**Drosophila** Mitochondrial Transcription Factor B2 Regulates Mitochondrial DNA Copy Number and Transcription in Schneider Cells*

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We report the cloning and molecular analysis of *Drosophila* mitochondrial transcription factor B2 (d-mtTFB2), a protein that plays a role in mitochondrial transcription and mitochondrial DNA (mtDNA) replication in *Drosophila*. An RNA interference (RNAi) construct was designed that reduces expression of d-mtTFB2 to 5% of its normal level in Schneider cells. RNAi knock-down of d-mtTFB2 reduces the abundance of specific mitochondrial RNA transcripts 2- to 8-fold and decreases the copy number of mtDNA -3-fold. In a corollary manner, we find that overexpression of d-mtTFB2 increases both the abundance of mitochondrial RNA transcripts and the copy number of mtDNA. In a comparative experiment, we find that overexpression of *Drosophila* mitochondrial transcription factor A (d-TFAM) increases mtDNA copy number with no significant effect on mitochondrial transcripts. This argues for a direct role for mtTFB2 in mitochondrial transcription and suggests that, if TFAM serves a role in transcription, its endogenous level limits mtDNA copy number but not transcription. Furthermore, we suggest that mtTFB2 increases mtDNA copy number by increasing the frequency of initiation of DNA replication, whereas TFAM serves to stabilize and package mtDNA in mitochondrial nucleoids. Our work represents the first study to document the function of mtTFB2 in vitro, establishing a dual role in regulation of both transcription and replication, and provides a benchmark for comparative biochemical studies in various animal systems.

The mitochondria of eukaryotic cells utilize a number of organelle-specific factors in DNA and RNA metabolism. In *Saccharomyces cerevisiae*, mitochondrial transcription is mediated by the yeast mtRNA1 polymerase, Rpo41p (1–3), and a specificity factor Mtflp (4–6), also known as mitochondrial transcription factor B (sc-mtTFB) (7). Deficiency in sc-mtTFB lowers the abundance of mitochondrial transcripts and reduces mtDNA copy number (5, 7). sc-mtTFB facilitates specific binding of mitochondrial RNA polymerase at numerous promoter sites in the yeast mitochondrial genome (4, 8, 9). Although sc-mtTFB is functionally similar to bacterial sigma factor (9–11), the two proteins do not share amino acid sequence (7, 12) or structural homology (13). Rather, the structure of mtTFB is analogous to bacterial rRNA methyltransferases (13).

Mammalian mitochondrial transcription utilizes mitochondrial RNA polymerase, and three distinct transcription factors: transcription factor A (TFAM; formerly referred to as mtTFAM) and two proteins homologous to sc-mtTFB, transcription factors mtTFB1 and mtTFB2 (3, 14–17). TFAM contains two high mobility group boxes and was shown in organello to bind non-specifically at regularly phased intervals to the control region of human mtDNA (18); it has recently been shown to package mtDNA in nucleoids (19, 20). h-TFAM was also shown to be required for specific initiation at mitochondrial promoters in *vitro* (14–17). The yeast homologue of TFAM is Abf2p, an abundant protein whose primary role is to stabilize and compact mtDNA (21–23); however, Abf2p is not required for transcription of yeast mtDNA (24). Both human mtTFB1 and mtTFB2 have been shown to activate transcription from mitochondrial promoters in *vitro*, although their specific roles in mitochondrial transcription remain unclear (14, 15, 25). Recent studies indicate that h-mtTFB1 has rRNA methylase activity (26) and that its *in vitro* transcriptional activation and methylase activities can be differentially inactivated by mutation (25).

Functional studies of the three animal mitochondrial transcription factors in *vitro* are very limited, and to date, only studies of TFAM have been reported. Gene-targeted disruption of mouse TFAM results in loss of mtDNA (27), and its overexpression in chicken DT40 cells increases the copy number of mtDNA (28). Depletion of *Drosophila* TFAM in cell culture by double-stranded RNAi results in a marked reduction of mtDNA content, without significant inhibition of mitochondrial transcription (29). Taken together, these physiological data support an important role for TFAM in mtDNA maintenance.

Here, we report the cloning and overexpression of *Drosophila* mtTFB2, and the knock-down phenotype of *Drosophila* Schneider cells treated with d-mtTFB2-targeted RNAi. Our results argue strongly that d-mtTFB2 is required both for efficient mitochondrial transcription and for maintenance of mtDNA.

