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SURVEY AND SUMMARY

The *Drosophila* termination factor DmTTF regulates *in vivo* mitochondrial transcription

Marina Roberti[1], Francesco Bruni[1], Paola Loguercio Polosa[1], Maria Nicola Gadaleta[1,2] and Palmiro Cantatore[1,2,*]

[1]Dipartimento di Biochimica e Biologia Molecolare 'Ernesto Quagliariello', Università di Bari and [2]Istituto di Biomembrane e Bioenergetica, CNR, Bari, Via Orabona 4, 70125, Bari, Italy

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ABSTRACT

DmTTF is a *Drosophila* mitochondrial DNA-binding protein, which recognizes two sequences placed at the boundary of clusters of genes transcribed in opposite directions. To obtain *in vivo* evidences on the role of DmTTF, we characterized a DmTTF knock-down phenotype obtained by means of RNA interference in D.Mel-2 cells. By a combination of RNase protection and real-time RT–PCR experiments we found that knock-down determines remarkable changes in mitochondrial transcription. In particular, protein depletion increases not only the level of (+) and (−)strand RNAs mapping immediately after of the two protein-binding site, but also that of transcripts located further downstream. Unexpectedly, depletion of the protein also causes the decrease in the content of those transcripts mapping upstream of the protein target sites, including the two rRNAs. The changes in transcript level do not depend on a variation in mitochondrial DNA (mtDNA) content, since mtDNA copy number is unaffected by DmTTF depletion. This work shows conclusively that DmTTF arrests *in vivo* the progression of the mitochondrial RNA polymerase; this is the first ever-obtained evidence for an *in vivo* role of an animal mitochondrial transcription termination factor. In addition, the reported data provide interesting insights into the involvement of DmTTF in transcription initiation in *Drosophila* mitochondria.

INTRODUCTION

Mitochondrial DNA (mtDNA) transcription in animals [for review see (1,2)] requires the catalytic activity of an organelle-specific RNA polymerase (mtRNApol), a monomeric protein showing significant similarity to bacteriophage RNA polymerases (3), and some accessory factors. They include TFAM and TFB1/2M, involved in transcription initiation, and a protein responsible for transcription termination. TFAM acts as a DNA-binding protein that recognizes promoters in a sequence-specific manner and stimulates transcription (4,5); moreover it possesses the property of non-specific DNA binding (6) and exerts an architectural role in the maintenance of mtDNA (7,8). TFB1/2M are two related factors that are required for basal transcription, with TFB2M being about two orders of magnitude more active in promoting specific transcription than TFB1M (5). The proposed model for transcription initiation requires TFBM proteins to bridge an interaction between mtRNApol and promoter-bound TFAM (9). So far, *in vivo* studies for TFB1/2M have been performed only in *Drosophila* that allowed ascribing a different role to each of them. In particular, TFB2M regulates mtDNA transcription and copy number (10), whereas TFB1M modulates mitochondrial translation but not transcription or mtDNA copy number in Schneider cells (11). The role of TFB1M in mitochondrial translation is probably related to the RNA methyltransferases activity described for this protein (12).

Transcription termination factors have been characterized in human (13,14), sea urchin (15,16) and *Drosophila* (17,18); they belong to a wide family of proteins identified in several animal *phyla* (19). Termination factors display sequence-specific DNA-binding activity and the capacity to arrest *in vitro* the progression of the mtRNApol in a bidirectional way. In spite of these common features, the location of target sites on mtDNA and the specific role accomplished by the termination factors seem to vary in the different species according to the peculiar mtDNA gene organization and the transcription mechanism. The human factor mTERF binds mtDNA immediately downstream of the 3′ end of the ribosomal gene unit and is thought to be responsible for an attenuation/termination event that causes the steady-state

*To whom correspondence should be addressed. Tel: +39 080 5443378; Fax: +39 080 5443403; Email: p.cantatore@biologia.uniba.it

level of rRNAs to be higher than that of the downstream mRNAs (20). Most recently, a second DNA-binding site has been detected for mTERF in the D-loop region of mtDNA (21). The sea urchin factor mtDBP recognizes two homologous sequences located in the main non-coding region and at the ND5/ND6 gene boundary, respectively. Also the *Drosophila* factor DmTTF recognizes two homologous sequences on mtDNA that lie at the boundary of clusters of genes transcribed in opposite direction, namely the boundary ND3/ND5 and cyt b/ND1. The position and the number of the binding sites point to the involvement of these factors in controlling the multiple transcription units existing in these organisms. Contrary to human mTERF, the sea urchin and *Drosophila* proteins do not bind at the 3′ end of the ribosomal genes and do not seem to be directly involved in the regulation of rRNA level. An additional role in mtDNA replication has been proposed for sea urchin mtDBP, that has been demonstrated *in vitro* to posses also a contrahelicase activity (22).

Despite the multiplicity of data produced by *in vitro* studies, no evidence has been reported so far demonstrating *in vivo* the capacity of a mitochondrial transcription termination factor to arrest mtRNApol, and, more in general, to affect transcription. With the aim to study *in vivo* the role of DmTTF, we used *Drosophila melanogaster* cultured cells as a model system to obtain a knock-down phenotype. Our results indicate that DmTTF depletion causes a general increase in the level of sense and antisense transcripts mapping downstream of DmTTF binding sites on either strands. This allows to conclude that removal of the protein facilitates the progression of mtRNApol in both directions. Moreover, we found that knock-down of DmTTF affects the level of transcripts mapping upstream of the protein-binding site.

