *C*). Interestingly, the TbIF2-Tb/TbMTF-RNAi cell line shows a much stronger growth phenotype (Fig. 3*D*) than either the TbIF2-Tb-RNAi cell line (Fig. 3*B*) it is derived from or the TbMTF-RNAi cell line alone (Fig. 3*C*). These results show that, although reduced MTF activity does not affect growth of wild-type cells, it will stop growth of cells having reduced concentrations of IF2.

The growth phenotype observed in IF2 RNAi cells is expected to be due to the lack of mitochondrial protein synthesis. It is difficult to directly measure mitochondrial translation in trypanosomatids (30, 31). Thus, as an alternative, we decided to measure the consequences that ablation of IF2 has on mitochondrial ATP production (Fig. 4). We have recently established a sensitive luciferase-based assay to quantify ATP production in digitonin-isolated mitochondria of *T. brucei* in response to different substrates (21, 25). There are two fundamentally different ways by which mitochondria can produce ATP: (i) by oxidative phosphorylation using the electron transport chain or (ii) by substrate-level phosphorylation linked to the citric acid cycle. Our assay allows us to measure both ATP production pathways separately. To measure oxidative phosphorylation, mitochondria are incubated with ADP and the respiratory substrate succinate. This mode of ATP production is expected to be sensitive to antimycin, an inhibitor of the bc1 complex. To measure substrate-level phosphorylation, succinate is replaced by the citric acid cycle intermediate α-ketoglutarate. Substrate-level phosphorylation induced by α-ketoglutarate is antimycin-resistant. Thus, atractyloside, a specific inhibitor of the ADP/ATP translocator, is used as a control. Atractyloside blocks import of ADP and therefore will inhibit both substrate-level and oxidative phosphorylation. The results in Fig. 4 show that ablation of IF2 selectively knocks down oxidative phosphorylation but does not interfere with substrate-level phosphorylation. This is expected, because all mitochondria-encoded proteins in *T. brucei* are either components of the respiratory chain or the mitochondrial translation system. Thus, although oxidative phosphorylation depends on both imported, as well as mitochondria-encoded proteins, substrate-level phosphorylation will only require nucleus-encoded components.

Finally, we have used the MTF-RNAi cell line to verify that only formylated $tRNA^{Met}$ is able to bind to IF2. Mitochondrial RNA isolated from uninduced and induced cells was incubated with the glutathione-Sepharose-bound IF2/GST fusion protein. Fig. 5 shows that, although a fraction of the elongator $tRNA^{Met}$ present in mitochondrial RNA isolated from uninduced cells was recovered in the pellet, this amount was reduced to ~20% when the RNA was isolated from MTF-ablated cells. Thus, these results confirm the ones shown in Fig. 2, *A* and *B*, and provide independent evidence that formylation of the methionyl-$tRNA^{Met}$ is essential for *in vitro* binding of IF2.

DISCUSSION

The aim of the present study was to elucidate the role trypanosomal IF2 plays in the mitochondrial translation initiation pathway, in light of the fact that all tRNAs it can use are imported and of eukaryotic type. Interestingly, *T. brucei* and mammalian mitochondria each have only a single type of $tRNA^{Met}$. This tRNA is used as an elongator in the unformylated and as an initiator in the formylated states. However, although in mammalian mitochondria, the single $tRNA^{Met}$ is of the initiator-type, sharing features from both bacterial-type and eukaryotic-type $tRNA^{Met\text{-}i}$ (5), the one present in trypanosomal mitochondria is clearly an elongator (16). The main fraction of this tRNA actually resides in the cytosol and functions in cytosolic translation elongation (15). Thus, the question is how an elongator-type $tRNA^{Met}$ can be recruited to function in mitochondrial translation initiation. We have recently shown that, in *T. brucei*, this is achieved by an atypical MTF, which unlike its counterparts in other organisms, selec-

