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Biochimica et Biophysica Acta 1757 (2006) 1199–1206

BIOCHIMICA ET BIOPHYSICA ACTA

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MTERF3, the most conserved member of the mTERF-family, is a modular factor involved in mitochondrial protein synthesis

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Received 28 February 2006; received in revised form 12 April 2006; accepted 20 April 2006
Available online 20 May 2006

Abstract

The MTERF-family is a wide family of proteins identified in Metazoa and plants which includes the known mitochondrial transcription termination factors. With the aim to shed light on the function of MTERF-family members in *Drosophila*, we performed the cloning and characterization of D-MTERF3, a component of the most conserved group of this family. D-MTERF3 is a mitochondrial protein of 323 amino acids. Sequence analysis in seven different organisms showed that the protein contains five conserved "mTERF-motifs", three of which include a leucine zipper-like domain. D-MTERF3 knock-down, obtained by RNAi in D.Mel-2 cells, did not affect mitochondrial replication and transcription. On the contrary, it decreased to a variable extent the rate of labelling of about half of the mitochondrial polypeptides, with ND1 being the most affected by D-MTERF3 depletion. These results indicate that D-MTERF3 is involved in mitochondrial translation. This role, likely based on protein–protein interactions, may be exerted either through a direct interaction with the translation machinery or by bridging the mitochondrial transcription and translation apparatus.

Keywords: mitochondria; mTERF; MTERF3; *Drosophila*; mitochondrial protein synthesis

1. Introduction

Mitochondrial transcription termination factors are monomeric proteins of about 40 kDa able to bind DNA in a sequence-specific manner. Three factors have been well characterized in animal systems: human mTERF [1], sea urchin mtDBP [2] and *Drosophila melanogaster* DmTTF [3]. They all share the capacity to arrest the progression of mitochondrial RNA polymerase in a bidirectional way, as demonstrated by *in vitro* transcription experiments [4–6]. On the other hand, the location of target sites on mtDNA and the specific role of these factors seem to be peculiar for each species. Human mTERF binds DNA at the 3′ end of the ribosomal gene unit and is thought to be responsible for an attenuation/termination event that causes the steady-state level of rRNAs to be higher than that of the downstream mRNAs. The recent discovery of a second binding site in proximity of the H-strand promoter has suggested a mechanism of transcription machinery recycling accounting for the high rate of rDNA transcription [7]. This mechanism could be associated with post-translational modifications of mTERF factor; protein phosphorylation was recently reported for rat mTERF [8]. The sea urchin factor mtDBP recognizes two mtDNA sequences located in the main non-coding region and at the ND5/ND6 gene boundary, respectively. In the light of recent findings, mtDBP seems to be a dual function protein: it terminates the multiple and partially overlapping transcription units described in sea urchin mitochondria and negatively regulates mtDNA replication by acting as a contrahelicase [9]. 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. Similarly to the sea urchin homologue, DmTTF seems to regulate the multiple transcription units

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doi:10.1016/j.bbabio.2006.04.026

existing in *Drosophila* mitochondria. All these factors share an overall amino acid similarity [3] and belong to a wide family of proteins identified in several Metazoa and plants but not so far in fungi [10]. Three mTERF paralogues have been identified in vertebrates; together with mTERF, they define four subfamilies, named MTERF1 through MTERF4, which include most of metazoan MTERF proteins. Sub-families MTERF1, which comprises the well-known human mTERF, and MTERF2 are restricted to vertebrates; sub-families MTERF3 and MTERF4 include members belonging also to insects and worms, thus suggesting that they could represent the ancestral MTERF genes in Metazoa. MTERF3 is the most conserved group of the MTERF-family. Although sea urchin mtDBP and *Drosophila* DmTTF do not belong to any of those sub-families, they seem to be evolutionary more close to MTERF1 and MTERF2 than MTERF3 and MTERF4 sub-families.

With the aim to shed light on MTERF-family components in *Drosophila*, we performed the cloning and the characterization of D-MTERF3. Here we show that D-MTERF3 is a modular protein and that its knock-down decreases the rate of synthesis of some mitochondrial polypeptides.

2. Materials and methods

2.1. Cloning of D-MTERF3 cDNA

D-MTERF3 cDNA was obtained by means of PCR amplification on a *D. melanogaster* cDNA library from 2–14 h embryos. Primers were CG-For (ATGTTTTGTTCAGCTCTACGTAA) and CG-Rev (TTATCTCGTTTT-CAAAAACAAATCA), nt positions 193022–193000 and 191899–191923 of the genomic sequence (AE003591.4), respectively. The amplification product (1065 bp) was cloned into the vector pGEMT (Promega) and sequenced.