MATERIALS AND METHODS

Drosophila cell culture and conditions for RNAi

Drosophila embryonic cell line D.Mel-2 (GIBCO-Invitrogen) was maintained in *Drosophila* SFM (GIBCO-Invitrogen) supplemented with 16 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin, at 28°C in 75 cm^2 flasks. For double-stranded RNA (dsRNA) treatment, the cells were diluted to a final concentration of 1.0×10^6 cells/ml in 10 ml of complete *Drosophila* SFM in 75 cm^2 flasks. dsRNA (15 μg per 10^6 cells) was added directly to the medium to a final concentration of 30 nM. Flasks were swirled by hand and the cells were incubated at 28°C for 1 h. Then, 10 ml of medium were added to obtain a cell density of 0.5×10^6 cells/ml, an additional incubation at 28°C for 72 h followed.

dsRNA production

The templates for the production of dsRNA were PCR-derived fragments carrying at both ends the T7 promoter sequence. Each primer used in the PCR contained a 5′ end sequence corresponding to the T7 polymerase promoter (TAATACGACTCACTATAGGGA), followed by a gene-specific sequence as indicated: DmTTF (accession no. AY196479) forward-primer 506–521, reverse-primer 1223–1207; odds-paired (accession no. NM_079504) forward-primer 270–285, reverse-primer 987–972. The PCR products were purified by the Wizard SV PCR Clean-Up System (Promega) and used as templates for the MEGAscript T7 transcription kit (Ambion) to produce dsRNA according to the manufacturer's instructions.

Protein extract preparation and western blotting analysis

dsRNA treated and untreated D.Mel-2 cells were harvested, centrifuged at 1000 *g* in the Eppendorf A-4-62 rotor for 4 min at 4°C and washed twice in D-PBS (Euroclone). To prepare total protein extract, pellets were resuspended in NP-40 lysis buffer [25 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 0.5 mM EDTA, 400 mM KCl, 20% glycerol, 1% Nonidet P-40, 1 mM DTT and protease inhibitors (Sigma)], homogenized and centrifuged at 130 000 *g* in the Beckman 70.1 Ti rotor for 30 min at 4°C. To obtain mitochondrial protein extract, cells were resuspended in 10 mM Tris–HCl (pH 6.7), 10 mM KCl, 0.1 mM EDTA. Mitochondria were prepared as described by Ausenda and Chomyn (23) and lysed in NP-40 lysis buffer in the same conditions used for total cell lysate. Total and mitochondrial lysates, containing 250 μg of proteins, were fractionated on 12% SDS–polyacrylamide gel and electroblotted for 1 h to PVDF membrane (Hybond-P, Amersham Pharmacia). Membranes were pre-incubated for 1 h with 1× Blotto [20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100] containing 5% non-fat dry milk, followed by incubation for 1 h, in the same solution, with polyclonal antibodies raised in guinea pig against recombinant DmTTF (Eurogentec) and in rabbit against recombinant D-TFAM and h-NDUFS4 (kindly provided by Y. Kitagawa, Nagoya University, Japan, and S. Scacco, University of Bari, Italy, respectively). Filters were washed four times for 15 min with 1× Blotto / 5% non-fat dry milk, incubated for 1 h with horseradish peroxidase (HRP)-coniugated anti-guinea pig IgG or anti-rabbit IgG (Santa Cruz Biotechnology) and washed with 1× Blotto. Protein bands were visualized using the ECL western blotting reagents (Amersham Pharmacia).

RNase protection assay

Total cellular RNA was extracted from treated and untreated D.Mel-2 cells by the RNeasy Midi Kit (Qiagen). The templates for the production of riboprobes Ribo-1 and Ribo-2 were PCR-derived fragments carrying at one end the T7 promoter polymerase sequence. The reverse primers used in the PCR contained a 5′ end sequence corresponding to the T7 promoter (TAATACGACTCACTATAGG), followed by a *D.melanogaster* mtDNA-specific sequence (accession no. NC_001709). Primer sequences were: forward-primer 11 637–11 661 and reverse-primer 11 931–11 908 for Ribo-1; forward-primer 11 637–11 661 and reverse-primer 11 855–11 834 for Ribo-2. The PCR products were purified by the Wizard SV PCR Clean-Up System (Promega) and used as templates to produce ^{32}P-labeled riboprobes with the MAXIscript T7 kit (Ambion), according to the manufacturer's instructions.

RNase protection assay was performed by using the RPA III kit (Ambion) with some modifications. For each sample, about 1.5×10^5 c.p.m. of gel-purified riboprobes were co-precipitated with 50 μg of *Drosophila* total RNA in the presence of 0.5 M ammonium acetate and 2.5 vol of absolute ethanol. RNA pellets were dissolved in 10 μl of hybridization

buffer [40 mM Hepes (pH 6.4), 1 mM EDTA, 400 mM NaCl, 50% ultrapure formamide], denaturated for 4 min at 95°C and incubated for 16 h at 37°C. Hybrids were digested in 150 µl of RNase Digestion III buffer (Ambion) in the presence of 2.5 U/ml of RNase A and 100 U/ml of RNase T1 for 30 min at 37°C. Digestion products were ethanol precipitated and pellets were resuspended in gel loading buffer II (Ambion), denatured and run on 10% polyacrylamide/7 M urea gels in TBE. Bands were visualized by Typhoon 8600 Phosphor Imaging System (Molecular Dynamics) and the quantitative analysis was performed with ImageQuant 5.2 software (Molecular Dynamics).