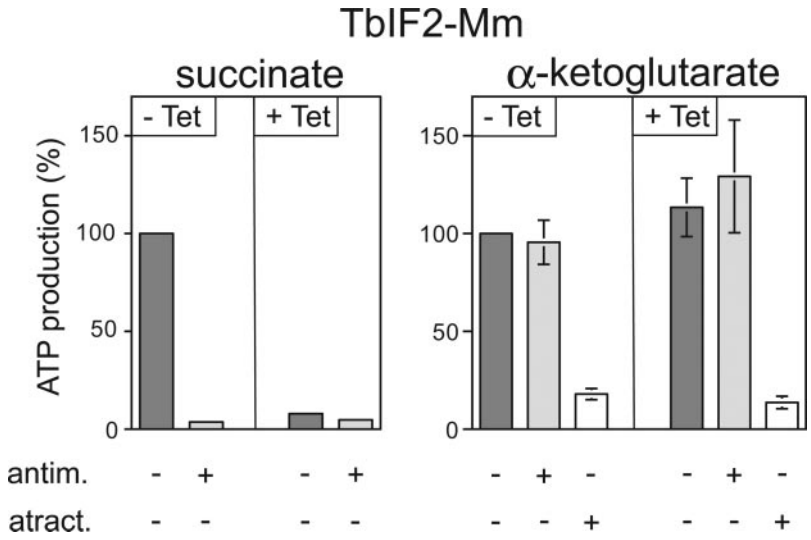

FIG. 4. **Succinate- and α-ketoglutarate-induced mitochondrial ATP production in the TbIF2-Mm RNAi cell line.** Uninduced cells (*−Tet*) are shown on the *left*, and induced cells (*+Tet*) on the *right* of each panel. The substrate tested is indicated at the *top* and additions of antimycin (*antim.*) and atractyloside (*atract.*) at the *bottom* of each panel. ATP productions in mitochondria isolated from uninduced cells tested without antimycin or atractyloside are set to 100%. The *bars* represent means expressed as percentages from three independent inductions. Standard errors are indicated.

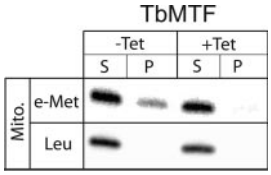

FIG. 5. **Mitochondrial tRNA^{Met} from the TbMTF RNAi cell line does not bind IF2.** IF2 binding assays using mitochondrial RNA (*Mito.*) isolated from uninduced (*−Tet*) and induced (*+Tet*) MTF-RNAi cells. Northern blots containing bound (*P*) and unbound (*S*) RNA fractions were probed for elongator tRNA^{Met} (*e-Met*) or tRNA^{Leu} (*Leu*). Quantifications of the signals for elongator tRNA^{Met} in the pellet fractions showed an ~5-fold reduction of binding in induced cells.

tively recognizes and formylates elongator tRNA^{Met} (16). In the present study, we were focusing on IF2, a component of the mitochondrial translation initiation pathway that acts after formylation of the tRNA^{Met}. IF2 promotes binding of the formylated tRNA^{Met} to mitochondrial ribosomes. Whether it does so by acting as a carrier or whether it interacts with the tRNA already bound to the small subunit of the ribosome is unknown. Using a homologous *in vitro* binding assay, we could show that, as predicted, the carboxyl-terminal domain of trypanosomal IF2 was able to specifically bind the elongator tRNA^{Met} when present in a mixture of total mitochondrial RNAs. The fact that only the elongator tRNA^{Met} in the mitochondrial RNA fraction was able to bind to IF2 (Fig. 2*A*), the relative enrichment of formyl-methionine in the bound RNA fraction (Fig. 2*B*), and the much reduced binding that is observed when mitochondrial RNA of the MTF-RNAi strain is used (Fig. 5) all strongly suggest that formylation of the methionyl-tRNA^{Met} is a prerequisite for binding to IF2. Furthermore, we showed that, when incubated with *E. coli* RNA, trypanosomal IF2 selectively recognized the heterologous tRNA$^{Met\text{-}i}$ (Fig. 2*C*). Trypanosomal elongator tRNA^{Met} (16) and *E. coli* tRNA$^{Met\text{-}i}$ (1) have very different structures; however, both of them are at least in part formylated, suggesting that the formylated methionine is the main recognition determinant for the trypanosomal protein.

