**Drosophila Mitochondrial Transcription Factor B1 Modulates Mitochondrial Translation or DNA Copy Number in Schneider Cells**

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We report the cloning and molecular analysis of *Drosophila* mitochondrial transcription factor (d-mtTF) B1. An RNA interference (RNAi) construct was designed that reduces expression of d-mtTFB1 to 5% of its normal level in Schneider cells. In striking contrast with our previous study on d-mtTFB2, we found that RNAi knockdown of d-mtTFB1 does not change the abundance of specific mitochondrial RNA transcripts, nor does it affect the copy number of mitochondrial DNA. In a corollary manner, overexpression of d-mtTFB1 did not increase either the abundance of mitochondrial RNA transcripts or mitochondrial DNA copy number. Our data suggest that, unlike d-mtTFB2, d-mtTFB1 does not have a critical role in either transcription or regulation of the copy number of mitochondrial DNA. Instead, because we found that RNAi knockdown of d-mtTFB1 reduces mitochondrial protein synthesis, we propose that it serves its primary role in modulating translation. Our work represents the first study to document the role of mtTFB1 in vivo and establishes clearly functional differences between mtTFB1 and mtTFB2.

Mitochondrial number and DNA content vary widely depending on cellular energy requirements, which are met in large part by ATP production by the oxidative phosphorylation pathway. Expression of the 13 polypeptides involved in oxidative phosphorylation that are encoded in the mtDNA1 genome is essential for this process. Transcription in animal mitochondria is thought to involve mitochondrial RNA polymerase and three distinct transcription factors (1, 2). Mitochondrial transcription factor A (formerly referred to as mtTFA) contains two HMG boxes and was shown in *organello* to bind nonspecifically at regularly phased intervals to the control region of human mtDNA (3) and to package mtDNA in nucleoids (4, 5). Human mitochondrial transcription factor A was also shown to be required for specific initiation at mitochondrial promoters in vitro (6–9). Two additional human transcription factors, mtTFB1 and mtTFB2, have also been shown to activate transcription from mitochondrial promoters in the presence of mitochondrial transcription factor A and mitochondrial RNA polymerase in vitro, and h-mtTFB2 is more active in promoting transcription than h-mtTFB1 (6, 10). Recent studies show that h-mtTFB1 has rRNA adenyl dimethyltransferase activity when expressed in bacteria (11) and that its in vitro transcriptional activation and methylase activities can be inactivated differentially by mutation (12).

Although in vitro studies show that both mtTFB1 and mtTFB2 support transcription from human mitochondrial promoters (6), their relative importance and specific physiological roles are not well understood. In a recent study (13), we showed that RNAi knockdown of d-mtTFB2 reduces the abundance of specific mitochondrial RNA transcripts and decreases the copy number of mtDNA in *Drosophila* cultured cells. This finding suggests that endogenous d-mtTFB1 cannot complement a deficiency in d-mtTFB2 and thus is not functionally redundant with d-mtTFB2, pointing to specialized roles for the two transcription factors in vivo. Here, we report the cloning and overexpression of *Drosophila* mtTFB1 and the knockdown phenotype of *Drosophila* Schneider cells treated with d-mtTFB1-targeted RNAi. Our results do not support an important role for mtTFB1 in either efficient mitochondrial transcription or maintenance of mtDNA. Rather, we found that mtTFB1 modulates mitochondrial translation.

**EXPERIMENTAL PROCEDURES**

Identification and Sequence Analysis of d-mtTFB1 cDNA—The amino acid sequence of d-mtTFB1 was used to search the Berkeley *Drosophila* Genome Project Data Base. One sequence (CG7319) was identified that has a high level of homology to d-mtTFB1. Full-length cDNA was prepared using the SMART RACE cDNA amplification kit (Clontech), total RNA from *Drosophila* Schneider S2 cells, and the following primers: 5′-TTACCAACTTGTCATCTTGTTG-3′ and 5′-GGCTCGAGCCACCTCGGTAAATTGGCTC-3′ for 5′-RACE, and 5′-CAAAATGACACCTGTCACAGG-3′ and 5′-GGGAATTCCTCTAGACACTTGATACCC-3′ for 3′-RACE. RACE products were purified from an agarose gel and sequenced, and sequence analysis was performed using MacDNASIS version 3.7 (Hitachi Software). Similarity searches against the non-redundant GenBank™ data base were performed using BLAST (14). The deduced amino acid sequence was checked for targeting signal peptides using iPSORT (15) and MITProt (16). Multiple sequence alignments were performed with ClustalW (17).