RT–PCR and real-time RT–PCR assays

Semiquantitative RT–PCR assay was performed using the Enhanced Avian HS RT–PCR Kit (Sigma) according to the manufacturer's instruction. Reactions contained 600 ng of *Drosophila* total RNA as template, and 100 pmol primers specific for *Drosophila* mtDNA or 28S RNA (endogenous control) in a final volume of 100 µl.

For real-time RT–PCR assay, RNA was reverse-transcribed using the Enhanced AMV Reverse Transcriptase (Sigma), according to the manufacturer's instruction. Each reaction was carried out in a final volume of 25 µl using 250 ng of total RNA and 25 pmol of mtDNA gene-specific primer. Forward or reverse primer was added to select the sense or antisense mitochondrial transcripts. Real-time PCR was performed using the SYBR® Green PCR MasterMix (Applied Biosystems) and the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Primers were designed using the Primer Express 2.0 software (Applied Biosystem) and sequence positions on *D.melanogaster* mtDNA were as follows: ND2-for 558–573 nt, ND2-rev 651–632 nt; $tRNA^{Cys}$-for 1322–1346 nt, $tRNA^{Cys}$-rev 1383–1357 nt; COI-for 1838–1862 nt, COI-rev 1932–1904 nt; COII-for 3569–3593 nt, COII-rev 3652–3631 nt; ATPase6-for 4409–4433 nt, ATPase6-rev 4481–4459 nt; COIII-for 5304–5328 nt, COIII-rev 5379–5355 nt; ND5-for 7040–7061 nt, ND5-rev 7114–7095 nt; cyt b-for 10 697–10 719 nt, cyt b-rev 10 766–10 748 nt; ND1-for 11 984–12 002 nt, ND1-rev 12 056–12 034 nt; lrRNA-for 12 827–12 845 nt, lrRNA-rev 12 906–12 887 nt; srRNA-for 14 407–14 434 nt, srRNA-rev 14 484–14 462 nt. Cytoplasmic 28S rRNA (accession no. M21017) was used as endogenous control; primer sequence positions were: 28S-for 1407–1429 nt, 28S-rev 1480–1462 nt.

Each reaction was run in triplicate and contained 1 µl of reverse transcription reaction (1 µl of a 1:300 dilution was used for 28S rRNA) along with 200 nM primers in a final reaction volume of 30 µl. Amplification conditions were: 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. To ensure that only a single product was amplified, the melting curve analysis was performed; it took an additional step of 20 min after the real-time PCR, and was carried out using the Dissociation Curves software (Applied Biosystem). All PCR products were run on a 2.5% agarose gel to confirm specificity. The amplification plots produced during real-time PCR were used to determine the amplification efficiency for each amplicon with the formula $E = 10^{-1/\text{slope}}$. The relative quantification of target transcripts in RNAi cells compared to untreated cells, all normalized to 28S rRNA, was performed according to the Pfaffl mathematical model (24). For each amplicon, the mean ratio value was obtained from at least four real-time RT–PCR assays, using RNA obtained from independent RNAi experiments. Statistical analysis was performed using paired two-tailed Student's *t*-test.

Southern blotting analysis

Total cellular DNA was extracted from treated and untreated D.Mel-2 cells by the Wizard Genomic DNA Purification kit (Promega). DNA was digested with XhoI, fractionated on a 0.7% agarose/TAE gel (5 µg/lane) and electrotransferred to nylon filters (Hybond-N^+, Amersham Biosciences). Blots were hybridized to ^{32}P-labeled probes for the ND3 gene (5720–5930 nt) and for the nuclear histone gene H2B (accession no. X14215, 416–787 nt). Hybridization was performed in 6× SSC [900 mM NaCl, 90 mM sodium citrate (pH 7.0)], 5× Denhardt's reagent, 0.5% SDS, 100 µg/ml of salmon sperm DNA at 68°C for 16 h. The membrane was washed twice at room temperature for 10 min in 2× SSC, 0.1% SDS, at 65°C for 30 min in 0.1× SSC, 0.1% SDS and finally at room temperature for 5 min in 0.1× SSC. Filters were analyzed as reported for RNase protection assay.

RESULTS

RNAi-mediated knock-down of DmTTF

To *in vivo* analyze the role of the *Drosophila* transcription termination factor DmTTF, we produced a knock-down phenotype in D.Mel-2 cultured cells by using the RNAi technique. DmTTF level was monitored by means of western blotting using specific polyclonal antibodies raised against the bacterial recombinant protein (17). The antiserum was tested with total cellular proteins and with mitochondrial protein extract (Figure 1A); in both cases, the antibodies detected a polypeptide showing an approximate molecular mass of 43 kDa. This result represents the first detection of the natural form of DmTTF and confirms the calculated mass of the mature protein. Mitochondrial lysate appears highly enriched in DmTTF as expected for a mitochondria-localized protein.