2.2. Bioinformatics

Multiple alignments were performed with ClustalW at NPS@ Web server of the PBIL (http://npsa-pbil.ibcp.fr) and were formatted with ESPript 2.2. Coiled-coils were predicted with the program COILS 2.2 [11], using the MTK matrix. Protein architecture analysis and graphical representation of the 'mTERF-motifs'' was carried out using the Web tool SMART in Normal mode (http://smart.embl-heidelberg.de/) and the tool HMM-Logos [12], respectively. Mitochondrial localization of D-MTERF3 was predicted using PSORT II Prediction program and MitoProt II [13].

2.3. 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 units/ml penicillin and 50 μg/ml streptomycin, at 28 °C in 75-cm^2 flasks. For 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.

2.4. 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: D-MTERF3 (accession: DQ414686) forward-primer nt 375–391, reverse-primer nt 947–931; *odds-paired* (accession: NM_079504) forward-primer nt 270–285, reverse-primer nt 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.

2.5. RT-PCR and Real-Time RT-PCR assays

Total cellular RNA was extracted from treated and untreated D.Mel-2 cells by the RNeasy Midi Kit (Qiagen). Semiquantitative RT-PCR assay was performed using the Enhanced Avian HS RT-PCR Kit (Sigma) according to the manufacturer's instructions. Reactions contained 300 ng of *Drosophila* total RNA as template, and 50 pmol of primers specific for D-MTERF3 (CG-For and CG-Rev, described above) or for 28S rRNA (endogenous control, see below) in a final volume of 50 μl.

For Real-Time RT-PCR assay, RNA was reverse-transcribed using the Enhanced AMV Reverse Transcriptase (Sigma), according to the manufacturer's instructions. 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 (accession: NC_001709) were as follows: COI-for nt 1838–1862, COI-rev nt 1932–1904; COII-for nt 3569–3593, COII-rev nt 3652–3631; ATPase6-for nt 4409–4433, ATPase6-rev nt 4481–4459; COIII-for nt 5304–5328, COIII-rev nt 5379–5355; ND3-for nt 5698–5717, ND3-rev nt 5767–5746; ND5-for nt 7040–7061, ND5-rev nt 7114–7095; cyt b-for nt 10697–10719, cyt b-rev nt 10766–10748; ND1-for nt 11984–12002, ND1-rev nt 12056–12034; lrRNA-for nt 12827–12845, lrRNA-rev nt 12906–12887; srRNA-for nt 14407–14434, srRNA-rev nt 14484–14462. Cytoplasmic 28S rRNA (accession: M21017) was used as endogenous control; primer sequence positions were: 28S-for nt 1407–1429, 28S-rev nt 1480–1462.

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 equation: $R=(E_T)^{\Delta Ct,T}/(E_C)^{\Delta Ct,C}$ [14], where E_T is the amplification efficiency of target gene transcripts; E_C is the amplification efficiency of endogenous control; $\Delta Ct,T$ and $\Delta Ct,C$ are the differences between Ct of the control and Ct 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. Statistical analysis was performed using paired two-tailed Student's t test ($P<0.05$).

2.6. Labelling of mitochondrial polypeptides

D.Mel-2 cell growth and RNAi procedure were performed as described above. After 3 days of incubation, about 5×10^6 control and treated cells were harvested at room temperature, washed twice with methionine-free Grace's insect culture medium (GIBCO-Invitrogen) and resuspended at 3×10^6 cells/ml in the same medium containing 200 μg/ml emetine and 100 μg/ml cycloheximide. Redivue™ L-[^{35}S]methionine (specific activity: 1000 Ci/mmol, Amersham Biosciences) was added at a concentration of 300 μCi/ml, then cells were incubated at 28 °C for 90 min. To test the specificity of mitochondrial protein labelling, control experiments were performed in the presence of 100 μg/ml chloramphenicol. After incubation,

two volumes of *Drosophila* SFM were added, then cells were washed with D-PBS. Cells were lysed in Laemmli buffer and total cellular proteins (200 μg/lane) were fractionated on 15–20% exponential gradient polyacrylamide SDS gel. After running, gel was fixed in 25% isopropanol and 10% acetic acid for 30 min and then treated with the Amplify™ Fluorographic Reagent (Amersham Biosciences) for 20 min. After 16 h exposure, the protein synthesis products were visualized by Typhoon 8600 Phosphor Imaging System (Molecular Dynamics) and the quantitative analysis was performed with ImageQuant 5.2 software (Molecular Dynamics). Statistical analysis was performed using paired two-tailed Student's *t*-test ($P<0.05$).