**EXPERIMENTAL PROCEDURES**

**Identification and Sequence Analysis of d-mtTFB2 cDNA**—The amino acid sequence of d-mtTFB1 was used to search the Berkeley...
Drosophila Genome Project database. One sequence (CG3910) was identified that has a high level of homology to d-mtTFB2. 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′-TATCCAACTTGGCAGCCTTGCAG-3′ and 5′-GGCCCCCGACGACATGTTGTTG-3′ for 5′ RACE, and 5′-CAAAATGAGCTGTCAGAATCTGAC-3′ for 3′ RACE. cDNA 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 nonredundant GenBank™ database were performed using BLAST (30). The deduced amino acid sequence was checked for targeting signal peptides using iPSORT (31) and MTFRTO (32). Multiple sequence alignments were calculated with Clustal W (33) and then analyzed with the 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 per lane) was cleaved with XhoI, fractionated in a 0.8% agarose gel/TBE and transferred to nylon membrane (Amersham Biosciences). Hybridization was performed as above. Filters were washed three times for 10 min at room temperature with 2× SSC containing 0.1% SDS, once for 30 min at 65 °C with 0.2× SSC containing 0.1% SDS, and then analyzed with a PhosphorImager (Amersham Biosciences). 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 estimate the relative copy number of mtDNA.

RESULTS

Cloning of Drosophila melanogaster mtTFB2—Two conserved proteins, h-mtTFB1 and h-mtTFB2, function in human mitochondrial transcription in vitro (14, 15, 25). The sequence of a putative d-mtTFB1 (14) was used to search the Berkeley Drosophila Genome Project database for a Drosophila homologue of h-mtTFB2. The search identified a predicted protein, CG3910, whose nucleotide sequence was used to identify and clone a full-length cDNA by rapid amplification of cDNA ends (RACE). The cDNA clone obtained was 1506 nucleotides and encoded a predicted polypeptide of 452 amino acids. Protein sequence analysis with MitoProt II and iPSORT indicate a high probability of mitochondrial localization via a 30-amino acid N-terminal mitochondrial targeting peptide, with the full-length and processed forms of d-mtTFB2 predicted to be 52.2 and 48.6 kDa, respectively. d-mtTFB2 was found to be 34% homologous to human mtTFB2 and 36% homologous to mouse d-mtTFB2 (Fig. 1). As was shown previously (15), we found bacterial rRNA methyltransferase to be a member of the same protein family (data not shown).

Rabbit antisera was raised against a recombinant, truncated form of d-mtTFB2 (Ala-13 to Ser-252). The antibody detects a single polypeptide in protein extracts from Drosophila Schneider cells with an electrophoretic mobility corresponding to an approximate molecular mass of 50 kDa (Fig. 2A). Immunoblot analysis of subcellular fractions of Drosophila melanogaster mitochondria demonstrated that d-mtTFB2 is localized to the mitochondria (data not shown).

RNAi-dependent Knock-down of d-mtTFB2 Reduces Cell Growth and Mitochondrial Transcription and Induces mtDNA Depletion—The abundance of d-mtTFB2 was reduced by expressing a metallothionein-inducible d-mtTFB2-targeted RNAi species (35–37) from the plasmid pMt/invB2/Hy. The RNA species produced forms a dsRNA hairpin homologous to d-mtTFB2. Previous studies indicate that dsRNA hairpins are efficient RNAi inhibitors (38–40).

Cells stably expressing pMt/invB2/Hy or the control plasmid pmr/invB2/Hy were cultured for 10 days in the absence or presence of 0.4 mM CuSO4. Immunoblot analysis of copper-treated cells showed that cells carrying pMt/invB2/Hy expressed 20-fold less protein than control cells.
d-mtTFB2 than cells carrying the control vector (Fig. 2B). Basal, uninduced expression from pMt/invB2/Hy (which results from metal contamination in the fetal bovine serum, see “Experimental Procedures”) also suppressed expression of d-mtTFB2 by 5-fold. In contrast, expression of the catalytic subunit of Drosophila mtTFB2 is critical for transcription of both strands of mtDNA, because Drosophila TFB2 is required for synthesis of RNA primers during replication of mtDNA.