DmTTF-targeted RNAi was accomplished by treating D.Mel-2 cells with dsRNA for DmTTF. After 72 h of incubation cells were harvested and mitochondrial proteins were extracted and tested by western blotting. As shown in Figure 1B, DmTTF was not detectable in mitochondrial lysates from DmTTF-dsRNA treated cells. The interference was specific since no effect was obtained in cells treated with dsRNA containing the sequence of Opa1 gene (mock control). Moreover, the intensity of the band corresponding to D-NDUFS4, a nuclear-encoded subunit of NADH dehydrogenase used as loading control, remained almost unchanged in all cases. A longer exposure of the membrane (data not shown) indicated that >90% depletion of DmTTF was obtained. In addition, we tested whether the knocking down of DmTTF might have affected the level of another mitochondrial transcription factor, namely D-TFAM. Figure 1B shows that D-TFAM content remained unchanged in treated and untreated cells, thus indicating that DmTTF knock-down did not affect D-TFAM expression. No changes in cell viability and morphology were observed following the treatment with dsRNA for DmTTF.

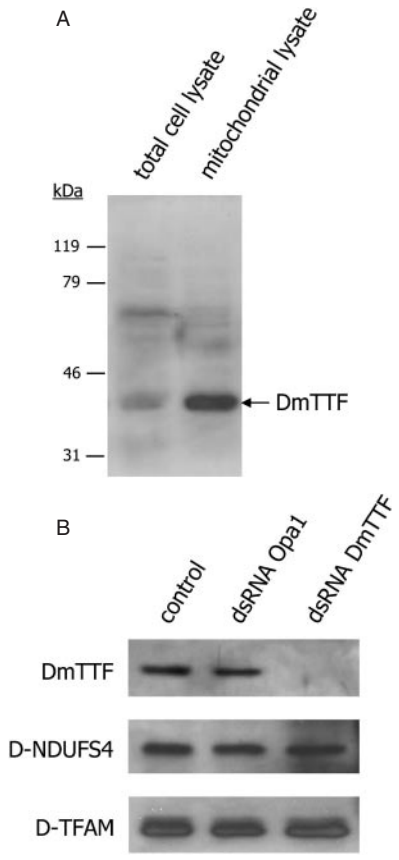

Figure 1. Effect of DmTTF-targeted RNAi in D.Mel-2 cells. (**A**) Western blotting analysis of D.Mel-2 total cell and mitochondrial lysate. A total of 250 μg of proteins were fractionated on a 12% SDS–polyacrylamide gel, electroblotted to PVDF filters and incubated with polyclonal antibodies against recombinant DmTTF. (**B**) D.Mel-2 cells were either untreated (control) or treated with odds-paired (Opa1) or DmTTF dsRNA. Mitochondrial lysate (250 μg of proteins) was probed with polyclonal antibodies against DmTTF, h-NDUFS4 or D-TFAM.

DmTTF depletion affects transcription in the cyt b/ND1 region

The molecular effect of DmTTF knock-down on mitochondrial transcription was investigated by means of different strategies. Firstly, we focused our attention on transcripts mapping in proximity of the cyt b/ND1-binding site. In particular, we performed an RNase protection assay using two overlapping riboprobes of 295 nt (Ribo-1) and 218 nt (Ribo-2), that are complementary to (−)strand transcripts and share the same 3′ end (Figure 2A). After RNase treatment, hybrids were sized by denaturing polyacrylamide gel electrophoresis. Figure 2B shows that, in control cells, Ribo-1 produced a prominent band of 67 nt corresponding to the $tRNA^{Ser(UCN)}$ that is entirely contained in the probe, and few additional faint bands having a higher molecular weight. In DmTTF knock-down cells, in addition to the 67 nt band, which is 30% more intense than in the control, many other higher molecular weight hybrids, that were about 5-fold more abundant than in the control, were detected. The size of the slowest migrating band, about 230 nt, was consistent with that of the portion of ND1-antisense transcript extending from DmTTF-binding site to the 5′ end of the probe. These results were confirmed by a similar experiment

