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

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Synopsis

The DNA Replication-related Element-binding Factor (DREF), which regulates transcription of a group of cell proliferation-related genes in *Drosophila*, controls also the expression of three genes involved in mitochondrial DNA (mtDNA) replication and maintenance. In this study, by *in silico* analysis, we have identified DNA Replication-related Elements (DRE) in the promoter region of a gene participating in mtDNA transcription, the *Drosophila* mitochondrial Transcription Termination Factor (DmTTF). Transient transfection assays in *Drosophila* S2 cells, with mutated versions of DmTTF promoter region, showed that DRE elements control DmTTF transcription; moreover, gel-shift and ChIP assays demonstrated that the analysed DRE sites interact with DREF either *in vitro* and *in vivo*. Accordingly, DREF knockdown in S2 cells by RNA interference induced a considerable decrease in DmTTF mRNA level. These results clearly demonstrate that DREF positively controls DmTTF expression. On the contrary, mitochondrial RNA polymerase (mtRNApol) lacks DRE elements 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 11th-12th. Territories where transcription occurred mostly were gut and Malpighi tubes for DmTTF, and 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 multiples roles in mtDNA transcription process, for which different levels of the protein with respect to mtRNApol are required.

Key words: DREF, DmTTF, mtRNApol, RNA interference, ChIP, embryogenesis

Short title: DmTTF transcription regulation

INTRODUCTION

Mitochondria are semi-autonomous cytoplasmic organelles whose biogenesis requires the co-ordinate expression of nuclear and mitochondrial genomes. The coding capacity of animal mitochondrial DNA (mtDNA) is limited to 13 polypeptides of the oxidative phosphorylation system (OXPHOS), 22 tRNAs and 2 rRNAs; thus, the majority of 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 in order to clarify the mechanisms that regulate the expression of nuclear genes encoding mitochondrial proteins. In mammals two nuclear transcription factors, namely NRF-1 and NRF-2 (Nuclear Respiratory Factor 1 and 2), have been shown to regulate, in cooperation with co-activators of the PGC-1 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 coordinate mitochondrial protein gene expression (4, 5). Recently, *in silico* analysis of *D. 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 electron transport chain, oxidative metabolism and mitochondrial biogenesis (6).

The DNA Replication-related Element Factor (DREF) is a *Drosophila* transcription regulatory protein consisting of an 80 kDa polypeptide forming an 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, being its binding sites 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 mitochondrial single-stranded DNA-binding protein (mtSSB), the β subunit of the mitochondrial DNA polymerase (pol γ - β) and the mitochondrial transcription factor A (TFAM), whose products are required for mtDNA replication and maintenance (16-18), thus providing evidence for a coordinated regulation of nuclear and mitochondrial DNA replication. No information concerning the control by DRE/DREF system on proteins that are specifically involved in mtDNA transcription are available yet; therefore we wished to investigate on the possible dependence on DREF of the genes for the single subunit mitochondrial RNA polymerase (mtRNApol) and for the accessory factor DmTTF. DmTTF is a nuclear encoded 43 kDa protein, member of the mTERF 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 convergent direction (21, 22). Moreover, it appears that DmTTF has a more 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 this paper, by a combination of *in vitro* and *in vivo* experiments, we demonstrate that DREF binds two DRE sequences located about 50 nt upstream of the DmTTF transcription initiation site and that positively regulates the expression of DmTTF. On the contrary, no control by the DRE/DREF system was observed for the mtRNApol gene. In addition, by analyzing 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 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 the transcription start point and being +113 the first nucleotide of the start codon) was obtained by PCR using total DNA from *D. melanogaster* derivative S2 cell line as template, and the forward primer dir(1339) (5'CCGGATCCCTTCAATTGTCACCTGTCATT3') and the reverse primer rev(818) (5'GGCTCGAGCCCGAATTGCTTAATTTACA3'). 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 of a series of deletion and mutant clones.