3. Results

3.1. D-MTERF3: a member of the mTERF-family in Drosophila

A bioinformatic approach, consisting of retrieval tools based on protein sequence similarity and domain architecture conservation, revealed the existence in *Drosophila melanogaster* of three proteins that, in addition to the characterized mitochondrial transcription termination factor DmTTF, exhibit similarity with the human factor mTERF. These polypeptides are annotated in FlyBase GadFly Genome Annotation Database as CG7175, CG5047 and CG15390, respectively.

CG7175 (560 amino acids), similarly to DmTTF, does not clearly fall in any of the four sub-families described in Metazoa by Linder et al. [10]. Evident orthologues of this protein were found only in *Drosophila pseudoobscura* and *Anopheles gambiae* so that CG7175 seems unique to insects. Protein CG5047 (354 amino acids) belongs to the MTERF3 sub-family whose members, identified in worms through vertebrates, show the highest degree of sequence conservation. Finally, CG15390 (275 amino acids) is included into the MTERF4 sub-family that also comprises members from worms and vertebrates, but displays a lower degree of conservation.

As a first step in studying the function of the proteins belonging to mTERF-family in *D. melanogaster,* we focused our attention on the most conserved member, CG5047, that from now on will be named D-MTERF3. As reported in FlyBase, D-MTERF3 gene is 1156 bp long; it consists of 2 exons and 1 intron and generates a transcript of 1097 nt. We cloned D-MTERF3 cDNA by means of PCR on a cDNA library of *D. melanogaster*. The sequence of the ORF (accession: DQ414686) differs from that reported in the *Drosophila* database because of 6 nucleotide substitutions which give rise to 5 amino acid changes (V, A, G, S and I instead of L, V, E, P and K, in position 42, 125, 301, 316 and 431, respectively). According to MitoProt II and PSORT II analysis, D-MTERF3 is predicted to localize to mitochondria, with a putative signal sequence of 31 residues according to the R(−2) rule. Therefore the predicted mature D-MTERF3 (323 amino acid long) should have a calculated molecular weight of 37.7 kDa and an isoelectric point of 8.67.

Fig. 1 shows the Clustal W alignment of the complete MTERF3 sequences from *D. melanogaster* and 6 more organisms belonging to mammals, birds, amphibians, fishes and nematodes, respectively. The *Drosophila* protein displays a shorter N-terminal portion, whereas the counterpart in *C. elegans* possesses an insertion of about 20 residues in the C-terminal region. The multiple alignment shows that 42 amino acid positions are conserved in all the organisms, corresponding to 9.5% amino acid identity; the similarity among the 7 proteins is rather uniformly distributed along the molecule with a higher conservation in the C-terminal region. The analysis performed using the SMART tool showed for all these proteins a modular architecture due to the repetition of a motif of about 30 amino acids, named "mTERF-motif". In all the organisms analyzed, MTERF3 contains 5 "mTERF-motifs" (modules 2–6) placed in corresponding position; a less conserved additional module (module 1) was identified only in human and mouse. It is noteworthy that most of the conserved amino acids are placed in the "mTERF-motifs"; this points to a critical role of this element for the structure and function of the protein.

Fig. 2A, B shows the multiple alignment of the "mTERF-motifs" 2–6 and the pattern of sequence conservation obtained by using the HMM-Logos tool. The most evident feature is the very high conservation of a proline residue at position 8. Interestingly, the presence of a leucine or another hydrophobic amino acid at positions 11, 18 and 25 suggests the existence of three repeats of leucine zipper-like heptad X_3LX_3 inside the "mTERF-motifs" (the residues in the heptad being designed a–g in Fig. 2B). In particular, inside the "mTERF-motifs" 2, 3 and 6, the leucine zipper-like heptads are not interrupted by proline residues in all the organisms examined. Therefore, MTERF3 appears to contain at least three leucine zipper-like domains (see Fig. 1). Moreover, according to the analysis with the COILS program, all the proteins are predicted to contain at least two sequences able to form coiled-coil structures in similar positions (not shown). Interestingly, two of these sequences overlap with the leucine zipper-like heptads detected in the "mTERF-motifs" 2 and 3.

3.2. Functional characterization of D-MTERF3

To gain information on the role of D-MTERF3, we produced a knock-down phenotype in D.Mel-2 cultured cells by the RNAi procedure. Cells were treated for 72 h with a dsRNA encompassing 573 nt of D-MTERF3 coding sequence. The depletion of D-MTERF3 mRNA was monitored by RT-PCR assay using two primers designed so as to produce the entire coding sequence, and not to amplify the double-stranded interfering RNA. The level of D-MTERF3 mRNA showed a decrease of about 80% in cells treated with D-MTERF3 dsRNA (Fig. 3); the decrease was specific since no effect was obtained in cells treated with dsRNA containing the sequence of Opa1 gene (mock control).