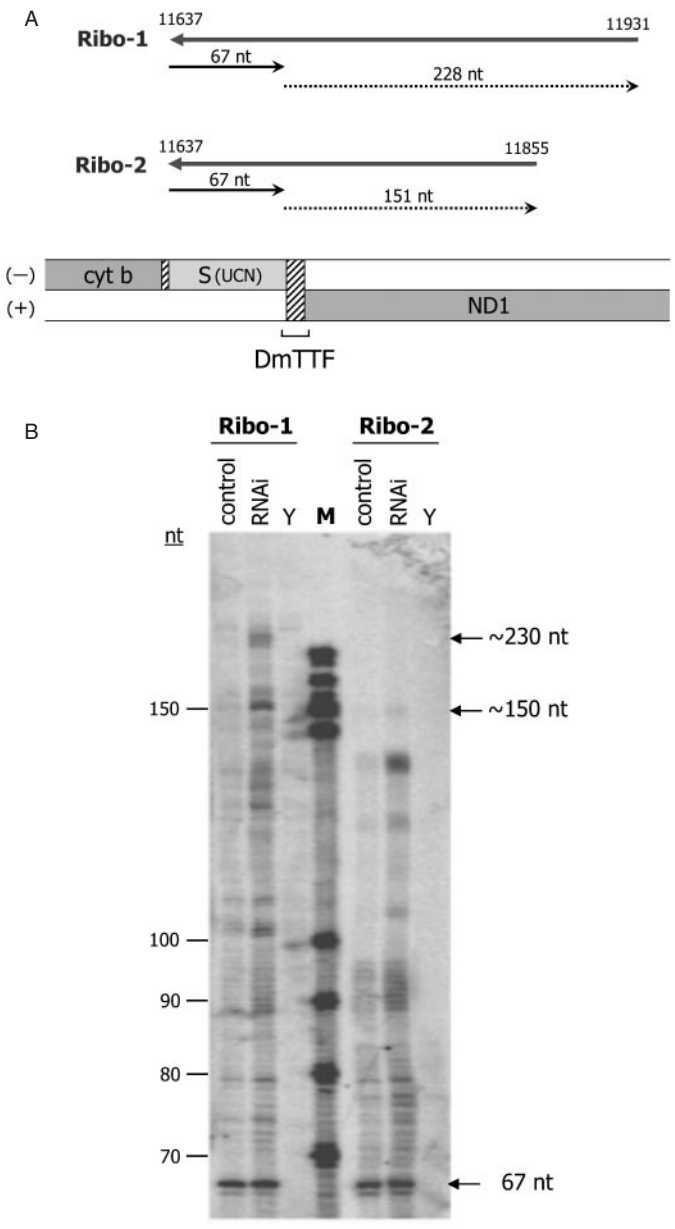

Figure 2. RNase protection assay on mitochondrial (−)strand transcripts mapping around the cyt b/ND1-binding site in DmTTF-depleted cells. (**A**) Schematic representation of digestion products using probes Ribo-1 (295 nt) and Ribo-2 (218 nt). Riboprobes (grey bold arrows), mature transcripts (continuous arrows) and read-through transcripts (dotted arrows) are indicated above the cyt b/ND1 region map. Dashed regions indicate non-coding sequences. (**B**) Total cellular RNA (50 μg), extracted from untreated (control) and DmTTF-dsRNA treated (RNAi) D.Mel-2 cells, was hybridized with about 1.5×10^5 c.p.m. of Ribo-1 and Ribo-2 probes and digested with RNase A and T1. Digestion products were denatured and run on a 10% polyacrylamide/7 M urea gel. Y, sample containing 50 μg of yeast total RNA. M, Decade RNA marker (Ambion).

using the shorter probe Ribo-2. In control cells we observed again a prominent band of 67 nt along with minor higher molecular weight products. However in knock-down cells, the 67 nt band, which was again 30% more abundant than in the control, was accompanied by additional slow migrating products that were about 5-fold more abundant and reached a maximum size of about 150 nt. Also in this case the dimension

corresponded to that of the portion of ND1-antisense RNA protected by Ribo-2. The low intensity of the highest molecular weight bands (230 and 150 nt) obtained with both riboprobes is very likely due to the high A:T content of *Drosophila* mtDNA sequence that could make hybrid ends unstable and therefore highly susceptible to RNase digestion. No precursor transcripts, including both $tRNA^{Ser(UCN)}$ and the portion of ND1-antisense contained in the probe, were detectable probably due to the very low abundance of unprocessed precursor molecules.

We used the more sensitive RT–PCR to detect RNA molecules spanning the cyt b/ND1-binding site in knock-down and control cells. By using two primers located respectively upstream and downstream of the termination site, we carried out a semi-quantitative analysis by taking samples at different time points (Figure 3). The amplification product of 360 bp, deriving from precursor transcripts encompassing the 3′ ends of cyt b and ND1 genes, appears almost five cycles earlier in treated cells than in the control. Although this analysis does not allow to distinguish between (+) and (−)strand transcripts, the obtained results indicate that mitochondria from both control and depleted cells contain unprocessed precursor transcripts spanning DmTTF-binding sites and that those molecules are more abundant in DmTTF-depleted cells.

DmTTF depletion causes a general alteration of mitochondrial transcription

The above reported data show that DmTTF knock-down causes the increase in the level of transcripts mapping beyond the cyt b/ND1-binding site. To examine whether protein depletion may have a wider impact on mitochondrial transcription, we performed a systematic analysis based on the measurement of the steady-state level of several mitochondrial RNA species by means of real-time RT–PCR. In particular, we determined the relative concentration of 11 sense and 9 antisense transcripts located upstream and downstream of the two DmTTF-binding sites, and we calculated for each transcript the ratio between knock-down and control cells.

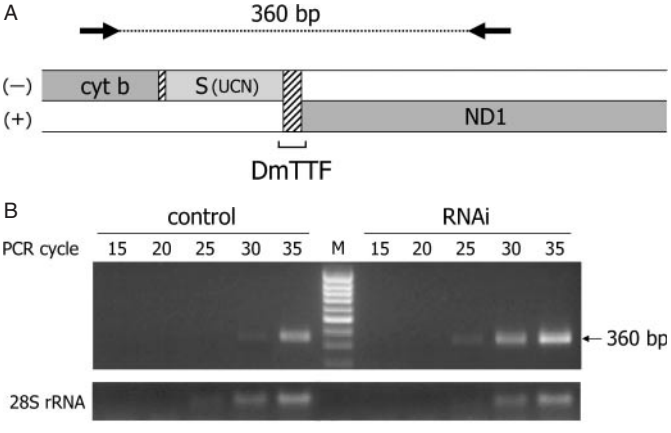

Figure 3. RT–PCR analysis on mitochondrial transcripts spanning the cyt b/ND1-binding site in DmTTF-depleted cells. (**A**) Schematic illustration of the cyt b/ND1-binding site. Black arrows represent the forward (11 494–11 520 nt) and reverse (11 855–11 834 nt) primers used for RT–PCR. Dashed regions indicate non-coding nucleotides. (**B**) Total RNA (600 ng) extracted from untreated (control) and treated (RNAi) D.Mel-2 cells was used as template in RT–PCR; 10 µl-samples were collected at the indicated cycles, run on a 1.5% agarose gel and stained with ethidium bromide. Nuclear encoded 28S rRNA was used as endogenous control.