Mutagenesis of DRE element at position -42 (5'TAACGATA3') on p2000DDD was achieved by three PCR steps and sub-cloning of the amplified fragment. In the first PCR p2000DDD (template) and primers dir(1339) and rev(820) (5'TCAAACAATTActaTTATCGGTTTTGAAAATAATGA3') were used. In the second PCR the same template and primers dir(1839) (5'TCATTATTTCAAACCCGATAAtagTAATTGTTTGA3') and rev(818) were used. Primers rev(820) and dir(1839) are complementary and contain the DRE element at -42 (underlined) mutated (changes are in lower case). A third PCR was carried out using as 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 to the original contained in p2000DDD except for the mutated DRE element at position -42. The final PCR product was cloned into BamHI and XhoI sites of pXp2 to generate p2000DDd. Mutagenesis of DRE element at position -48 (5'AACCGATA3') was carried out as for DRE at -42, using the mutation containing primers rev(821) (5'TCAAACAATTATCGTTActaGTTTTGAAAATAATGA3') for the first PCR and dir(1840) (5'TCATTATTTCAAACtagTAACGATA3') 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 element (-42/-48) containing construct was obtained in the same manner, using primers rev(822) (5'TCAAACAATTActaTTActaGTTTTGAAAATAATGA3') for the first PCR and dir(1841) (5'TCATTATTTCAAACtagTAAtagTAATTGTTTGA3') for the second. Primer sequences show DRE at -42 in italic and that at -48 underlined and mutations on DRE elements 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 template and primers dir(1299) (5'CCAAGCTTAAGTAACTAGTTAACGCAATTAAGCAGC3'; 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'CCAAGCTTTGTTCAAATCCTACAAGTC3'; HindIII site in italic). Mutation of proximal DRE elements (-42/-48) on p400DDD was carried out using p2000Ddd as 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 template and primers dir(2875) (5'CCAAGCTTTGTTCAAATCCTACAAGTCTTTGTTTAgtagTAATGTTCCG3'; 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 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) following manufacturer's recommendations. Promoter activities were calculated 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) following 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-³²P-ATP and T4 polynucleotide kinase and then purified using G-50 nick columns (Pharmacia). Binding reactions were carried out combining 10 µg of nuclear protein extract, 50000 cpm (corresponding to 30 fmoles) of DNA probe and 1 µg poly(dI-dC)·poly(dI-dC) (Pharmacia) in binding buffer (20% glycerol, 1 mM dithiothreitol, 20 mM HEPES pH 9.0, 5 mM MgCl₂, 0.2 mM EDTA, 200 mM KCl). After an incubation of 30 min at 4 °C, the reaction products were electrophoresed onto a 6% polyacrylamide gel in 0.5X TBE. A 200-fold molar excess of non-radioactive wild type or DRE mutated oligonucleotides was used as homologous competitor. DRE mutations in cold 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 L. Kaguni, Michigan State University) 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 assays

Chromatin immunoprecipitation 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 5 times (10 seconds continuous pulses at 7 amplitude microns power) in a MSE Soniprep 150 sonifier with a microtip 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 antiserum anti-DREF from rabbit and anti-Eyegone from guinea pig and their respective preimmune serum were used. Chromatin extracts were immunoprecipitated twice, initially with the corresponding preimmune 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' AAACAGCTGATATGTTGCGG 3') as forward and reverse primers, respectively, for amplifying a 225 bp fragment containing the -42/-48 DRE site; Fw-361 (5' ATGTGGAGTAGTGCTTGTGA 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 seconds; 58°C/30 seconds; 72°C/30 seconds) were resolved by gel electrophoresis and visualized by ethidium bromide staining.