**Bacterial Overexpression of d-mtTFB1 and Preparation of d-mtTFB1 Antibody**—To express d-mtTFB1 in *Escherichia coli*, a PCR fragment of d-mtTFB1 cDNA encoding amino acid residues Gly-12 to Leu-330 was cloned into pET-28a (Novagen) cleaved with EcoRI and XhoI. Bacterial cells harboring the plasmid were grown in LB medium containing 100 μg/mL ampicillin at 37 °C. Cells were harvested, sonicated, and analyzed by SDS-PAGE. Purified recombinant d-mtTFB1 was used to generate a rabbit antibody by routine procedures.
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μg/ml kanamycin, and expression was induced with 1 mM isopropylthiogalactoside. The C-terminal His6 tag was utilized for nickel-nitrotriacetic acid affinity purification of recombinant d-mtTFB1 according to the manufacturer's instructions (Qiagen). Rabbits were immunized by injection of column-purified d-mtTFB1 in Freund's complete adjuvant, followed by secondary immunization in Freund's incomplete adjuvant. To prepare affinity-purified antisera, purified d-mtTFB1 (100 μg) was bound to a polyvinylidene difluoride membrane, and the filter was preincubated for 1 h with 5% skim milk in PBS, incubated for 1 h with crude antisera (1 ml in 5 ml of PBS containing 0.1% Tween 20), washed four times with PBS containing 0.1% Tween 20, and eluted with 0.2 M glycine-HCl (pH 2.4) (~5 ml). The eluted antibody was neutralized with 1 M Tris base.

Preparation of Inducible Plasmids Expressing d-mtTFB1 and d-mtTFB1-targeted RNAi—The plasmid pMt/B1/Hy, in which d-mtTFB1 cDNA is regulated by the metallothionein promoter, was constructed as follows: a fragment of d-mtTFB1 cDNA was amplified by PCR using 5'-GCGCAGTACGCTGCGAGGATCCATCCCAACAG-3' as 5'-primer and 5'-GGGCTCGAGGAAGTCGTTGACAACAG-3' as 3'-primer. The PCR fragment was cleaved by XhoI and SpeI and subcloned. The plasmid pMt/invB1/Hy carries an inverted repeat of a thiogalactoside. The C-terminal His6 tag was utilized for nickel-nitrotriacetate precipitation. The two PCR products were ligated and cloned into the pMt/Hy vector cleaved by XhoI and SpeI.

Generation and Induction of Stable Cell Lines—Drosophila Schneider S2 cells were cultured at 25 °C in Drosophila Schneider Medium (Invitrogen) supplemented with 10% fetal bovine serum. Cells were subcultured to 3 × 106 cells/ml every third day. Cells were transfected using Effectene (Qiagen). Hygromycin-resistant cell lines were selected by growth of the cell culture in the presence of 0.05, 0.1, and 0.4 mM hygromycin. Cells were passaged at least five times in hygromycin-containing medium and then cultured in standard medium. The C-terminal His6 tag was utilized for nickel-nitrotriacetate precipitation. The cell lines were grown to a density of 3 × 106 cells/ml and then treated with 0.4 mM CuSO4 to induce high level expression from the metallothionein promoter (18). Overexpression of d-mtTFB1 was effected by growth of the cell culture in the presence of 0.05, 0.1, and 0.4 mM CuSO4.

Analysis of Mitochondrial Protein Synthesis—Cell lines were grown in the presence or absence of 0.4 mM CuSO4 for 10 days. Cells were harvested at room temperature, washed twice with methionine-free Grace's insect culture medium (Invitrogen), and resuspended at 3 × 106 cells/ml in methionine-free Grace's insect culture medium supplemented with 10% fetal bovine serum, 200 μg/ml emetine, 100 μg/ml cycloheximide. Five minutes after cell resuspension, 35S-Trans-label (ICN) was added to 300 μCi/ml, and the cells were incubated for 2 h at 25 °C. After incubation, the cells were diluted with 2 volumes of Schneider Medium and then washed twice with PBS. The cells were lysed in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 1% SDS. Total cellular protein (30 μg/lane) was fractionated by 15–20% gradient SDS-PAGE. The gels were stained with Coomassie Brilliant Blue and dried. The gels were autoradiographed by exposure to x-ray film (Kodak) for 3–10 days; the mitochondrial proteins indicated at right in Fig. 5 were quantitated from two independent experiments using the Kodak 1D program software.

