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The *Drosophila* nuclear factor DREF positively regulates the expression of the mitochondrial transcription termination factor DmTTF

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The DREF [DRE (DNA replication-related element)-binding factor], which regulates the transcription of a group of cell proliferation-related genes in *Drosophila*, also controls the expression of three genes involved in mtDNA (mitochondrial DNA) replication and maintenance. In the present study, by *in silico* analysis, we have identified DREs in the promoter region of a gene participating in mtDNA transcription, the DmTTF (*Drosophila* mitochondrial transcription termination factor). Transient transfection assays in *Drosophila* S2 cells, with mutated versions of DmTTF promoter region, showed that DREs control DmTTF transcription; moreover, gel-shift and ChIP (chromatin immunoprecipitation) assays demonstrated that the analysed DRE sites interact with DREF *in vitro* and *in vivo*. Accordingly, DREF knock-down in S2 cells by RNAi (RNA interference) induced a considerable decrease in DmTTF mRNA level. These results clearly demonstrate that DREF positively controls DmTTF expression. On the other hand, mtRNApol (mitochondrial RNA polymerase) lacks DREs in its promoter and is not regulated *in vivo* by DREF. *In situ* RNA hybridization studies showed that DmTTF was transcribed almost ubiquitously throughout all stages of *Drosophila* embryogenesis, whereas mtRNApol was efficiently transcribed from stages 11–12. Territories where transcription occurred mostly were the gut and Malpighi tubes for DmTTF, and the gut, mesoderm, pharyngeal muscle and Malpighi tubes for mtRNApol. The partial overlapping in the temporal and spatial mRNA expression patterns confirms that transcription of the two genes is differentially regulated during embryogenesis and suggests that DmTTF might play multiple roles in the mtDNA transcription process, for which different levels of the protein with respect to mtRNApol are required.

Key words: chromatin immunoprecipitation (ChIP), DNA replication-related element (DRE)-binding factor (DREF), *Drosophila* mitochondrial transcription termination factor (DmTTF), embryogenesis, mitochondrial RNA polymerase (mtRNApol), RNA interference (RNAi).

INTRODUCTION

Mitochondria are semi-autonomous cytoplasmic organelles whose biogenesis requires the co-ordinate expression of nuclear and mitochondrial genomes. The coding capacity of animal mtDNA (mitochondrial DNA) is limited to 13 polypeptides of the OXPHOS (oxidative phosphorylation system), 22 tRNAs and two rRNAs; thus most of the mitochondrial proteins including respiratory complex polypeptides, metabolic enzymes and proteins involved in mtDNA replication and expression are nuclear encoded [1,2]. Many efforts have been made to clarify the mechanisms that regulate the expression of nuclear genes encoding mitochondrial proteins. In mammals, two nuclear transcription factors, namely NRF-1 (nuclear respiratory factor-1) and NRF-2, have been shown to regulate, in co-operation with co-activators of the PGC-1 (peroxisome-proliferator-activated receptor γ co-activator) family, the expression of many nuclear genes encoding proteins involved in mitochondrial biogenesis and function [3]. In *Drosophila*, *erect wing*, the NRF-1 orthologue that regulates myogenesis and neurogenesis, has never been demonstrated to co-ordinate mitochondrial protein gene expression [4,5]. Recently, *in silico* analysis of the *Drosophila melanogaster* genome has led to the identification of a single, ten-nucleotide long sequence, named NRG (nuclear respiratory gene) element, in more than 50 % of genes with mitochondrial function, including the electron transport chain, oxidative metabolism and mitochondrial biogenesis [6].

The DREF [DRE (DNA replication-related element)-binding factor] is a *Drosophila* transcription-regulatory protein consisting of an 80 kDa polypeptide forming a homodimer that specifically binds DRE sequences [7]. They constitute an eight-nucleotide long palindromic consensus sequence that was initially shown to be required for the expression of genes involved in nuclear DNA replication and cell cycle control [8–11]. DREF may either function as a *bona fide* transcription factor, as its binding sites are located up to more than 1 kb upstream of the transcription start point of the controlled genes [12–14], or may be part of the basal transcription machinery involved in recognizing core promoters [15].

Interestingly, it has been demonstrated that DREF also controls the expression of genes, such as the mtSSB (mitochondrial single-stranded DNA-binding) protein, the β-subunit of the mitochondrial DNA polymerase (pol γ-β) and the TFAM (mitochondrial transcription factor A), whose products are required for mtDNA replication and maintenance [16–18], thus providing evidence for a co-ordinated regulation of nuclear and mitochondrial DNA replication. No information concerning the

Abbreviations used: ANT, adenine nucleotide translocase; ChIP, chromatin immunoprecipitation; DmTTF, *Drosophila* mitochondrial transcription termination factor; DRE, DNA replication-related element; DREF, DRE-binding factor; dsRNA, double-stranded RNA; hDREF, human DREF; mTERF, mitochondrial termination factor; mtDNA, mitochondrial DNA; mtRNApol, mitochondrial RNA polymerase; NRF, nuclear respiratory factor; RNAi, RNA interference; RT–PCR, reverse transcription–PCR; UTR, untranslated region.

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control by the DRE/DREF system on proteins that are specifically involved in mtDNA transcription are available yet; therefore we wished to investigate the possible dependence on DREF of the genes for the single-subunit mtRNApol (mitochondrial RNA polymerase) and for the accessory factor DmTTF (*Drosophila* mitochondrial transcription termination factor). DmTTF is a nuclear encoded 43 kDa protein member of the mTERF (mitochondrial termination factor) protein family [19,20]. It acts as a transcription termination factor in *Drosophila* mitochondria by arresting the progression of mtRNApol at its two DNA-binding sites, both of which are located at the 3′-end of clusters of genes transcribed in a convergent direction [21,22]. Moreover, it appears that DmTTF has a broad effect on mitochondrial transcription, since its depletion alters the level of those mitochondrial RNAs that map upstream of its binding sites, thus pointing towards a possible function also in transcription initiation [22].

In the present paper, by a combination of *in vitro* and *in vivo* experiments, we demonstrate that DREF binds two DRE sequences located approx. 50 nt upstream of the DmTTF transcription initiation site and that it positively regulates the expression of DmTTF. On the other hand, no control by the DRE/DREF system was observed for the mtRNApol gene. In addition, by analysing the temporal and spatial accumulation of both DmTTF and mtRNApol mRNAs during *Drosophila* embryogenesis, we found that the two mRNAs show common patterns of significant expression in the gut and Malpighi tubes and distinct profiles in other regions.

MATERIALS AND METHODS

Promoter constructs

Construction of p2000DDD and mutant derivatives

A 2076 bp DNA fragment upstream of the *D. melanogaster* DmTTF coding region (from −1964 to +112, considering +1 as the transcription start point and +113 being the first nucleotide of the start codon) was obtained by PCR using total DNA from the *D. melanogaster* derivative S2 cell line as a template, and the forward primer dir(1339) (5′-CC*GGATCC*CTTCAATTG-TCACTTGTCATT-3′) and the reverse primer rev(818) (5′-GG*CTCGAG*CCCGAATTTGCTTAATTTACA-3′). Primers include the recognition sequence (in italic) for BamHI and XhoI respectively. The PCR product was digested with these enzymes and cloned into the corresponding sites of pXp2 vector, upstream of the luciferase reporter gene. The resulting construct (p2000DDD) was used to generate a series of deletion and mutant clones.