RNA interference and western blotting assay

Templates for the production of dsRNAs 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'TAATACGACTCACTATAGGGA3'), followed by a gene-specific sequence as indicated: DREF (accession number NM_078805) forward-primer nt 720-736, reverse-primer nt 1321-1306; LacZ (pUC18 vector accession number L09136) forward-primer nt 2512-2528, 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% FBS (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 (no supplemented) in a 75-cm² flask. dsRNAs (15 µg per 10^6 cells) were added directly to the medium and cells were incubated at 25° C for 1 hour. Then, 10 ml of complete medium were added to obtain a cell density of 0.5×10^6 cells/ml followed by an additional incubation at 25° C for 72 hours.

dsRNA treated and untreated S2 cells were then harvested, centrifuged at 1000 g for 4 min at 4°C and washed twice in PBS. Pellets were resuspended in 1X Laemmli buffer and proteins were fractionated on 12% SDS polyacrylamide gel and electroblotted onto PVDF membrane (Immobilon-P, Millipore). Membranes were incubated with affinity-purified anti-DREF antibodies (from M. Yamaguchi, Kyoto Institute of Technology) and with anti-actin antibodies (Sigma), it followed 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 assay

Total cellular RNA was extracted from dsRNA treated and untreated S2 cells by the RNeasy Midi Kit (Qiagen); 500 ng were reverse-transcribed in a final volume of 25 µl 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 Biosystem) resulting sequence positions as follows: nt 583-602 (forward), nt 683-663 (reverse) for DmTTF (accession number AY196479); nt 2352-2371 (forward), nt 2452-2434 (reverse) for mtRNA pol (accession number NM_134721); nt 1407-1429 (forward), nt 1480-1462 (reverse) for cytoplasmic 28S rRNA (accession number M21017), which was used as 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 sec 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 Biosystem). All PCR products were run on a 2.5% agarose gel to confirm specificity. Analysis of data was performed as previously described (22).

In situ hybridization

In situ analysis of mtRNAPol and DmTTF expression on *Drosophila* embryos was performed 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 in (24) with minor modifications: in point 2.3.3., PBT solution was replaced by PBS containing 0.1% (v/v) Tween-20 and the developing solution by 100 mM NaCl, 50 mM MgCl₂, 100 mM Tris-HCl, pH 9.0, 0.1% (v/v) Tween-20.

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RESULTS

DRE elements are present in DmTTF promoter and are conserved in different *Drosophila* species.

With the aim to characterize the involvement of DREF in controlling transcription of DmTTF and mtRNAPol genes in *D.melanogaster*, we searched for DRE consensus sequence TATCGATA in the 2 kb DNA region upstream and around DmTTF and mtRNAPol transcription initiation site 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 mtRNAPol promoter, no DREF binding motifs were identified. On the contrary, the analysis of DmTTF promoter allowed the identification of ten putative DRE elements scattered between position -42 and -1899, according to the transcription start point, which keep at least six out of the eight nucleotides of the DRE consensus sequence (see Fig. 2). Three of these elements are located within the proximal 400 bp region, specifically at -42, -48 and -361, and matches 7, 6 and 6 nucleotides out of the 8 forming DRE consensus, respectively (see Fig. 1A). The element in position -42 overlaps by two nucleotides 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 DRE elements (Fig. 1A) as well as in the relative position of the two most proximal, with the position of the third DRE sequence being very similar (Fig. 1B).

Role of DRE sites in DmTTF promoter activity

Transient transfection experiments in S2 cells were carried out in order to analyze 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 Fig. 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 as 100%.

Deletion mutant analysis showed that the removal up to 1577 bp from the distal end of the promoter sequence did not alter significantly transcription efficiency, since p1300DDD and p400DDD mutants show approximately the same luciferase activity than p2000DDD. The 400 bp promoter proximal region, that 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 (Fig. 2) indicates that mutation of DRE at either -42 (p2000DDd) or -48 (p2000DdD) site causes a significant reduction of the promoter activity (about 30%); the reduction reaches about 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, approximately 60%, of the reporter gene expression than that in p2000Ddd. Finally, mutation of the distal -361 DRE sequence (p400dDD) causes 40% of 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) DRE elements are involved in DmTTF transcription, (ii) there are three functional DRE elements in 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 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 (Fig. 3) shows the formation of two protein-DNA complexes (lane 2) that were completely abolished by an excess of homologous cold competitor (lane 3). Moreover, we used as cold competitor oligonucleotides containing a mutated version of each or both DRE sites. As shown in Fig. 3, the oligonucleotide containing mutation at -42 site still keeps a high competition capacity (lane 5), whereas the oligonucleotide mutated at -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 a clear evidence of the involvement of DREF in binding to DmTTF promoter, we performed the binding reaction in the presence of anti-DREF serum: as shown in Fig. 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 cold competitor nor in the sample containing the pre-immune serum. The obtained results clearly demonstrate that the two DNA-protein complexes contain DREF.