RT-PCR Analysis—RT-PCRs were performed to determine mtTFB1 mRNA expression in Schneider cells. Reactions contained 5 μg of total RNA, 50 mM KCl, 10 mM Tris-HCl (pH 7.9), 2.5 mM MgCl2, 100 μg/ml bovine serum albumin, 1 mM deoxynucleotide triphosphates, and 20 units of RNASin (Promega) in a total volume of 50 μl. First-strand cDNA was synthesized using 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen) with 20 pmol of dNTP primers. Five microliters of the reaction was added to PCR mix (50 μl) containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl2, 0.1% Triton X-100, 10% DMSO, and 0.2 units of Taq DNA polymerase (PerkinElmer Life Sciences). PCR amplification (94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min (35 cycles for mtTFB1 and 25 cycles for RP49), with an initial step of 94 °C for 1 min and a final step of 72 °C for 2 min) was performed with primer combinations of 5'-d-mtTFB1 (5'-TGCAGTTCATGAGAAGCT-GATC-3') and 3'-d-mtTFB1 (5'-AGTCTGTCAGTTCACCACG-3') and 5'-RP49 (5'-GGACATCCGCGCCACGATA-3') and 3'-RP49 (5'-AGAACGAGCGACCCCTGGT-3'). RT-PCR products were separated by electrophoresis on 1.2% agarose gels and detected by staining with ethidium bromide.

Immunoblotting—Total cellular protein (20 μg/lane) was fractionated by 10.5% SDS-PAGE and transferred to nitrocellulose filters. Filters were preincubated for 1 h with 5% skim milk in PBS, followed by incubation for 1 h with d-mtTFB1 antibody (1:20) in PBS containing 0.1% Tween 20. Filters were washed four times with PBS containing 0.1% Tween 20, incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG (Bio-Rad), and washed with PBS containing 0.1% Tween 20. Protein bands were visualized using ECL Western blotting reagents (Amersham Biosciences). Polyclonal antibody against Drosophila mtTFB2 was prepared and used as described in Ref. 13.

Northern and Southern Blotting—Total cellular RNA was extracted using TRIzol reagent (Invitrogen). RNA (5 μg/lane) was fractionated in a 1.2% agarose/formaldehyde gel, blotted onto Hybond-N+ nylon membrane (Amersham Biosciences), and hybridized to 32P-labeled probes for each of the following four genes: ribosomal protein 49 (RP49), cytochrome b (Cytb), NADH dehydrogenase subunit 4 (ND4), and 12S rRNA. Hybridization was carried out for 16 h at 42 °C in 5× SSPE, 150 mM NaCl, 10 mM sodium phosphate (pH 7.4), and 1 ml of 5x EMTA, 0.5% SDS, 5× Denhardt's solution, and 50% formamide. The membrane was washed twice at room temperature with 2× SSC containing 0.1% SDS, washed once with 0.1% SDS, washed once with 0.1% SDS, and then analyzed with a PhosphorImager (Amersham Biosciences). The signal for RP49 was used to normalize mitochondrial transcripts.

Genomic DNA was purified from Drosophila Schneider S2 cells by standard methods. DNA (5 μg/lane) was cleaved with XhoI, fractionated in a 0.8% agarose/TBE gel, and transferred to nylon membrane. Hybridization was performed as described above. Filters were washed with 0.1% SDS, incubated at 65 °C for 1 h, washed once with 0.1% SDS, washed once for 30 min at 65 °C with 0.2% SSC containing 0.1% SDS, and then analyzed with a PhosphorImager. Blots were probed with radiolabeled DNAs for the mitochondrial gene Cytb and the nuclear histone gene cluster. The ratio of the signals for these two genes was used to determine the relative copy number of mtDNA.