Mutagenesis of DRE at position −42 (5′-TAACGATA-3′) on p2000DDD was achieved by three PCR steps and subcloning of the amplified fragment. In the first PCR, p2000DDD (template) and primers dir(1339) and rev(820) (5′-TCAAACAATTActaTTA TCGGTTTTGAAAATAATGA-3′) were used. In the second PCR, the same template and primers dir(1839) (5′-TCATTATTTCAA-AACCGATAAtagTAATTGTTTGA-3′) and rev(818) were used. Primers rev(820) and dir(1839) are complementary and contain the DRE at −42 (underlined) mutated (changes are in lower-case). A third PCR was carried out using as a template an equimolar mixture of the two obtained products and primers dir(1339) and rev(818); it yielded a 2076 bp fragment that is identical with the original contained in p2000DDD except for the mutated DRE at position −42. The final PCR product was cloned into BamHI and XhoI sites of pXp2 to generate p2000DDd. Mutagenesis of DRE at position −48 (5′-AACCGATA-3′) was carried out as for DRE at −42, using the mutation containing primers rev(821) (5′-TCAAACAATTATCGTTActaGTTTTGAAAATAATGA-3′) for the first PCR and dir(1840) (5′-TCATTATTTTCAAAACtag-TAACGATA-3′) for the second. Primer sequences show DRE at −48 underlined and mutation in lower-case. The product was finally cloned to generate p2000DdD.

The double mutant DRE (−42/−48)-containing construct was obtained in the same manner, using primers rev(822) (5′-TCAAACAAT*TActaTT*ActaGTTTTGAAAATAATGA-3′) for the first PCR and dir(1841) (5′-TCATTATTTTCAA AACtag-*TAAtagTA*ATTGTTTGA-3′) for the second. Primer sequences show DRE at −42 in italic and that at −48 underlined and mutations on DREs in lower-case. The product was finally cloned in pXp2 to generate p2000Ddd.

Construction of p1300DDD, p400DDD and mutant derivatives

Construct p1300DDD was made by PCR using p2000DDD as a template and primers dir(1299) (5′-CC*AAGCTT*AACTAG-TTAACGCAATTAAGCAGC-3′; HindIII site in italic) and rev(818). The PCR product was digested with HindIII and XhoI and cloned into the corresponding sites of pXp2 vector.

Construct p400DDD was made in the same manner, using as forward primer dir(1298) (5′-CC*AAGCTT*TGTTC-AAAATCCTACAAGTC-3′; HindIII site in italic). Mutation of proximal DREs (−42/−48) on p400DDD was carried out using p2000Ddd as a template, and primers dir (1299) and rev(818) yielding p400Ddd. To construct p400dDD (containing only the mutated form of DRE at −361) a PCR reaction was carried out using p2000DDD as a template and primers dir(2875) (5′-CC*AAGCTT*TGTTCAAAATCCTACAAGTCTTTGTTTAGtag-TAATGTTCCG-3′; HindIII site in italic; DRE site underlined; mutation in DRE in lower-case) and rev(818). Finally, p400ddd (containing mutated DRE at −361, −48 and −42) was made by PCR using p2000Ddd as a template and primers dir(2875) and rev(818). The product was digested with HindIII and XhoI and cloned into the corresponding sites of pXp2 vector. All constructs were confirmed by sequencing.

Transient transfection assay

Transient transfection assays in *Drosophila* S2 cells were performed as described in [17] using 5 μg of the luciferase gene containing plasmids. To measure luciferase activity, cells were harvested by centrifugation and washed with PBS. Cell lysis and luciferase measurements were carried out using the Luciferase Assay System (Promega) according to the manufacturer's recommendations. Promoter activities were calculated by normalizing luciferase activities by the β-galactosidase activity from plasmid pAc5.1/V5-His/LacZ (Invitrogen), 1 μg of which was co-transfected. β-Galactosidase activity was measured using the Beta-Glo Assay System (Promega) according to the manufacturer's instructions.

Gel mobility-shift assay

For the mobility-shift assay, nuclear protein extract from S2 cells (prepared as reported in [17]) was incubated with a double-stranded 36-mer oligonucleotide (from positions −61 to −26). The probe was 5′-end-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase and then purified using G50 NICK™ columns (Amersham Biosciences). Binding reactions were carried out by combining 10 μg of nuclear protein extract, 50000 c.p.m. (corresponding to 30 fmol) of DNA probe and 1 μg of poly(dI-dC)·poly(dI-dC) (Amersham Biosciences) in binding buffer [20 % (v/v) glycerol, 1 mM dithiothreitol, 20 mM Hepes, pH 9.0, 5 mM $MgCl_2$, 0.2 mM EDTA and 200 mM KCl]. After

incubation for 30 min at 4 °C, the reaction products were electrophoresed on a 6 % polyacrylamide gel in 0.5 × TBE (Tris/borate/EDTA; 1 × TBE = 45 mM Tris/borate and 1 mM EDTA). A 200-fold molar excess of non-radioactive wild-type or DRE mutated oligonucleotides was used as a homologous competitor. DRE mutations in unlabelled competitors corresponded to the same nucleotides changed in constructs for luciferase assays. In order to produce a super-shifted band, specific anti-DREF serum (from Laurie Kaguni, Department of Biochemistry, Michigan State University, East Lansing, MI, U.S.A.) was added (1:200 dilution in PBS) after 15 min of binding reaction. Pre-immune serum was used at the same dilution as a negative control.

ChIP (chromatin immunoprecipitation) assays

ChIP assays were carried out with DNA obtained from 0.45 g of *D. melanogaster* overnight embryos as previously described [23], with some minor modifications. Homogenized embryos were sonicated five times (10 s continuous pulses at 7 amplitude microns power) in an MSE Soniprep 150 sonifier with a micro-tip probe at 4 °C, with 30 s cooling on ice between pulses, yielding DNA fragments mostly between 200 and 700 bp. Immunoprecipitation was made on chromatin extract without filtering through a centricon column.

For immunoprecipitation, polyclonal anti-DREF sera from rabbit and anti-Eyegone from guinea-pig and their respective pre-immune sera were used. Chromatin extracts were immunoprecipitated twice, initially with the corresponding pre-immune serum for 6 h (IgG control to remove non-specific interactions) and then overnight at 4 °C with polyclonal antiserum.