To analyse the *in vivo* binding of DREF to 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 and -361 DRE elements (Fig. 4). As a negative control, PCR amplifications were also made on chromatin immunoprecipitated with anti-Eyegone serum (25). As shown in Fig. 4, PCR amplified a clear, single and intense band for -42/-48 and -361 DRE elements when 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 preimmune and anti-Eyegone serum. These results demonstrated that DREF binds indeed *in vivo* to proximal DRE elements on DmTTF promoter region. ChIP experiments on SL2 cells also produced similar results to those made on embryos (data not shown).

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 also mtRNAPol expression could be indirectly regulated by the DRE/DREF system, we tested the effect of DREF depletion also on mtRNAPol mRNA. To this purpose, we used RNAi procedure to obtain DREF depletion in S2 cells. After treating cells for 72 hours with a dsRNA encompassing 601 nt of DREF coding sequence, the level of DREF polypeptide was monitored by western blotting assay. As reported in Fig. 5A, a decrease of more than 90% of 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 LacZ gene (mock control).

Then, we measured the steady-state level of DmTTF and mtRNAPol mRNAs using Real-Time RT-PCR. As shown in Fig. 5B, results obtained from five independent RNAi experiments indicate that in DREF-depleted S2 cells the level of DmTTF mRNA is about 55% of that in non-depleted cells. On the contrary, no effect of DREF depletion was observed on mtRNAPol mRNA level. The decrease of DmTTF mRNA is consistent with the data obtained from the transient transfection assay, and definitively ensures 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. Fig. 6A shows that DmTTF mRNA has a significant maternal deposit; until stage 12th, the signal appears to be quite uniform except for ectoderm and polar cells, where there is no expression (only stage 6th is shown; polar cells data are not shown). On stage 12th, DmTTF mRNA level is increased in midgut (digestive tube) over a general background. From stage 13th, gut and Malpighi tubes show a higher content of DmTTF transcript than the rest of the embryo. Fig. 6B shows that mtRNApol mRNA also has a significant maternal deposit that disappears before stage 4th. Zygotic transcription starts approximately at stage 11th, when hybridization signal appears exclusively in gut primordia. Approximately from stage 12th a strong signal is present in gut, pharyngeal muscles and mesoderm and seems to be absent or undetectable in the rest of the embryo. Finally, from stage 13th mtRNApol transcript is relatively highly expressed in gut, pharyngeal muscles, Malpighi tubes and somatic mesoderm.

DISCUSSION

DREF is a *Drosophila* key transcription factor which regulates the expression of a set of genes involved in nuclear DNA replication 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 to provide 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 searching for DRE elements. This allowed the identification of ten DRE sequences in the promoter region of DmTTF gene, the three most proximal of which are 100% conserved in the *Drosophila* species belonging to the *melanogaster* subgroup. No significant DRE elements were found in mtRNApol promoter. Afterwards, by both *in vivo* and *in vitro* experiments we clearly demonstrate that DRE elements are required for DmTTF promoter activity and that DREF positively regulates the expression of the mitochondrial 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 contrary, our data rule out the possibility that DREF may control, although indirectly, mtRNApol gene at least in S2 cells. Some of us found that DREF also stimulates the expression of another transcription factor, namely TFB2M, that is involved in transcription initiation (manuscript in preparation). Therefore our findings on mtDNA transcription machinery parallel what observed for 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. The last polypeptide was 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 (unpublished observations), 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 the adenine nucleotide translocase (ANT) gene expression (28).

It was reported that hDREF, 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 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 human and *Drosophila*.