The results are shown in Figure 4A. Starting from the $tRNA^{Ile}$ gene and proceeding in the direction of (−)strand transcription, we found that the level of several transcripts mapping upstream of the ND3/ND5-binding site, namely ND2, $tRNA^{Cys}$-antisense, COI, COII, COIII, was 1.5- to 2-fold reduced in knock-down cells. The only exception was ATPase6/8 transcript that remained substantially unchanged. On the contrary, the content of transcripts mapping downstream of the ND3/ND5-binding site, namely ND5-antisense and cyt b, almost doubled in knock-down cells with respect to the control. A higher increase (about 3-fold) of the level of ND1-antisense transcript, which maps downstream of the cyt b/ND1-binding site, occurred in knock-down cells. The relative increase in the level of ND1-antisense transcript measured by real-time RT–PCR is in agreement with that deduced by RNase protection assay.

The level of (+)strand transcripts displayed a trend similar to that of the (−)strand RNA molecules. Starting from the srRNA and moving in the (+)strand transcription direction, we observed that in knock-down cells the level of srRNA, lrRNA and ND1 transcripts, all mapping upstream of the cyt b/ND1-binding site, declined by a factor of 2. On the contrary the content of cyt b-antisense and ND5 transcripts, which are placed downstream of the protein-binding site, is markedly increased as it doubles with respect to the control. Finally, we measured the abundance of transcripts mapping downstream of the ND3/ND5-binding site. We found that, with the only exception of COIII-antisense that remained unchanged, the level of ATPase6/8-antisense, COII-antisense, COI-antisense, $tRNA^{Cys}$ and ND2-antisense transcripts was markedly increased in depleted cells, with ND2-antisense transcript level reaching a value that was about 10-fold than in the control. We conclude that DmTTF depletion has a wide effect on mitochondrial transcription, causing the increase in the level of transcripts mapping downstream of one or both target sites, and the decrease in the level of transcripts mapping upstream of both target sites.

To evaluate the possibility that DmTTF depletion could affect mtDNA copy number, we determined the ratio between mtDNA and nuclear DNA, in both control and DmTTF depleted cells. For this purpose, total cellular DNA was cleaved with XhoI and analyzed by Southern blotting using a probe for the mitochondrial ND3 gene and a probe for the nuclear H2B histone gene. The results of a representative experiment are shown in Figure 4B. It appears that there is no significant change in mtDNA level in knock-down cells; this was confirmed by measuring the relative mtDNA content by real-time PCR (data not shown). This result indicates that changes in the concentration of mitochondrial RNAs are due to a direct effect of DmTTF depletion on mitochondrial transcription rather than to changes in mtDNA content.

DISCUSSION

Mitochondrial transcription termination factors are sequence-specific DNA-binding proteins characterized in mammals (13), echinoderms (15) and *Drosophila* (17). Their function has been inferred from the capacity of arresting *in vitro*