Immunoprecipitated DNA was used for PCR amplification of proximal DRE sites using the following primers: Fw-42 (5′-TTGTGTATGAACCAGCACAC-3′) and Rev-42 (5′-AAACAG-CTGATATGTTGCGG-3′) as forward and reverse primers respectively for amplifying a 225 bp fragment containing the −42/−48 DRE site; Fw-361 (5′-ATGTGGAGTAGTGCTTGT-GA-3′) and Rev-361 (5′-CATTCTTAGACAGAGAGTCC-3′) as those for amplifying a 175 bp fragment including the −361 DRE site. The PCR products (after 30 cycles of 94 °C/30 s; 58 °C/30 s; 72 °C/30 s) were resolved by gel electrophoresis and visualized by ethidium bromide staining.

RNAi (RNA interference) and Western blotting assay

Templates for the production of dsRNAs (double-stranded RNAs) were PCR-derived fragments carrying at both ends the T7 promoter sequence. Each primer contained a 5′-end sequence corresponding to the T7 polymerase promoter (5′-TAATACGACTCACTATAGGGA-3′), followed by a gene-specific sequence as indicated: DREF (accession number NM_078805) forward-primer nt 720–736 and reverse-primer nt 1321–1306; LacZ (pUC18 vector accession number L09136) forward-primer nt 2512–2528 and reverse-primer nt 362–348. The PCR products were phenol-extracted, ethanol-precipitated and used as templates for the MEGAscript® RNAi kit (Ambion) to produce dsRNAs according to the manufacturer's instructions.

S2 cell were maintained in Schneider's *Drosophila* medium (Gibco–Invitrogen) supplemented with 10 % (v/v) foetal bovine serum (Gibco–Invitrogen), 50 units/ml penicillin and 50 mg/ml streptomycin, at 25 °C. For dsRNA treatment, cells were diluted to a final concentration of 1.0×10^6 cells/ml in 10 ml of Schneider's *Drosophila* medium (not supplemented) in a 75-cm^2 flask. dsRNAs (15 μg per 10^6 cells) were added directly to the medium and cells were incubated at 25 °C for 1 h. Then, 10 ml of complete medium was added to obtain a cell density of 0.5×10^6 cells/ml followed by an additional incubation at 25 °C for 72 h.

dsRNA-treated and -untreated S2 cells were then harvested, centrifuged at 1000 ***g*** for 4 min at 4 °C and washed twice with PBS. Pellets were resuspended in 1 × Laemmli buffer and proteins were fractionated on SDS/12 % (w/v) polyacrylamide gel and electroblotted on to a PVDF membrane (Immobilon-P; Millipore). Membranes were incubated with affinity-purified anti-DREF antibodies (from Masamitsu Yamaguchi, Kyoto Institute of Technology, Kyoto, Japan) and anti-actin antibodies (Sigma), followed by an incubation with anti-rabbit IgG (Santa Cruz Biotechnology). Protein bands were visualized using the ECL® Plus Western Blotting Detection System (GE Healthcare).

Real-time RT–PCR (reverse transcription–PCR) assay

Total cellular RNA was extracted from dsRNA-treated and -untreated S2 cells by using the RNeasy Midi kit (Qiagen); 500 ng of it was reverse-transcribed in a final volume of 25 μl by using the Enhanced AMV Reverse Transcriptase kit (Sigma), according to the manufacturer's instructions. Real-Time PCR was performed using the Power SYBR® Green PCR Master Mix (Applied Biosystems) and the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Primers were designed using Primer Express 2.0 software (Applied Biosystems) and the resulting sequence positions were as follows: nt 583–602 (forward), nt 683–663 (reverse) for DmTTF (accession number AY196479); nt 2352–2371 (forward) and nt 2452–2434 (reverse) for mtRNA pol (accession number NM_134721); nt 1407–1429 (forward) and nt 1480–1462 (reverse) for cytoplasmic 28S rRNA (accession number M21017), which was used as an endogenous control.

Each reaction was run in triplicate and contained 1 μl of reverse transcription reaction (1 μl of a 1:200 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 using the Dissociation Curves software (Applied Biosystems). All PCR products were run on a 2.5 % agarose gel to confirm specificity. Analysis of the results was performed as described previously [22].

In situ hybridization

In situ analysis of mtRNApol and DmTTF expression on *Drosophila* embryos was performed by incubating previously treated embryos with antisense riboprobes, using the corresponding sense riboprobes as negative controls.

To prepare the mtRNApol riboprobe, a fragment of its cDNA (positions 2067–4277, according to FlyBase annotation symbol CG4644) was previously PCR-amplified and cloned into the EcoRV site of pGEMT-easy vector (Promega). In the same manner, to prepare the DmTTF riboprobe, a fragment of its cDNA (positions 113–1346 according to FlyBase annotation symbol CG18124) was cloned into the EcoRV site of pBluescript-KS$^+$ vector.

Preparation of embryos, riboprobes and *in situ* experiments were carried out as described in [24] with minor modifications: in point 2.3.3. 'Hybridization, developing and visualization', subpoint a., PBT (phosphate buffer/0.1 % Tween 20) solution was used, and in subpoint g., developing solution containing 100 mM NaCl was used.