The copy number of mtDNA was also reduced in cells expressing d-mtTFB2-targeted RNAi. Total cellular DNA was isolated from cells carrying no plasmid, pMt/Hy or pMt/invB2/Hy, cleaved with XhoI, and analyzed by Southern blot. Blots were hybridized sequentially with probes for the mitochondrial transcripts 12S rRNA, ND4, and CytB and the nuclear transcript Rp49. Relative transcription efficiency was quantitated by normalizing mitochondrial transcript abundance to that of Rp49.

The copy number of mtDNA was also reduced in cells expressing d-mtTFB2-targeted RNAi. Total cellular DNA was isolated from cells carrying no plasmid, pMt/Hy or pMt/invB2/Hy, cleaved with XhoI, and analyzed by Southern blot. Blots were hybridized sequentially with probes for the nuclear histone gene cluster and for the mitochondrial gene CytB (Fig. 4A). Relative mtDNA copy number was estimated from the ratio of CytB hybridization to histone cluster hybridization (Fig. 4B). After induction of d-mtTFB2-targeted RNAi for 10 days, relative mtDNA copy number was 38% of the control; without induction, the basal level of d-mtTFB2-targeted RNAi reduced the mtDNA copy number to 64% of control. This result suggests that d-mtTFB2 is also required for synthesis of RNA primers during replication of mtDNA.

Overexpression of d-mtTFB2 Increases Mitochondrial Transcription and mtDNA Copy Number—d-mtTFB2 was subcloned into the inducible expression vector pMt/Hy under the control of the metallothionein promoter. The resulting expression vector, pMt/d-mtTFB2/Hy, was introduced into Schneider cells, and
increase in the presence of copper, immunoblot analysis indicated a 40-fold growth or viability (data not shown). The effect of d-mtTFB2 overexpression on mitochondrial transcript abundance was evaluated by Northern blots. Blots were probed for transcripts from the mitochondrial ND4, Cytb, and 12S rRNA genes and for nuclear RP49 as a control (Fig. 5B). The results demonstrate that the level of all three mitochondrial RNA transcripts decreases ~2-fold in cells that overexpress d-mtTFB2, whereas the level of nuclear RP49 transcript is unchanged. Relative copy number of mtDNA was also increased ~2-fold in cells that overexpress d-mtTFB2 (Fig. 5C).

**DISCUSSION**

*Drosophila* mtTFB2 shares homology with human mtTFB2 that has been shown to be important for mitochondrial tran-
scription in vitro (15). In Drosophila Schneider cells treated with RNAi, d-mtTFB2 is expressed at 5% of the endogenous level and induces cellular phenotypes of slow growth, reduced viability, 2- to 8-fold reduction in abundance of specific mitochondrial transcripts, and 3-fold reduction in mtDNA copy number. Overexpression of d-mtTFB2 modestly stimulates transcription of mitochondrial genes and increases mtDNA copy number 2-fold. These data provide the first evidence that animal mtTFB2 plays an important role in both mitochondrial transcription and mtDNA replication in vivo.

We found that mitochondrial transcript levels depend both on the abundance of d-mtTFB2 and on the specific mitochondrial gene examined. Basal-level expression of RNAi in Schneider cells was sufficient to reduce 4-fold the level of d-mtTFB2, whereas its induced expression reduced d-mtTFB2 levels by 20-fold. By comparison, mitochondrial mRNA abundance was reduced 1.6- to 3-fold under basal conditions and 6- to 8-fold under induced conditions. Under both induced and uninduced conditions, the level of the 12S rRNA transcript was substantially less sensitive to the abundance of d-mtTFB2 than were the levels of two mRNA species. The reason for this pattern is not clear, but it may indicate a faster turnover of mitochondrial mRNA as compared with mitochondrial rRNA (41). Although information on transcriptional mechanism in Drosophila remains limited, several polycistronic transcripts were identified and mapped by Berthier et al. (42), suggesting that Drosophila mtDNA is transcribed polycistronically as in other organisms, and similar to mammals, mitochondrial rRNAs were found to be more abundant than mRNAs (42). Recently, Drosophila mitochondrial transcription termination factor was identified that mediates termination of transcription of the ribosomal unit, accounting for the excess production of mitochondrial rRNAs (43). Overall, we found that mRNA levels were reduced more than the levels of mtDNA, and this, too, may reflect a higher turnover rate for mRNAs as compared with that of mtDNA. Interestingly, a similar result was obtained in an RNA knock-down analysis of mitochondrial RNA polymerase in trypanosomes (44). In any case, because of the differential effects we observed on mtDNA copy number and transcription in the overexpression analyses of d-mtTFB2 and d-TFAM, it is apparent that the effect of d-mtTFB2 on transcription is not a simple consequence of mtDNA dose.