The molecular effect of D-MTERF3 knock-down on the mitochondrial metabolism was investigated by measuring the content of mtDNA and mitochondrial transcripts, and by analyzing the pattern of mitochondrial protein synthesis. MtDNA copy number was determined as mtDNA/nDNA ratio in control and RNAi cells by means of Southern blotting and Real-Time PCR assays; this analysis revealed that mtDNA content did not vary significantly (data not shown). To

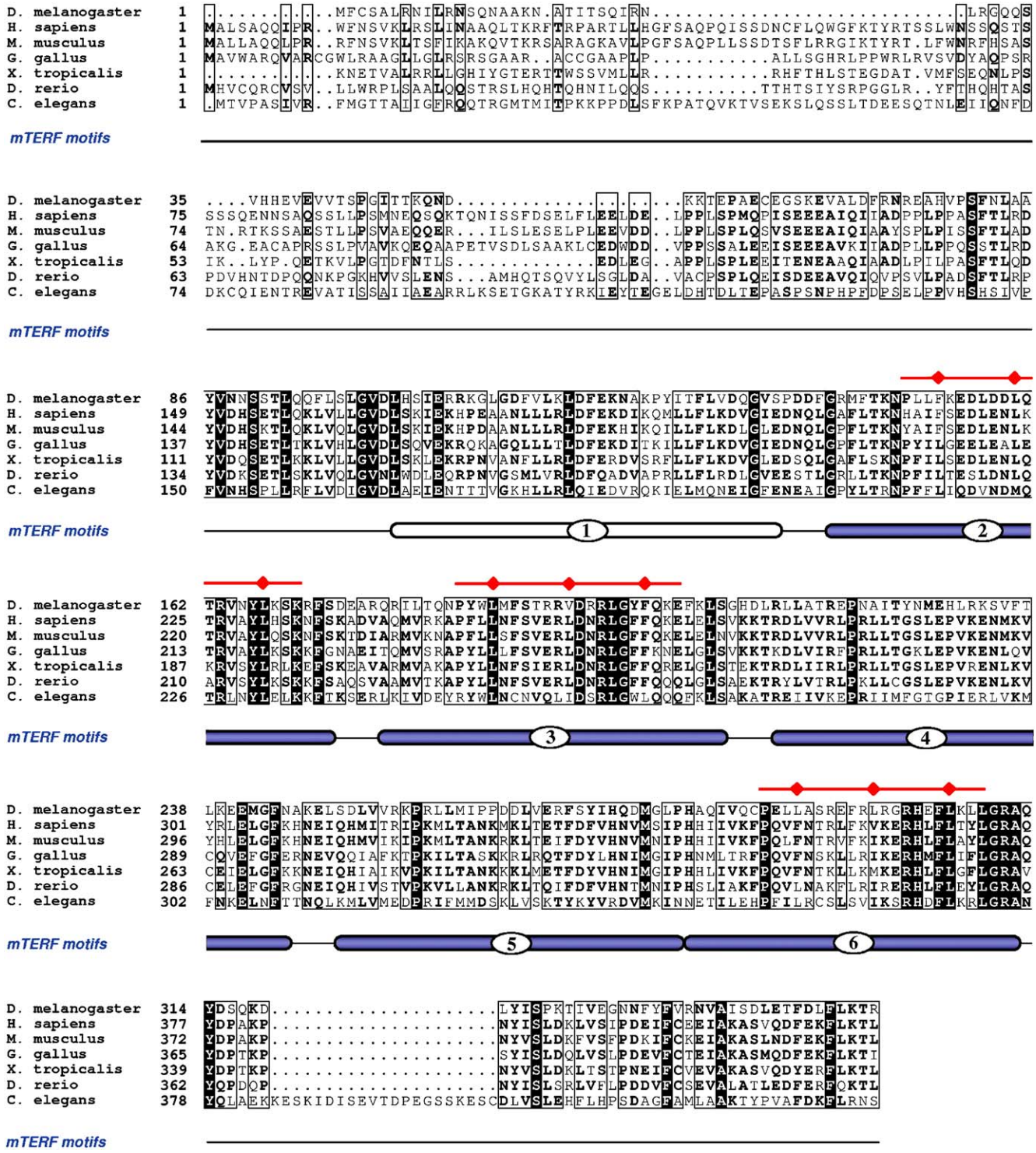

Fig. 1. ClustalW alignment of the complete sequence of *Drosophila melanogaster* D-MTERF3 with its orthologue in *Homo sapiens* (UniprotKB/TrEMBL accession: Q96E29), *Mus musculus* (UniprotKB/TrEMBL accession: Q8R3J4), *Gallus gallus* (Ensembl accession: ENSGALP00000025725), *Xenopus tropicalis* (UniprotKB/TrEMBL accession: Q6P619), *Danio rerio* (UniprotKB/TrEMBL accession: Q6DHJ7) and *Caenorabditis elegans* (UniprotKB/TrEMBL accession: Q18746). The multiple alignment was performed at NPS@ Web server of the PBIL (http://npsa-pbil.ibcp.fr) using the GONNET weight matrix and formatted with the Web tool ESPript 2.2 (http://espript.ibcp.fr). Identical amino acids in all sequences are shaded in black, similar amino acids are bolded. Blue shaded bars below the alignment show the mTERF-motifs predicted in all species; the open bar indicates the mTERF-motif predicted only in *H. sapiens* and *M. musculus*; positions of putative leucine zipper-like heptads are shown in red above the alignment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

investigate the effect of D-MTERF3 knock-down on mitochondrial transcription, we measured the steady-state level of several mitochondrial transcripts by using Real-Time RT-PCR. We first determined the relative steady-state concentration of most of the mitochondrial RNA species in control cells. The data reported in Table 1 show a remarkable