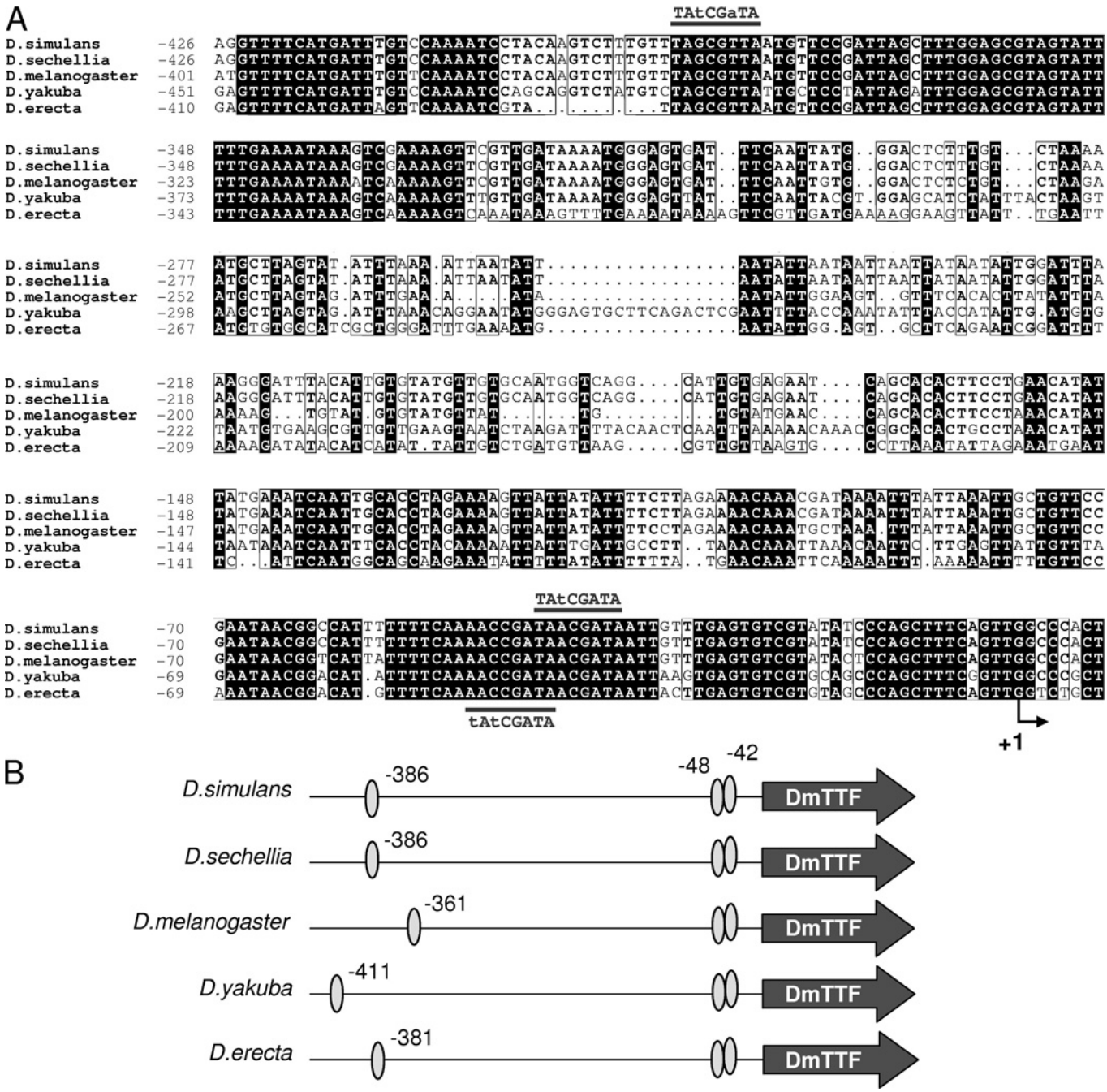

Figure 1 Multialignment of DmTTF promoters from different *Drosophila* species and location of DRE sequences

(**A**) Alignment of DmTTF proximal promoter regions from the *Drosophila* species of the *melanogaster* subgroup. Nucleotide sequences were obtained from FlyBase. The multiple alignment was performed at NPS@ web server of the PBIL (http://npsa-pbil.ibcp.fr) using the IUB (International Union of Biochemistry) weight matrix and formatted with the ESPript 2.2 web tool (http://espript.ibcp.fr). The arrow indicates the transcription start point according to *D. melanogaster* +1 site described in FlyBase. Nucleotides conserved in all sequences are shaded in black, and nucleotides conserved in four out of five sequences are boxed. DRE sequences within the promoters are indicated by solid bars; DRE consensus sequence is also shown with the conserved and the non-conserved nucleotides indicated in upper- and lower-case respectively. (**B**) Schematic representation of the location of DRE sites in DmTTF promoter-proximal regions from the different *Drosophila* species. DRE sequences are represented by ellipses. Positions are relative to the *D. melanogaster* transcription start point.

RESULTS

DRE sequences are present in the DmTTF promoter and are conserved in different *Drosophila* species

With the aim of characterizing the involvement of DREF in controlling transcription of DmTTF and mtRNApol genes in *D. melanogaster*, we searched for the DRE consensus sequence TATCGATA in the 2 kb DNA region upstream and around the DmTTF and mtRNApol transcription initiation sites that, according to FlyBase (http://www.flybase.org/), are located 112 and 168 nt upstream of the translation start codon respectively. In the case of the mtRNApol promoter, no DREF-binding motifs

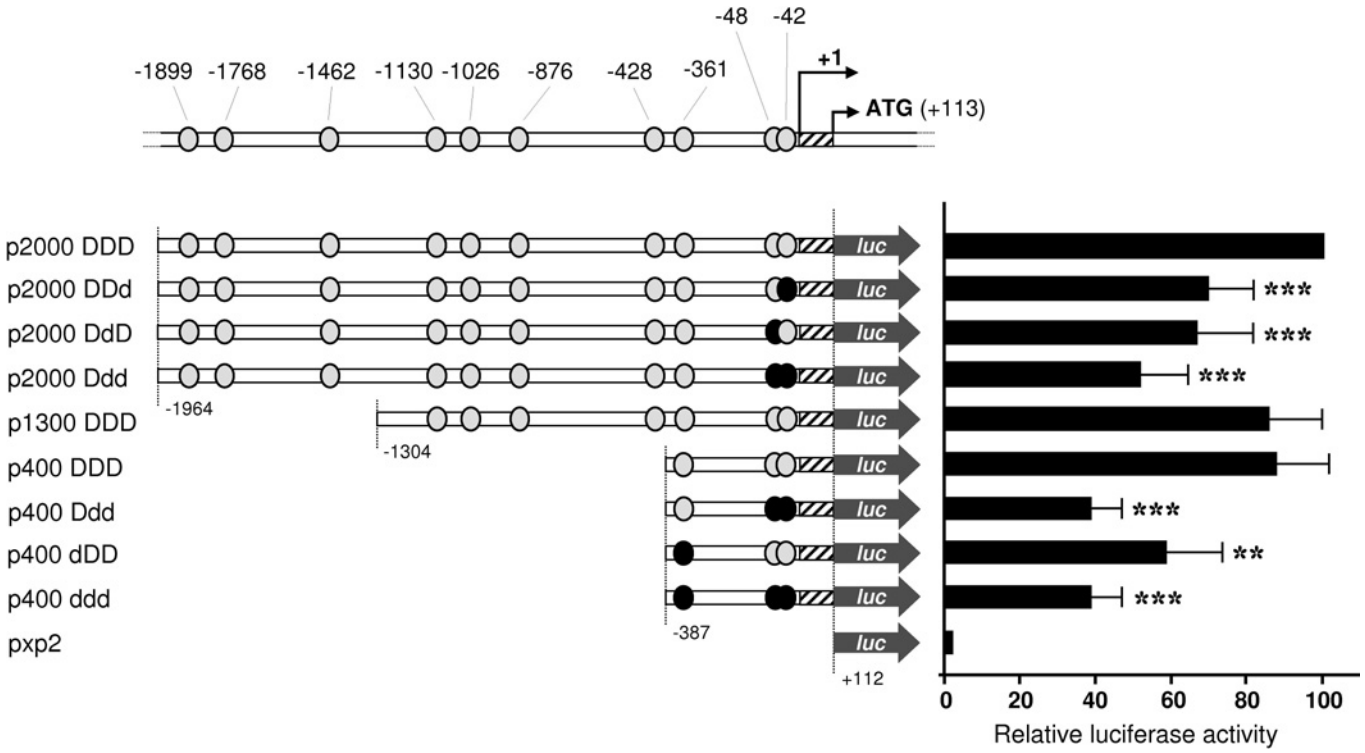

Figure 2 Functional analysis of the DmTTF promoter region

A schematic representation of the DmTTF promoter region containing the putative DRE sites is shown (top panel). The hatched box represents the 5′-UTR. Transcription and translation initiation sites are indicated by thin arrows. DRE sequences are represented as grey ellipses and numbers indicate their positions with respect to the transcription initiation site. Constructs used for the analysis of promoter activity are shown (bottom panel); the position of the ends of the fragments cloned upstream of the luciferase (luc) reporter gene (dark grey arrow) is indicated; a black ellipse indicates that the DRE sequence is mutated. The relative promoter activities measured in the luciferase assay are represented by the corresponding black horizontal bars. Luciferase activity of the 2 kb promoter region was defined as 100 %. Luciferase activity was normalized to β-galactosidase activity for each transfection. Results are means $\pm$ S.D. ($n \geqslant 6$). Statistical analysis was carried out using one-way ANOVA and the Bonferroni post-test (***$P < 0.001$ and **$P < 0.01$).