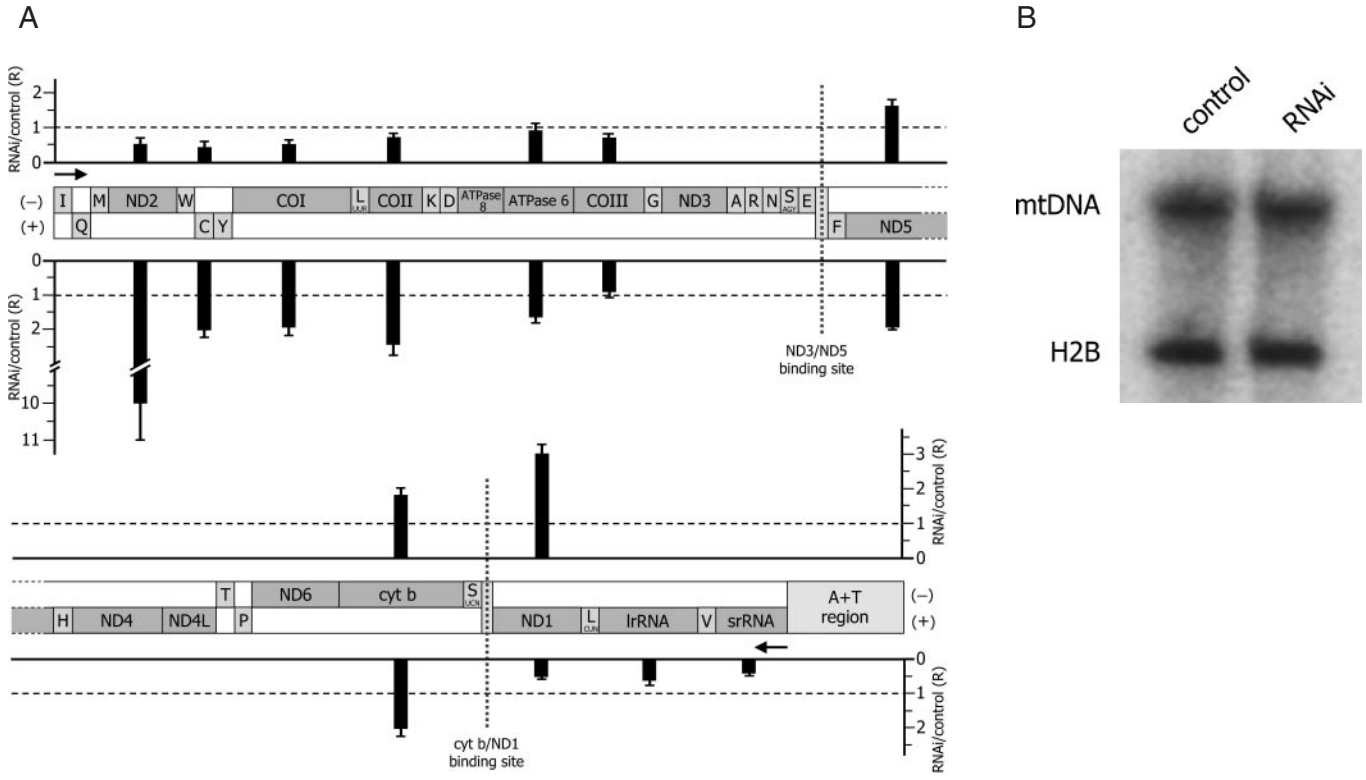

Figure 4. Effect of DmTTF depletion on the level of mitochondrial sense and antisense transcripts, and on mtDNA copy number. (**A**) Relative quantification of mitochondrial RNAs by real-time RT–PCR. MtDNA molecule is represented as a linear map. Transcription direction of (−) and (+)strand is indicated by black arrows; the two DmTTF-binding sites are marked by vertical dotted lines. Black bars reported above (−)strand and below (+)strand, in correspondence of the analyzed genes, indicate the relative content (R) of sense and antisense transcripts, normalized to 28S rRNA (endogenous control), in DmTTF depleted (RNAi) with respect to control cells, fixed as value 1. The relative quantification was performed according to the Pfaffl equation $R = (E_T)^{\Delta Ct,T}/(E_C)^{\Delta Ct,C}$ (24), where E_T is the amplification efficiency of target gene transcripts; E_C is the amplification efficiency of endogenous control; ΔC_t,T and ΔC_t,C are the differences between C_t of the control and C_t of the RNAi sample for target gene transcripts and for endogenous control, respectively. For each amplicon, the mean ratio value was obtained from at least four real-time RT–PCR assays, using RNA obtained from independent RNAi experiments; standard deviations are indicated on the top of the bars. Statistical analysis showed that the differences between RNAi and control are all significant ($P < 0.05$) except for ATPase6/8 and COIII-antisense transcript. (**B**) Southern blotting analysis of mtDNA. Total DNA (5 μg) from untreated (control) and treated (RNAi) D.Mel-2 cells was digested with XhoI, fractionated on a 0.7% agarose gel and electroblotted to nylon membrane. The filter was hybridized with radiolabeled probes for both mitochondrial ND3 gene and nuclear H2B histone gene (endogenous control). The amount of c.p.m. used for each probe was such to obtain a comparable signal for mitochondrial and nuclear DNA.

transcription catalyzed by mtRNApol (14,16,18), however this role has never been demonstrated *in vivo*. We have previously reported that DmTTF, the transcription termination factor of *Drosophila* mitochondria, recognizes two sequences placed at the boundary of clusters of genes transcribed in opposite directions and stops the progression of human mtRNApol in a bidirectional way. In the present work, we provide *in vivo* evidences on the role of DmTTF in transcription by characterizing a DmTTF knock-down phenotype obtained by means of RNAi in *Drosophila* cultured cells.

By a combination of RNase protection and RT–PCR experiments, we found that DmTTF depletion greatly affects the level of mitochondrial transcripts mapping around the cyt b/ND1-binding site. We showed that (−)strand processed transcripts extending beyond the putative termination site and precursor RNA molecules spanning DmTTF-binding site are both remarkably more abundant in knock-down cells. These findings constitute the first *in vivo* indication that DmTTF represents a hindrance to the progression of mtRNApol. A further support to this view was provided by the results of the extensive analysis performed on sense and antisense transcripts by real-time RT–PCR. With a few exceptions, we found in depleted cells an increase of about 2-fold in the level of those transcripts that map on either strand downstream of one or both DmTTF-binding sites. This increase is even more significant if compared to the 50% reduction of the level of the transcripts mapping upstream of both DmTTF target sites on either strand. Therefore, our results show a clear correlation between the removal of the protein and an enhanced read-through transcription downstream of the protein-binding sites, thus providing conclusive evidence of the *in vivo* role of DmTTF as transcription termination factor. RNase protection experiments also indicated that DmTTF-mediated transcription termination is not responsible for the production of the 3′ terminus of those RNA molecules mapping just upstream of the protein-binding site, since depletion of the protein did not affect the size of $tRNA^{Ser(UCN)}$. On this basis, contrary to what suggested earlier (25), it is conceivable that mitochondrial termination factors are not involved in the generation of the 3′ end of RNA molecules. Recently in *Drosophila* it has been characterized RNase Z, an endonucleolytic activity that is responsible for the specific cleavage at the 3′ end of mitochondrial tRNAs (26). A further interesting result derives from real-time RT–PCR experiments. As mentioned above, in

knock-down cells the amount of (+) and (−)strand transcripts mapping upstream of both DmTTF-binding sites was, in most of the cases, reduced of about 50%. A possible explanation for this decrease could be a direct involvement of DmTTF in transcription initiation since the affected transcripts map downstream of the A+T region (27), which should contain at least one initiation site for each strand. Very recently it has been demonstrated the capacity of the human mitochondrial transcription termination factor mTERF to bind mtDNA in the D-loop region, in proximity of the H-strand promoter, and to stimulate rRNAs synthesis via a mtRNApol recycling process (21). A similar mechanism could take place for DmTTF, considering that several sequences highly homologous to DmTTF-binding site are present in the A+T region. Alternatively, the decreased transcription could be due to the reduced availability of mtRNApol molecules for initiation events since in knock-down cells the enzyme could be engaged in aberrant transcription extending beyond the termination sites, or forced to pause by the collision of the oppositely moving transcription apparatus. Finally, we cannot rule out that secondary, indirect effects acting at posttranscriptional level also play a role in determining mitochondrial RNA levels in DmTTF knock-down cells. They may result in an altered stability of RNA molecules, possibly as a consequence of compensative response mechanisms (28). These effects could explain the observed exceptions and fluctuations in the variations of RNA levels.