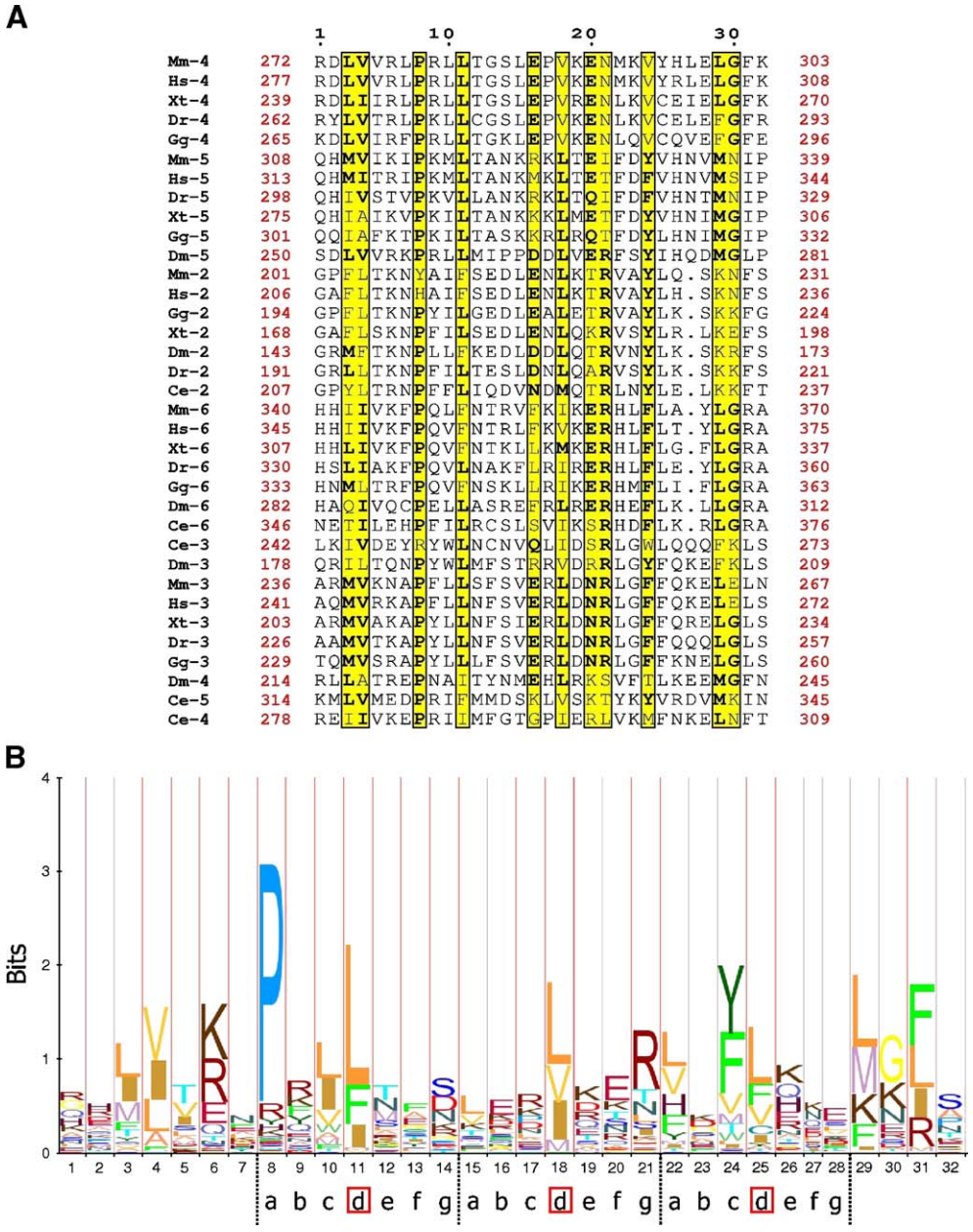

Fig. 2. (A) Multiple alignment of the conserved "mTERF-motifs". Initials of species name together with the "mTERF-motif" number (see Fig. 1) are reported to the left, sequence positions are also indicated. Similar amino acids are bolded, conserved blocks are shaded in yellow. (B) Graphical representation of the aligned "mTERF-motifs" shown in (A) as sequence logo; the information content of the motifs is expressed in bits. The relative size of the letters is a measure of the relative frequencies of the amino acids in the given positions; letters are sorted in descending order depending on their probability. The amino acid position in the putative leucine zippers (a–g) are also indicated.

variation in the concentration of *Drosophila* mitochondrial mRNAs. In particular, COIII is the most abundant mRNA, with its level being only 2-fold lower than that of large and small rRNAs; the less represented mRNAs are COI and ND1, whose level is about 8-fold less than COIII. These data are in agreement with early observations by Berthier et al. [15] based on northern blotting analysis. Then, we determined the relative concentration in knock-down and control cells of 7 sense and 5 antisense RNAs, namely the (−)strand transcripts COI, COII, ATPase8/6, ND5-antisense, cyt b, ND1-antisense, and the (+)strand transcripts lrRNA, ND1, cyt b-antisense, ND5, ATPase8/6-antisense and COI-antisense. The choice of analyzing antisense transcripts was dictated by the observation that the knock-down of the termination factor DmTTF causes in *Drosophila* cells a general alteration of mitochondrial transcription determining decreased and increased levels of sense and antisense RNAs [16]. Here, we found that the level of all the analyzed transcripts did not vary significantly in D-MTERF3 depleted cells with respect to control (not shown). From these data it can be concluded that D-MTERF3 depletion did not alter mtDNA and mitochondrial RNA level.

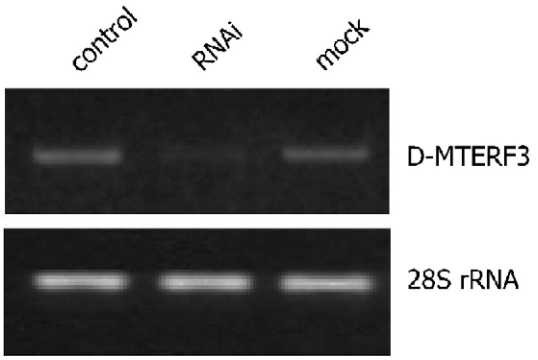