were identified. On the contrary, the analysis of the DmTTF promoter allowed the identification of ten putative DREs scattered between positions -42 and -1899, according to the transcription start point, which retain at least 6 out of the 8 nt of the DRE consensus sequence (see Figure 2). Three of these elements are located within the proximal 400 bp region, specifically at -42, -48 and -361, and match 7, 6 and 6 nt out of the 8 forming DRE consensus respectively (see Figure 1A). The element in position -42 overlaps by 2 nt with that in position -48. Comparison of the promoter proximal sequence in five *Drosophila* species (*D. simulans*, *D. sechellia*, *D. melanogaster*, *D. yakuba* and *D. erecta*) showed 100 % conservation in the sequence of all the three DREs (Figure 1A) as well as in the relative positions of the two most proximal, with the position of the third DRE sequence being very similar (Figure 1B).

Role of DRE sites in DmTTF promoter activity

Transient transfection experiments in S2 cells were carried out in order to analyse the role of DRE sequences in DmTTF promoter function. For this purpose, different deleted and/or point mutated versions of DmTTF upstream sequences were linked to a luciferase reporter gene in the pXp2 vector. As shown in Figure 2, the luciferase activity promoted by a fragment of 2076 bp (p2000DDD), spanning positions -1964 to $+112$ according to the transcription start point, was fixed at 100 %.

Deletion mutant analysis showed that the removal of up to 1577 bp from the distal end of the promoter sequence did not significantly alter transcription efficiency, since p1300DDD and p400DDD mutants show approximately the same luciferase activity as p2000DDD. The 400 bp promoter proximal region, which retains almost 90 % of the total promoter activity, contains the three conserved DRE sequences located at positions -42, -48 and -361.

Different combinations of these proximal DRE sites containing changes in the tri-nucleotide core of the consensus (positions 4–6) were generated in p2000DDD and p400DDD and used in transient transfection assays. Comparison of luciferase activity obtained with the wild-type and mutated versions of p2000 (Figure 2) indicates that mutation of DRE at either the -42 (p2000DDd) or the -48 (p2000DdD) site causes a significant reduction in the promoter activity (approx. 30 %); the reduction reaches approx. 50 % when both sites are mutated (p2000Ddd). The relevance of the proximal double $-42/-48$ DRE sequences is confirmed by the double mutation in p400Ddd, which causes an even higher reduction, approx. 60 %, of the reporter gene expression than that in p2000Ddd. Finally, mutation of the distal -361 DRE sequence (p400dDD) causes 40 % reduction in promoter efficiency, showing that this third DRE site also participates in DmTTF transcription in S2 cells.

In conclusion, this analysis indicates that (i) DREs are involved in DmTTF transcription, (ii) there are three functional DREs in

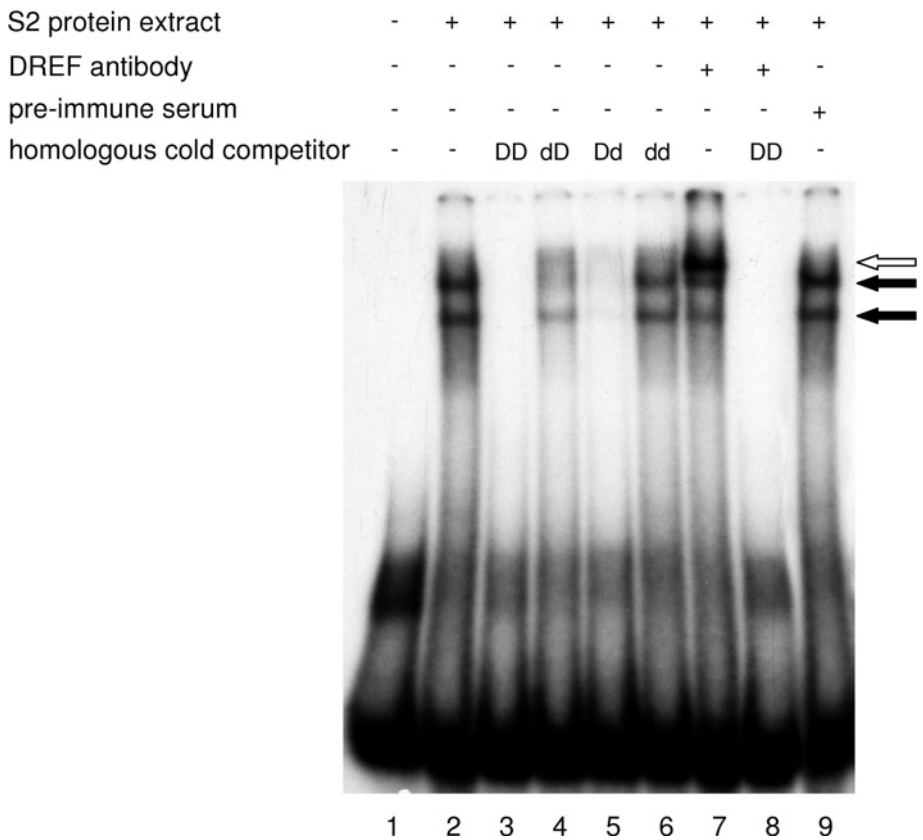

Figure 3 Binding of DREF to the DRE sites in the DmTTF promoter region

DREF binding was evaluated by gel-shift analysis using a nuclear extract (10 μg of protein) from S2 cells and a radiolabelled 36-mer double-stranded oligonucleotide (positions −61 to −26, relative to the DmTTF transcription initiation site) containing the −42 and −48 DRE sites. Homologous cold competitors (where cold indicates unlabelled), added to the assay in a 200-fold molar excess, were different versions of the same 36-mer oligonucleotide used as a probe. With respect to the wild-type (DD), the versions mutated in the −48 site (dD), in the −42 site (Dd) or in both sites (dd) contain the same three nucleotide changes used for the luciferase assay constructs. The black arrows indicate the protein–DNA complexes, the white arrow indicates the super-shifted band produced by adding anti-DREF serum to the binding reaction.

the DmTTF promoter and (iii) mutations on proximal DRE sites abolish 50–60 % of the promoter activity.

Binding of DREF to DmTTF promoter

To demonstrate that DRE sequences in the DmTTF promoter are recognized by DREF, gel mobility-shift and ChIP assays were carried out.