We found that the abundance of d-mtTFB2 influences modestly the efficiency of mtDNA replication, where 2- to 3-fold effects were observed over a broad range of 20-fold underexpression to 40-fold overexpression. In human and mouse cells, initiation of mtDNA replication was proposed to involve synthesis of an RNA primer by transcription from the light strand promoter, followed by RNA processing and then extension of the RNA primer by DNA polymerase γ in a unidirectional and strand asymmetric manner (45, 46). Our study suggests that d-mtTFB2 is involved in the synthesis of RNA primer(s) for mtDNA replication, because mtDNA copy number changes in parallel with both increasing and decreasing the levels of d-mtTFB2, and this is accompanied by corresponding changes in mitochondrial transcripts.

Relatively little is known about the mechanism of mtDNA replication in Drosophila (47), and new experimental approaches have led to a reconsideration of the long standing model proposed for mouse and man (48–50). In the recent model, mtDNA replication is proposed to initiate bidirectionally within a broad zone and proceed symmetrically, becoming unidirectional when one fork encounters a barrier in the vicinity of the previously designated leading strand origin (51–53). In considering both models, it remains possible that only one primer is made for leading and one for lagging DNA strand synthesis, or that multiple primers are synthesized on one or both DNA strands. Whatever the case, our data would suggest that at least one of these is made by mtRNA polymerase functioning together with d-mtTFB2, either at a promoter or elsewhere. Because we find d-mtTFB2 abundance regulates mtDNA copy number, we would suggest that this priming event is likely critical to initiation of mtDNA replication.

In vitro studies show that both mtTFB1 and mtTFB2 support transcription from human mitochondrial promoters (15), but their relative importance and specific roles are not well understood. That RNAi knock-down of d-mtTFB2 lowers the efficiency of mitochondrial transcription in Drosophila-cultured cells is particularly significant, because the data argue strongly that endogenous mtTFB1 cannot complement a deficiency in mtTFB2. Thus, mtTFB1 is not functionally redundant with mtTFB2, pointing to specialized roles for the two transcription factors in vivo. In this regard, it has been reported that h-mtTFB1 has RNA methyltransferase activity (26) and that h-mtTFB2 is at least one order of magnitude more active in promoting transcription in vitro than h-mtTFB1 (15).

The physiological role of TFAM in mitochondrial transcription is less clear. Our data show that overexpression of d-TFAM increases mtDNA copy number without increasing mitochondrial transcripts, consistent with the finding of Kitagawa and colleagues (29), who showed that deficiency of d-TFAM reduces markedly mtDNA copy number without significant inhibition of mitochondrial transcription in Drosophila cultured cells. One possible explanation for these results is that the endogenous level of d-TFAM is in excess of that needed for mitochondrial transcription but limits mtDNA copy number, such that its residual level upon RNA knock-down is sufficient to support constitutive levels of transcription but is insufficient for mtDNA maintenance. In this regard, recent studies show that the h-TFAM:mtDNA ratio is 1700:1 in human HeLa cells (54), yet it fully activates promoter-specific transcription in the presence of either h-mtTFB1 or B2 in vitro at a level corresponding to a h-TFAM:DNA template ratio of 10–100:1 (15). An alternate explanation is that, in Drosophila mitochondrial transcription, d-TFAM does not function together with d-mtTFB2 (or B1), reminiscent of the case in yeast, in which Abi22p (sc-TFAM), which is required for mtDNA stability and packaging (21, 55), is not required for transcription of yeast mtDNA (24). In any case, our data suggest differential roles for mtTFB2 and TFAM in mtDNA maintenance, with mtTFB2 regulating the frequency of initiation of replication and TFAM stabilizing and packaging mtDNA via dynamic, histone-like interactions in mitochondrial nucleoids. Clearly, more detailed physiological and biochemical studies are needed to understand the mechanisms of mitochondrial transcription, mtDNA replication, and the regulation of mitochondrial biogenesis. Such studies will help to differentiate the functional roles of the three animal mitochondrial transcription factors.

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