Fig. 3. D-MTERF3-targeted RNAi in D.Mel-2 cells monitored by RT-PCR. Total RNA (300 ng) extracted from control cells, and from cells treated with D-MTERF3 dsRNA (RNAi) or with Opa1 dsRNA (mock) was used as template in RT-PCR reactions. Samples were run on a 1.5% agarose gel and stained with ethidium bromide. Nuclear-encoded 28S rRNA was used as endogenous control.

To examine whether D-MTERF3 knock-down could affect the rate of mitochondrial protein synthesis, we incubated control and RNAi cells with [^{35}S] methionine for 90 min, in the presence of emetine and cycloheximide, two specific inhibitors of cytoplasmic protein synthesis. After protein separation (Fig. 4A), we observed the typical profile of the mitochondrial-encoded translation products; ATPase 8 polypeptide was not detected due to its small size. Protein labelling was specific since it was abolished by the addition of chloramphenicol, that inhibits mitochondrial protein synthesis (not shown). A densitometric analysis of the protein labelling profile in control cells allowed us to determine the relative labelling of each polypeptide. This value was divided by the relative concentration of the respective mRNA so to calculate the translation efficiency of each mRNA (see Table 2). The data obtained showed a wide variation covering almost one order of magnitude between the most efficiently (COI, ND3) and the least efficiently (COII, COIII) translated mRNAs.

When we compared the profile of mitochondrial translation products in knock-down and control cells, we did not observe any qualitative difference. On a quantitative basis, the rate of labelling of about half of the mitochondrial polypeptides exhibited a decline in knock-down cells, which ranged from about 20% for ND4L to about 45% for ND1 (Fig. 4B). Data concerning the relative labelling of each polypeptide and the translation efficiency of each mRNA in D-MTERF3 depleted cells are also reported in Table 2. In conclusion, the obtained results indicate that D-MTERF3 depletion affects the rate of mitochondrial protein synthesis.