Similar to the situation in bacteria, formylated tRNA$^{Met\text{-}i}$ has long been known to be the preferred ligand for mitochondrial IF2s. However, there is evidence that, to a limited extent, unformylated tRNA$^{Met\text{-}i}$ may be able to bind as well. The extent to which this can occur depends on the organism. Thus, whereas in an *in vitro* binding assay, bovine mitochondrial IF2 showed a strong preference (20–50-fold) for formylated tRNA$^{Met\text{-}i}$ (11), much less specificity (4-fold) is seen when yeast IF2 is used (9). Furthermore, the fact that a yeast strain deleted for MTF did not show a growth phenotype on non-fermentable carbon sources (6, 7) shows that *in vivo* mitochondrial translation initiation can also, in principle, occur without formylated tRNA$^{Met\text{-}i}$. Thus, it appears that the bovine IF2 shows a much stronger preference for formylated tRNA$^{Met\text{-}i}$ than the yeast protein. This makes sense when one considers that yeast mitochondria have distinct genes for the elongator and tRNA$^{Met\text{-}i}$, whereas mammalian mitochondria have only a single one. For mammals, the formyl group is therefore the only distinguishing feature between the elongator and the initiator tRNA^{Met}. However, it should be mentioned that a recent study has shown that the bovine IF2 could replace yeast IF2 in a MTF deletion strain, indicating that, at least *in vivo*, it is must bind unformylated tRNA$^{Met\text{-}i}$ (12).

RNAi-mediated ablation of trypanosomal IF2 causes a growth arrest (Fig. 3*A*) and a concomitant loss of oxidative phosphorylation (Fig. 4). This is expected as oxidative phosphorylation requires mitochondria-encoded protein and is known to be essential for procyclic *T. brucei*. Surprisingly, however, even though MTF and IF2 are known to act in the same pathway, growth of a trypanosomal MTF-RNAi knockdown cell line was not affected (16). How can this be explained? Do we have the same situation as in yeast, where IF2, under most growth conditions, can efficiently use unformylated tRNA^{Met} (6, 7)? We think this is unlikely, because in the *in vitro* assay, trypanosomal IF2 showed a strong preference for formylated tRNA^{Met}. In fact, for unformylated tRNA^{Met}, only a background level of binding was observed (Fig. 2*A*). However, RNAi-mediated ablation is known to not completely remove the ablated gene product. After induction of RNAi in the MTF-RNAi strain, ~10–20% of the MTF activity was retained (16). We therefore think that the residual amount of formylated tRNA^{Met} is sufficient to support normal growth. If, on the other hand, MTF activity was ablated in a cell line that already had limited amounts of IF2 (using the IF2-RNAi cell with the slow growth phenotype shown in Fig. 3*B*), a growth arrest was observed. This indicates that, under these conditions, both components of the translation initiation pathway, IF2 and MTF, are essential.

Initiation of protein synthesis in *E. coli* depends strictly on formylated tRNA$^{Met\text{-}i}$ (32), whereas *Pseudomonas aeruginosa* is able to use unformylated tRNA (33). Elegant experiments have shown that translation initiation in *E. coli* can be rendered formylation-independent by complementing an *E. coli* IF2 mutant with the *P. aeruginosa* IF2 (34). Furthermore, a single amino acid substitution (H774K) in the IF2 of *P. aeruginosa*, which introduces the corresponding amino acid found in the *E. coli* protein, resulted in a formylation-dependent protein. Structural modeling suggests that the mutation increases the

positive charge in the aa binding cleft of *P. aeruginosa* IF2. Surprisingly, in the *T. brucei* IF2, the aa corresponding to the His-774 of the *P. aeruginosa* protein is an uncharged alanine and thus reduces the positive charge potential. The *T. brucei* protein nevertheless shows great specificity for formylated methionine *in vitro*, indicating that this position is not the sole determinant involved in binding specificity.

We have previously shown that, using an elongator-type $tRNA^{Met}$ in translation initiation, *T. brucei* mitochondria requires a MTF with a unique substrate specificity (16). In this study, we showed that, once formylated, the elongator $tRNA^{Met}$ can bind to a rather conventional trypanosomal IF2 that is also able to selectively recognize *E. coli* $tRNA^{Met\text{-}i}$.

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