Gel mobility-shift experiments were performed using S2 cell nuclear extracts and a ^{32}P-labelled 36-mer double-stranded oligonucleotide containing the −42 and −48 DRE sites. The electrophoretic analysis (Figure 3) shows the formation of two protein–DNA complexes (lane 2) that were completely abolished by an excess of homologous unlabelled competitor (lane 3). Moreover, we used as unlabelled competitor oligonucleotides containing a mutated version of each or both DRE sites. As shown in Figure 3, the oligonucleotide containing mutation at the −42 site still keeps a high competition capacity (lane 5), whereas the oligonucleotide mutated at the −48 site shows a reduced capacity to compete (lane 4). As expected, the double mutant oligonucleotide is almost completely unable to compete (lane 6). These results suggest that the protein contacting the DNA probe is DREF; moreover, they indicate that the −48 element has a higher affinity for the protein than that at −42. To produce clear evidence of the involvement of DREF in binding to the DmTTF promoter, we performed the binding reaction in the presence of anti-DREF serum: as shown in Figure 3 (lanes 7–9), we observed a clear super-shift of the retarded bands; super-shift was observed neither in the presence of an excess of homologous unlabelled competitor nor in the sample containing the pre-immune serum. The results obtained clearly demonstrate that the two DNA–protein complexes contain DREF.

To analyse the *in vivo* binding of DREF to the DmTTF promoter, we performed ChIP experiments on *D. melanogaster* whole embryos. Chromatin was immunoprecipitated with anti-DREF antibodies and the bound DNA was identified by PCR amplification with primers specific for either the −42/−48 or −361 DREs (Figure 4). As a negative control, PCR amplifications were also made on chromatin immunoprecipitated with anti-Eyegone serum [25]. As shown in Figure 4, PCR amplified a clear, single and intense band for −42/−48 and −361 DREs when the template was the chromatin immunoprecipitated by anti-DREF, but no amplification, or a very faint band for −42/−48 region, was observed when the template was the chromatin immunoprecipitated by pre-immune and anti-Eyegone serum. These results demonstrated that DREF binds *in vivo* to proximal DREs on the DmTTF promoter region. ChIP experiments on SL2 cells also produced similar results to those of ChIP experiments on embryos (results not shown).

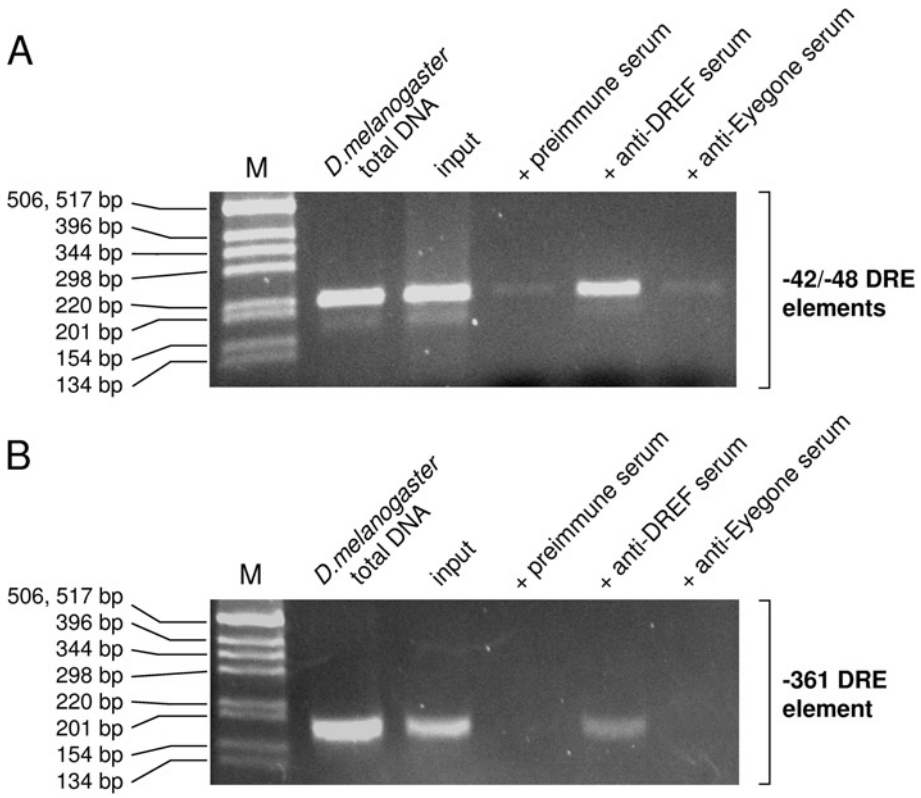

Figure 4 DREF binds to proximal DRE sites in the DmTTF promoter *in vivo*

Overnight *D. melanogaster* embryos were fixed to cross-link proteins to DNA. Embryos were homogenized, cells were lysed and their DNA fragmented by sonication. Cell lysates were immunoprecipitated with control IgG (pre-immune serum) or polyclonal antiserum raised against DREF or Eyegone. Immunoprecipitated DNAs were amplified by PCR with specific primers for −42/−48 DRE sites (**A**) and for −361 DRE site (**B**). DNA templates, immunoprecipitated in different conditions, are indicated above each lane. Input lanes show the PCR product derived from chromatin prior to immunoprecipitation. M corresponds to 1 kb DNA ladder (Invitrogen) and the sizes of the fragments are indicated on the left-hand side (in bp). The experiment was repeated twice, yielding similar results.

Effect of DREF knock-down on DmTTF and mtRNApol expression in S2 cultured cells

We finally investigated the effect of DREF knock-down on DmTTF endogenous gene transcription in *Drosophila* cultured cells. Moreover, since DREF is known to be involved in a complex network of regulatory pathways [11], and it could not be excluded that mtRNApol expression could also be indirectly regulated by the DRE/DREF system, we also tested the effect of DREF depletion on mtRNApol mRNA. To this purpose, we used the RNAi procedure to obtain DREF depletion in S2 cells. After treating cells for 72 h with a dsRNA encompassing 601 nt of DREF coding sequence, the level of DREF polypeptide was monitored by Western blotting assay. As reported in Figure 5(A), a decrease of more than 90 % in DREF level was obtained; the effect of DREF RNAi was specific as no effect was observed in cells treated with dsRNA containing the sequence of the LacZ gene (mock control).

Then, we measured the steady-state level of DmTTF and mtRNApol mRNAs using real-time RT–PCR. As shown in Figure 5(B), results obtained from five independent RNAi experiments indicate that in DREF-depleted S2 cells the level of DmTTF mRNA is approx. 55 % of that in non-depleted cells. On the other hand, no effect of DREF depletion was observed on the mtRNApol mRNA level. The decrease in DmTTF mRNA is consistent with the results obtained from the transient transfection assay, and confirms that DREF is a transcription factor controlling DmTTF gene expression.