RESULTS

RNAi-dependent Knockdown of d-mtTFB1 Does Not Reduce Mitochondrial Transcription and Does Not Induce mtDNA Depletion in Schneider Cells—The sequence of a putative d-mtTFB1 (10) was used to identify and clone a full-length cDNA by RACE. The cDNA clone obtained is 1530 nucleotides and encodes a predicted polypeptide of 330 amino acids. Rabbit antisera was raised against a recombinant, truncated form of d-mtTFB1 (Gly-12 to Leu-330). The antibody detects a single polypeptide in mitochondrial extracts from Drosophila Schneider cells with an electrophoretic mobility corresponding to an approximate molecular mass of 38 kDa (Fig. 1A). Immunoblot analysis of subcellular fractions of Drosophila Schneider cells demonstrated that d-mtTFB1 is localized to the mitochondria (data not shown).

The abundance of d-mtTFB1 was reduced by expressing a metallothionein-inducible d-mtTFB1-targeted RNAi species (19–21) from the plasmid pMt/invb1/Hy. The RNA species produced forms a double-stranded RNA hairpin homologous to d-mtTFB1 RNAi in cells carrying the plasmid pMt/invb1/Hy. Previous studies indicate that double-stranded RNA hairpins are efficient RNAi mediators. Cells stably expressing pMt/invb1/Hy or the control plasmid pMt/Hy were cultured for 10 days in the presence or absence of 0.4 mM CuSO4. Immunoblot analysis of copper-treated cells showed no detectable level of d-mtTFB1 in cells carrying pMt/invb1/Hy (Fig. 1B). Basal, uninduced expression from pMt/invb1/Hy also repressed expression of d-mtTFB1 by 6-fold. In contrast, expression of d-mtTFB2 was unchanged under all experimental conditions. RT-PCR analysis of copper-treated cells showed that cells carrying pMt/invb1/Hy expressed 20-fold less d-mtTFB1 RNA than cells carrying the control vector, and basal, uninduced expression from pMt/invb1/Hy also suppressed expression of d-mtTFB1 RNA by 7-fold (Fig. 1C). Copper-treated cells carrying pMt/invb1/Hy showed moderate growth retardation, but copper treatment...
had no adverse effects on growth of control cells (data not shown). This finding contrasts with the poor growth and reduced viability phenotype we observed upon similar reduction of d-mtTFB2 using the RNAi strategy (13).

The biological function of d-mtTFB1 was examined in cells expressing d-mtTFB1-targeted RNAi. Transcription of several mitochondrial genes and mtDNA copy number were measured in cells with no plasmid and cells carrying pMt/Hy or pMt/invB1/Hy. Northern blots were used to quantify the relative expression of the Cytb, ND4, and 12S rRNA genes in cells grown for 10 days in the presence or absence of copper. Neither basal nor induced expression of d-mtTFB1 or d-mtTFB2 as indicated. C, total RNA was extracted from Schneider cells (control) or Schneider cells carrying pMt/Hy (vector) or pMt/invB1/Hy (RNAiB1) after 10 days of culture in the presence or absence of 0.4 mM CuSO₄. RNAs were analyzed by RT-PCR. PCR products were run on a 1.2% agarose gel and stained with ethidium bromide. The relative abundance of the d-mtTFB1 PCR product was evaluated by densitometric scanning and normalizing its level to that of nuclear RP49 as a control.

from the ratio of Cytb hybridization to histone cluster hybridization (Fig. 3B). After induction of d-mtTFB1-targeted RNAi for 10 days, relative mtDNA copy number was almost the same as the control. This result suggests that d-mtTFB1 is not critical for maintenance of mtDNA.

Overexpression of d-mtTFB1 Has No Effect on Mitochondrial Transcription or mtDNA Copy Number—d-mtTFB1 was subcloned into the inducible expression vector pMt/Hy under the control of the metallothionein promoter. The resulting expression vector, pMt/dmtTFB1/Hy, was introduced into Schneider cells, and stable cell lines harboring this plasmid were cultured for 10 days, relative mtDNA copy number was almost the same as the control. This result suggests that d-mtTFB1 is not critical for maintenance of mtDNA.

overexpression of d-mtTFB1 had no effect on cell growth or viability (data not shown). The effect of d-mtTFB1 overexpression on mitochondrial transcript abundance was evaluated by Northern blots. Blots were probed for transcripts from the mitochondrial 12S rRNA, ND4, and Cytb genes and for nuclear RP49 gene as a control (Fig. 4B). We found that the levels of all three mitochondrial RNA transcripts were unchanged. Similarly, we found that the relative copy number of mtDNA was also unchanged in cells that overexpress d-mtTFB1 (Fig. 4C).