Table 1
Level of mitochondrial RNAs in control D.Mel-2 cells

Gene	Relative transcript concentration[a]
COI	2.7
COII	6.5
ATPase6	4.5
COIII	21.0
ND3	4.0
ND5	3.5
cyt b	5.0
ND1	2.8
lrRNA	41.0
srRNA	39.0

[a] Determined by Real-Time RT-PCR as relative to 28S rRNA.

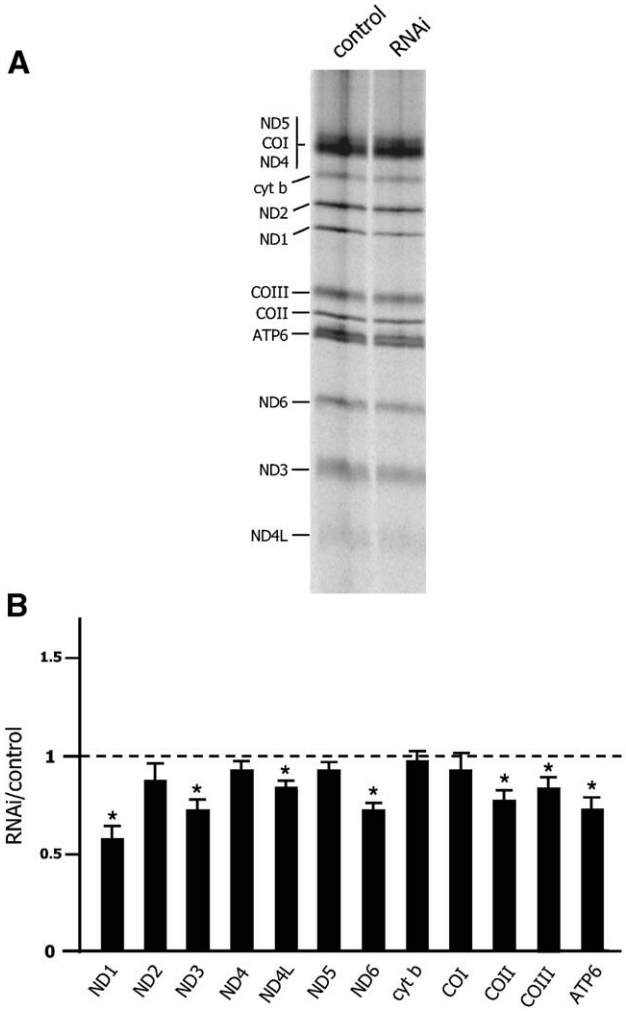

Fig. 4. Effect of D-MTERF3 depletion on mitochondrial protein synthesis. (A) Pulse-labelling of mitochondrial polypeptides was performed by incubating control and RNAi D.Mel-2 cells with L-[^{35}S]-methionine for 90 min in the presence of emetine and cycloheximide. Total cell lysates (200 μg of proteins) were fractionated on 15–20% exponential gradient polyacrylamide gel; following autoradiography, the gel was stained with Coomassie Brilliant Blue to normalize polypeptide band signals. (B) Histogram showing the quantification of polypeptide rate of synthesis in RNAi cells relative to control cells, fixed as 1-value. Data represent the mean ratio of five independent experiments; standard deviations are indicated on the top of the bars. Statistically significant differences ($P<0.05$) are marked with an asterisk.

4. Discussion

Mitochondrial gene expression in animal cells is regulated at different levels. Although in some cases modulation of mtDNA copy number has been described [17], it appears that regulation takes place mainly at transcriptional level. A plenty of information has been accumulated concerning transcriptional

Table 2
Rate of translation of mitochondrial mRNAs in control and RNAi D.Mel-2 cells

Polypeptide	Relative labelling of polypeptide product [a]		Relative efficiency of mRNA translation [b]	
	Control	RNAi	Control	RNAi
COI	2584	2518	4.0	3.9
COII [c]	1342	1095	0.9	0.7
ATPase6 [c]	2585	1959	2.4	1.8
COIII [c]	3990	2886	0.8	0.6
ND3 [c]	3617	2763	3.8	2.9
ND5	840	825	1.0	1.0
cyt b	1189	1220	1.0	1.0
ND1 [c]	1170	696	1.8	1.0

[a] Determined after a 90 min ^{35}S-methionine pulse, expressed as band intensity (see Materials and methods) and normalized with respect to methionine content.
[b] Calculated by dividing the relative labelling of each polypeptide by the relative concentration of the respective mRNA. The obtained results were normalized to cyt b value.
[c] Polypeptides exhibiting a statistically significative variation ($P<0.05$) of mRNA translation efficiency between control and RNAi cells.