Spatial and temporal expression of DmTTF and mtRNApol genes during *D. melanogaster* development

To study the spatial and temporal expression of DmTTF and mtRNApol during embryogenesis, we used whole-mount *in situ* RNA hybridization. Figure 6(A) shows that DmTTF mRNA has a significant maternal deposit; until stage 12, the signal appears to be quite uniform except for ectoderm and polar cells, where there is no expression (only stage 6 is shown; the polar cells results are not shown). At stage 12, DmTTF mRNA level is increased in midgut (digestive tube) over a general background. From stage 13, the gut and Malpighi tubes show a higher content of DmTTF transcript than the rest of the embryo. Figure 6(B) shows that mtRNApol mRNA also has a significant maternal deposit that disappears before stage 4. Zigotic transcription starts approximately at stage 11, when the hybridization signal appears exclusively in gut primordia. Approximately from stage 12, a strong signal is present in the gut, pharyngeal muscles and mesoderm and seems to be absent or undetectable in the rest of the embryo. Finally, from stage 13, the mtRNApol transcript is relatively highly expressed in the gut, pharyngeal muscles, Malpighi tubes and somatic mesoderm.

DISCUSSION

DREF is a *Drosophila* key transcription factor that regulates the expression of a set of genes involved in nuclear DNA replication

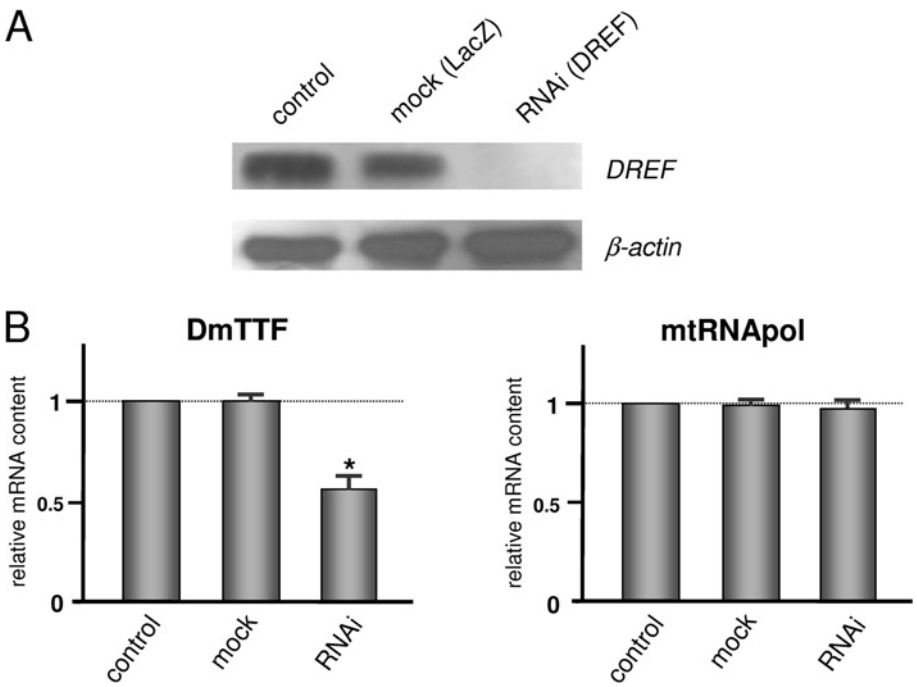

Figure 5 Effect of DREF knock-down on DmTTF and mtRNApol mRNA levels

(**A**) DREF protein was knocked down in S2 cells by means of RNAi. Cells, either untreated (control) or treated with LacZ (mock) or DREF dsRNA, were harvested and proteins were subjected to SDS/PAGE and Western-blot analysis with polyclonal antibodies against DREF and β-actin. (**B**) Total RNA was extracted from treated and untreated cells and relative quantification of mRNAs was carried out by real-time RT–PCR. Bars indicate the relative content of transcripts, normalized to 28S rRNA (endogenous control), in treated with respect to control cells fixed at the value 1. The relative quantification was performed according to the Pfaffl equation [26]. Values are expressed as a ratio and results are means $\pm$ S.D. ($n = 5$). Statistical analysis was performed using paired two-tailed Student's t test (*$P < 0.05$).

and cell cycle control [8–11]. Moreover, transcription regulation by DREF was demonstrated for the expression of protein factors directly involved in mtDNA replication and maintenance [16–18].

With the aim of providing a contribution to the study of mitochondrial biogenesis in *D. melanogaster*, we focused our attention on the putative regulation by DREF of two nuclear genes coding for mitochondrial proteins belonging to the mtDNA transcription apparatus: mtRNApol and the transcription termination factor DmTTF.

We first analysed *in silico* the 5′-flanking region of the DmTTF and mtRNApol genes by searching for DREs. This allowed the identification of ten DRE sequences in the promoter region of the DmTTF gene, the three most proximal of which are 100 % conserved in the *Drosophila* species belonging to the *melanogaster* subgroup. No significant DREs were found in the mtRNApol promoter. Afterwards, by both *in vivo* and *in vitro* experiments we clearly demonstrate that DREs are required for DmTTF promoter activity and that DREF positively regulates the expression of the transcription termination factor. Our study thus provides the first experimental evidence of a control exerted by DREF on a factor involved in mtDNA transcription. On the other hand, our results rule out the possibility that DREF controls, although indirectly, the mtRNApol gene at least in S2 cells. It has been determined recently that DREF also stimulates the expression of another transcription factor, namely TFB2M, which is involved in transcription initiation (M. Á. Fernández-Moreno, unpublished work). Therefore our findings on the mtDNA transcription machinery parallel what was observed for the mtDNA replication apparatus, that is, the accessory factors are controlled by DREF, whereas the catalytic polypeptides, mtRNApol and pol γ-α [17], are not.

The transcription termination factor DmTTF belongs to the mTERF protein family that includes three more members from *D. melanogaster* [20,27]; as reported in FlyBase, they are indicated as CG7175, CG15390 and CG5047, with the last polypeptide being named D-MTERF3 and was shown to be involved in mitochondrial protein synthesis [27]. We found that multiple DRE sequences are also present in the 5′-flanking sequences of the genes for all these three proteins (F. Bruni, unpublished work), thus suggesting the existence of a control by DREF on all the genes belonging to the mTERF family and pointing towards a more general role of DREF in the control of mitochondrial biogenesis in *Drosophila*. This hypothesis is further supported by the recent finding that the DRE/DREF system is also a crucial regulator of ANT (adenine nucleotide translocase) gene expression [28].

It has been reported that hDREF (human DREF), the human orthologue of the *Drosophila* factor, regulates cell proliferation and expression of ribosomal protein genes [29,30]. Interestingly, we found that the gene for the human mitochondrial transcription termination factor mTERF contains a putative recognition site for hDREF in the 5′-UTR (5′-untranslated region). A similar observation has been reported also for the human ANT gene [28]. These findings suggest that hDREF might have a role in controlling the expression of these genes and that some regulatory patterns of mitochondrial biogenesis could be shared by humans and *Drosophila*.