It has been recently reported that mtDBP, the sea urchin homologue of DmTTF, besides to function as transcription termination factor, can regulate mtDNA replication since it also possesses a contrahelicase activity (22). We found that in *Drosophila* mitochondria the knock-down of DmTTF did not change mtDNA copy number. On this basis we conclude that the observed variations in the mitochondrial transcript level do not depend on changes of mtDNA content. On the other hand a direct involvement of DmTTF in mtDNA replication cannot be completely ruled out since in our experimental system duration and penetrance of RNAi could be not sufficient to reveal differences in mtDNA level given the lower turnover rate of mtDNA as compared to mitochondrial RNAs.

With a few exceptions, *Drosophila* mitochondrial genes are arranged to form four clusters alternatively distributed on the two strands. Despite the extensive knowledge about transcription factors, information on transcription mechanism of *Drosophila* mtDNA is very limited. The data here reported show that both strands are entirely transcribed, since in control cells we detected all the antisense RNAs we investigated. They also allow proposing two possible models of transcription. According to the first (see model I in Figure 5), transcription of each strand should depend on only one promoter located in the A+T region. This model is in agreement with the complete transcription of mtDNA and requires the two DmTTF–DNA complexes to function mostly as attenuators rather than terminators. As shown in Figure 5, DmTTF bound to ND3/ND5 site would act as attenuator in both directions. At cyt b/ND1 site DmTTF would act as attenuator only in the direction of the (+)strand transcription, whereas in the opposite direction, the protein could serve as true terminator since no coding sequences are present downstream of the protein-binding site. The attenuation function of the human termination factor mTERF has been shown to be responsible for the difference of

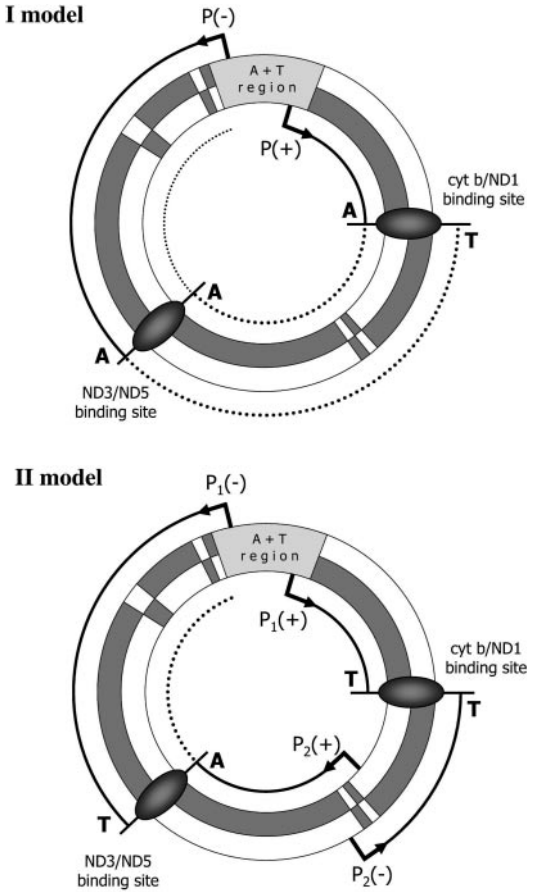

Figure 5. Proposed models of mtDNA transcription mechanism in *Drosophila*. Grey areas show the coding sequences on either strands; arrows indicate directions of (+) and (−)strand transcription initiating from the respective promoters. Dotted lines indicate attenuated transcription. T, termination; A, attenuation. See text for explanation.

the levels of mitochondria rRNAs relative to mRNAs (29). According to the second model, transcription of each strand should require at least two promoters, each located at the 5′ end of a gene cluster (see model II in Figure 5). In this case, DmTTF bound to cyt b/ND1 site would function as true terminator in both directions, since (+)strand transcription reinitiates downstream of that site; at ND3/ND5 site, the factor should function as attenuator only in the direction of (+)strand transcription to permit the synthesis of $tRNA^{Tyr}$, $tRNA^{Cys}$ and $tRNA^{Gln}$. This model is in agreement with the results of mitochondrial RNA mapping experiments early reported by Berthier *et al*. (30). *In vitro* transcription experiments showed that DmTTF, as well as its sea urchin and human homologues, terminates transcription with a biased efficiency depending on the direction of elongating RNA polymerase (14,16,18). This result, which could reflect a structural asymmetry of the protein–DNA complex, is in agreement with the capacity of the protein to function *in vivo* as both attenuator and terminator. The different behaviours of DmTTF might also depend on interactions with other factors. DmTTF belongs to a family of proteins most of which have a still unknown function (19); one or more of these proteins could selectively interact with DmTTF and determine its role as attenuation or termination factor.

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