regulation: gene expression is controlled at level of transcription initiation *via* the protein factors TFAM and TFB1/2M. TFAM binds specifically to mitochondrial promoters and activates transcription [18,19], moreover, it behaves as a histone-like protein in mtDNA maintenance [20]. While TFB2M seems to act as specific transcription initiation factor, TFB1M is involved in transcription activation and in the modification of rRNA as it possesses an RNA methyltransferase activity [19,21]. It has been recently reported that knock-down of TFB1M in *Drosophila* cells affected translation but not transcription or replication [22]. Transcription is regulated also at termination level by means of protein factors which have been shown to arrest RNA synthesis *in vitro* [1–6]. We have recently found that depletion of DmTTF, the *Drosophila* termination factor, causes a general alteration of the mitochondrial transcript pattern, thus suggesting an involvement of this factor in transcription termination as well as in transcription initiation [16]. Regulation of mtDNA transcription in *Drosophila* seems to occur also at post-transcriptional level. As reported in Table 1, the abundance of mitochondrial transcripts shows a wide variation. In particular, there is an about 7-fold difference between the level of COIII and COI mRNAs that belong to the same transcription unit; this likely depends on the different stability of these transcripts. The value of the mitochondrial rRNA/mRNA ratio in *Drosophila* is the lowest found in animal organisms.

Besides the known regulatory factors, other still unknown proteins could be involved in the regulation of mitochondrial gene expression. The mTERF-family is a protein family that includes the mitochondrial transcription termination factors and several paralogues whose function is still unknown [10]. The multiplicity of these proteins might be related to the complex panorama of regulation of the mitochondrial gene expression. To gain information on the possible regulatory function of those protein factors, we studied MTERF3, the most conserved member of the mTERF-family, using *Drosophila* as a model system. We applied RNAi procedure to obtain a knock-down phenotype for D-MTERF3 and tested whether protein depletion could affect mitochondrial gene expression.

Our results indicate that D-MTERF3 should not be directly involved in mitochondrial replication since mtDNA copy number is not affected by its depletion. Moreover, the careful analysis performed on sense and antisense transcripts scattered along the mtDNA molecule revealed no significant changes in knock-down cells. On this basis, we tend to rule out a direct involvement of D-MTERF3 in transcription. Interestingly, the protein knock-down exerts a depressing effect on mitochondrial translation, decreasing to a variable extent the translation rate of half of the mitochondrial mRNAs. Since D-MTERF3 depletion does not alter the level of ribosomal and messenger RNAs, it is very likely that the decrease in the protein synthesis rate reflects a direct role of the protein in the mitochondrial translation process.

Regulation at translational level has been described in different systems. Protein synthesis efficiency was found to depend on the availability of mRNAs in rat brain synaptosomes [23] and on the translation rate of mitochondrial mRNAs in human [24]. The latter situation seems to occur also in *Drosophila* where we observed a wide variation in mRNA translation efficiency covering almost one order of magnitude. The mechanisms that control translational efficiency in human are not well known. In yeast it has been reported that the translation of individual mRNAs depends on the interaction of activating factors with ribosomal proteins or with the 5′ UTR region of mRNAs [25]. None of these factors has been identified in animal mitochondria, where mRNAs lack a 5′ non-coding region and mitochondrial initiation factors seem to interact with the 5′ coding portion of the mRNAs [26]. Interestingly, ND1 mRNA, whose translation is the most affected by D-MTERF3 depletion, has the longest (10 nt) 5′ non-coding sequence, as predicted by the mtDNA sequence analysis; this non-coding region might be a signal for the binding of translation regulatory factors. In addition to mechanisms regulating the single steps of mitochondrial gene expression, there is growing evidence pointing to a functional coupling between transcription and translation [27]; this should take place through the action of proteins that bridge the transcribing mitochondrial RNA polymerase with the membrane-bound translational apparatus. The effect of D-MTERF3 depletion on mitochondrial translation might be explained hypothesizing that this protein plays a role in these regulatory mechanisms. D-MTERF3 may control mitochondrial translation by means of protein–protein interactions, as supported by the inspection of its primary structure. In fact, sequence analysis of D-MTERF3 and its orthologues reveals the existence of leucine zipper-like domains, including three heptads, inside the “mTERF-motifs”. These domains could be involved either in intra-molecular interactions, needed by the protein to assume the functionally active conformation, or in homologous or heterologous dimerization. Further studies on the role of D-MTERF3 in mitochondrial metabolism are under way and should contribute to shed light on these hypotheses.

Acknowledgments

This work was supported by grants from: M.I.U.R.—COFIN PRIN 2005; M.I.U.R.—Contributo straordinario di ricerca/ Aree obiettivo 1 (to I.B.B.E.); Piani di Potenziamento della Rete Scientifica e Tecnologica, Legge 488/92 Cluster C03; Università di Bari—Progetto di Ricerca di Ateneo.

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