In situ RNA hybridization studies on *Drosophila* embryos reported here show that DmTTF mRNA is transcribed almost ubiquitously and constitutively; mtRNApol expression seems to be more efficient and restricted to specific embryonic territories. However, in the late stages of embryogenesis, DmTTF transcription becomes, over a general background, more efficient in

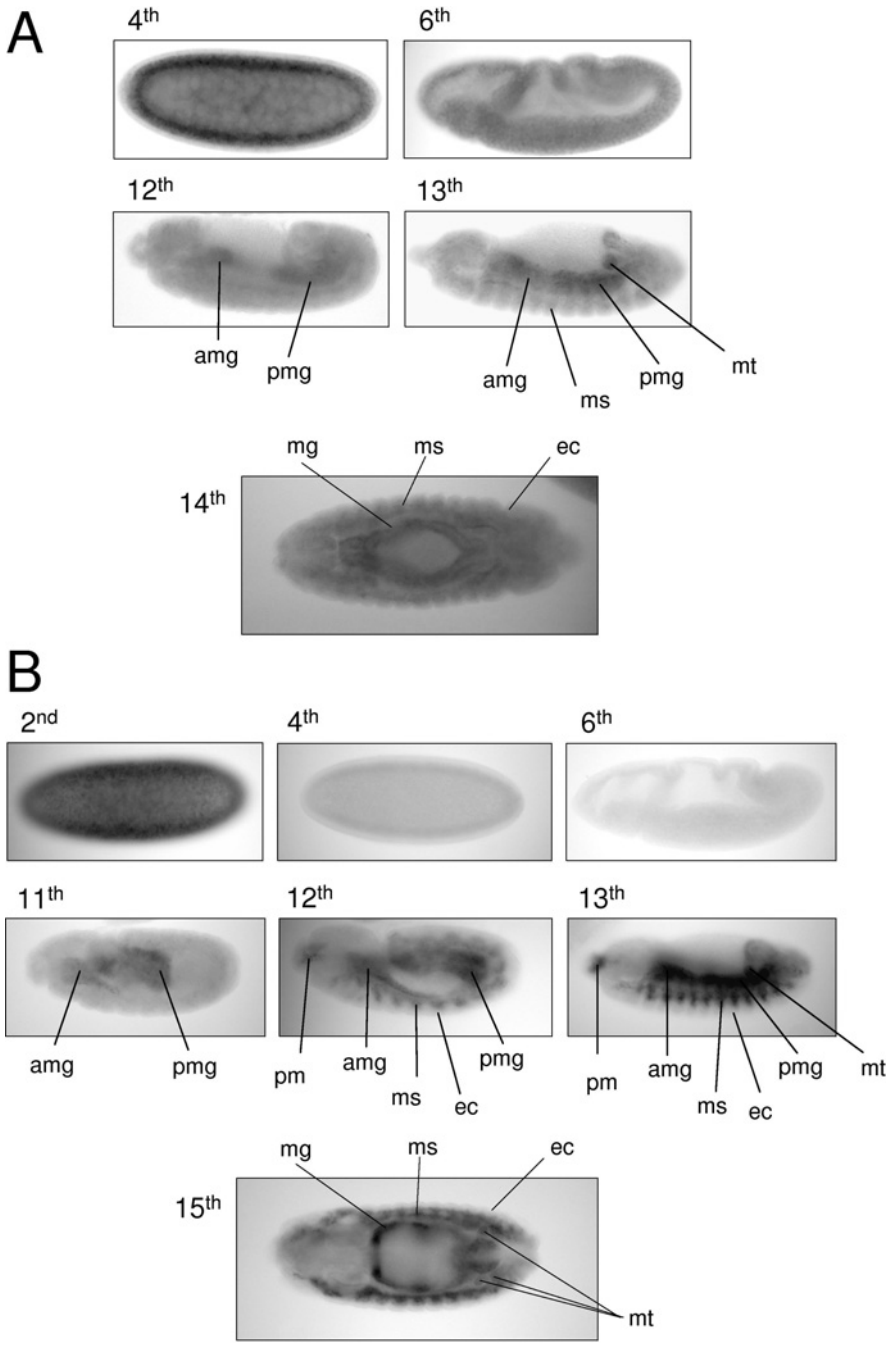

Figure 6 DmTTF and mtRNApol expression during *Drosophila* embryogenesis

The temporal and spatial distribution of *Drosophila* DmTTF (**A**) and mtRNApol (**B**) was examined by whole-mount *in situ* hybridization with digoxigenin-labelled antisense RNA as described in the Materials and methods section. The staining pattern of embryos is shown at the indicated stages. All stages are shown in lateral view with the dorsal at the top, except for the 14th and 15th stages that are shown in dorsal view. Both mRNAs display a significant maternal deposit. DmTTF mRNA is present in all stages and almost ubiquitously; after the 12th stage, it is transcribed more efficiently in the gut, and after the 13th stage, it is transcribed in the gut and Malpighi tubes (the latter are out of focus in the dorsal view). mtRNApol mRNA maternal deposit disappears before the 4th stage until the 11th approximately when it is efficiently transcribed in the gut. In late stages of the process, mtRNApol is efficiently and specifically transcribed in the gut, mesoderm, Malpighi tubes and pharyngeal muscles (the latter are out of focus in the dorsal view). amg, anterior midgut; ec, ectoderm; mg, midgut; ms, mesoderm; mt, Malpighi tubes; pm, pharyngeal muscles; pmg, posterior midgut.

Malpighi tubes and the gut (digestive tube), regions in which mtRNApol is also transcribed. Thus it is interesting that the mRNAs for DmTTF and mtRNApol, two proteins involved in the same process, share some expression regions but do not show identical temporal and spatial expression patterns during embryogenesis; this confirms that transcription of the two genes is differentially regulated. Moreover, the data obtained imply that higher levels of DmTTF might be required in territories such as Malpighi tubes and the gut. A similar situation can be inferred from the data for DmTTF and mtRNApol gene transcription in *Drosophila* larvae and adult tissues reported in the FlyAtlas database (http://www.flyatlas.org/) [31]. In particular,

the enrichment of mtRNApol in some adult tissues with respect to the whole fly is slightly different from that of DmTTF (1.1 versus 0.8 in brain, 0.8 versus 1.2 in crop and 0.6 versus 1.0 in tubule). This imbalance is higher in larval tissues such as larval tubule (0.2 versus 1.0) or larval fat body (0.2 versus 1.1).

Our findings and those from FlyAtlas suggest the possibility that DmTTF could play multiple roles in the mtDNA transcription process, for which different levels of the protein with respect to mtRNApol are required. This possibility is also supported by DmTTF RNAi data [22], which indicate a possible function of the termination factor also in transcription initiation. Therefore we suggest that variation in the relative amount of DmTTF might control, for example, the balance of different groups of mitochondrial transcripts during embryo development and, possibly, in larvae. Further experimental work is necessary to gather evidence in support of this proposal.

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