RNAi-dependent Knockdown of d-mtTFB1 Reduces the Efficiency of Mitochondrial Translation—The apparent lack of mo-
DISCUSSION

Drosophila mtTFB1 shares homology with human mtTFB1, which has been shown to stimulate mitochondrial transcription in vitro (6) and to have rRNA adenine dimethyltransferase activity in bacteria (11). In Drosophila Schneider cells treated with RNAi, d-mtTFB1 is expressed at 5% of the endogenous level. Although abundance of mitochondrial transcripts and mtDNA copy number were unchanged, RNAi treatment reduces the level of mitochondrial protein synthesis. Overexpression of d-mtTFB1 does not stimulate transcription of mitochondrial genes, nor does it increase mtDNA copy number. These data provide the first evidence that animal mtTFB1 plays a significant role in mitochondrial translation but apparently does not serve a critical function in mitochondrial transcription or mtDNA maintenance in vivo.

In an earlier report (13), we demonstrated that the abundance of d-mtTFB2 influences both mitochondrial transcription and the efficiency of mtDNA replication. It is particularly significant that RNAi knockdown of d-mtTFB2 lowers the efficiency of mitochondrial transcription in Drosophila cultured cells because the data suggest strongly that endogenous mtTFB1 cannot complement a deficiency in mtTFB2. In striking contrast, neither RNAi knockdown nor overexpression of mtTFB1 affects the levels of mitochondrial transcripts or mtDNA copy number in Drosophila cultured cells. In vitro studies have shown that both mtTFB1 and mtTFB2 support transcription from human mitochondrial promoters (6), but their relative importance and specific roles are not well understood. Our data argue strongly that either mtTFB1 does not play an important role in mitochondrial transcription in vivo or endogenous mtTFB2 is functionally redundant with the role of mtTFB1 in transcription. In this regard, it has been reported that h-mtTFB1 has rRNA methyltransferase activity (11) and that h-mtTFB2 is at least 1 order of magnitude more active in promoting transcription in vitro than h-mtTFB1 (6).

In composite, our current and previous data (13) document functional differences between mtTFB1 and mtTFB2 in vivo. Both mtTFB1 and mtTFB2 are related in primary sequence to a family of rRNA adenine dimethyltransferases that modify two adjacent adenine bases near the 3′-end of small subunit rRNA to produce N6,N6′-dimethyladenosine (25), and Shadel and co-workers (11) have shown that h-mtTFB1 can methylate E. coli small rRNA in vivo. Fig. 6 shows an amino acid sequence alignment between bacterial (KsgA and ErmC′) and yeast (Dim1) rRNA adenine dimethyltransferases and mtTFBs. KsgA and ErmC′ are well-studied rRNA adenine dimethyltransferases (26–29), and the three-dimensional structure of se-mtTFB (30) is very similar to those of KsgA (31) and ErmC′ (32). Thus, we aligned KsgA, ErmC′, and se-mtTFB based on their structures, and the mtTFB1s and mtTFB2s are aligned based on sequence homology with KsgA and ErmC′. The N-terminal domains of KsgA and ErmC′ share a negatively charged pocket that corresponds to residues in the canonical S-adenosyl-l-methionine (S-AdoMet)-binding site, and 12 residues in ErmC′ were shown to interact directly with S-AdoMet.
The physiological role of mitochondrial transcription factor B1 in Saccharomyces cerevisiae Dim1 has only one mtTFB gene, sc-mtTFB, and mitochondrial 12S rRNA is not methylated on the two adenosine bases (25, 39). It is particularly significant that RNAi knockdown of d-mtTFB1 lowers the efficiency of mitochondrial translation in *Drosophila* cultured cells because the data suggest strongly that endogenous mtTFB2 cannot complement a deficiency in mtTFB1 in its role in translation. These data are consistent with a lack of adenine dimethyltransferase activity in sc-mtTFB and mtTFB2.

Taken together, our data suggest strongly that the primary roles of mtTFB1 and mtTFB2 differ in vivo; mtTFB1 is an RNA adenine dimethyltransferase that influences the efficiency of mitochondrial translation, and mtTFB2 is a critical mitochondrial transcription factor.

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[FIG. 6. Structure-based sequence alignment of *Drosophila* mtTFB1 with other mtTFBs and rRNA adenine dimethyltransferases.](image)
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