In situ RNA hybridization studies on *Drosophila* embryos here reported 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 Malpighi tubes and 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 obtained data imply that higher levels of DmTTF might be required in territories such as Malpighi tubes and gut. A similar situation can be inferred from the data for DmTTF and mtRNAPol gene transcription in *Drosophila* larvae and adult tissues reported in 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 vs 0.8 in brain, 0.8 vs 1.2 in crop and 0.6 vs 1.0 in tubule). This imbalance is higher in larval tissues such as larval tubule (0.2 vs 1.0) or larval fat body (0.2 vs 1.1).

Our findings and those from FlyAtlas suggest the possibility that DmTTF could play multiple roles in 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 the variation of DmTTF relative amount might control, for example, the balance of different groups of mitochondrial transcripts during embryo development and, possibly, in larvae. Further experimental work is necessary for gaining evidences towards this direction.

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FIGURE LEGENDS

Fig. 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 weight matrix and formatted with the ESPript 2.2 Web tool (<http://esprict.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, 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 cases and in lower cases, respectively. (B) Schematic representation of DRE sites location in DmTTF promoter proximal regions from the different *Drosophila* species. DRE sequences are represented by ellipses. Positions are relative to *D. melanogaster* transcription start point.

Fig. 2 – Functional analysis of DmTTF promoter region. A schematic representation of DmTTF promoter region containing the putative DRE sites is shown on the top. The barred box represent 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 below; the position of the ends of the fragments cloned upstream of the luciferase reporter gene (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. Values are means \pm DS of at least six independent experiments. Statistical analysis was carried out using One-Way Anova and the Bonferroni post-test (** indicates $P < 0.001$ and * indicates $P < 0.01$).

Fig. 3 – Binding of DREF to the DRE sites in DmTTF promoter region. DREF binding was evaluated by gel-shift analysis using a nuclear extract (10 μ g of proteins) from S2 cells and a radiolabelled 36-mer double-stranded oligonucleotide (positions -61 to -26, relative to DmTTF transcription initiation site) containing the -42 and -48 DRE sites. Homologous cold competitors, added to the assay in a 200-fold molar excess, were different versions of the same 36-mer oligonucleotide used as 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 open arrow indicates the super-shifted band produced by adding of anti-DREF serum to the binding reaction.

Fig. 4 – DREF binds to proximal DRE elements in DmTTF promoter *in vivo*.

Overnight *D. melanogaster* embryos were fixed to cross-link proteins to DNA. Then, they were homogenized, cells were lysed and their DNA fragmented by sonication. Cell lysates were immunoprecipitated with control IgG (preimmune serum) or polyclonal antiserum raised against DREF or Eyegone. Immunoprecipitated DNAs were amplified by PCR with specific primers for -42/-48 DRE elements (panel A) and for -361 DRE element (panel B). DNA templates, immunoprecipitated in the 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 sizes of the fragments are indicated to the left. The experiment was repeated twice yielding similar results.

Fig. 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 blotting 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 as 1-value. The relative quantification was performed according to the Pfaffl equation (26). Data represent the mean ratio of five independent experiments; standard deviations are indicated on the top of the bars. Statistical analysis was performed using paired two-tailed Student's *t*-test (* indicates $P < 0.05$).

Fig. 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 Materials and Methods. The staining pattern of embryos is shown at the indicated stages. Abbreviations are the following: mg, midgut; amg, anterior midgut; pmg, posterior midgut; pm, pharyngeal muscles; ms, mesoderm; ec, ectoderm; mt, Malpighi tubes. All stages are shown as lateral view with dorsal at the top, except 14th and 15th that are shown as dorsal view. Both mRNAs display a significant maternal deposit. DmTTF mRNA is present in all stages and almost ubiquitously; after 12th stage is transcribed more efficiently in gut and after 13th stage it is done in gut and Malpighi tubes (the latter are out of focus in the dorsal view). MtRNApol mRNA maternal deposit disappears before 4th stage until 11th approximately when it is efficiently transcribed in gut. In late stages of the process, mtRNApol is efficiently and specifically transcribed in gut, mesoderm, Malpighi tubes and pharyngeal muscles (the latter are out of focus in the dorsal view).

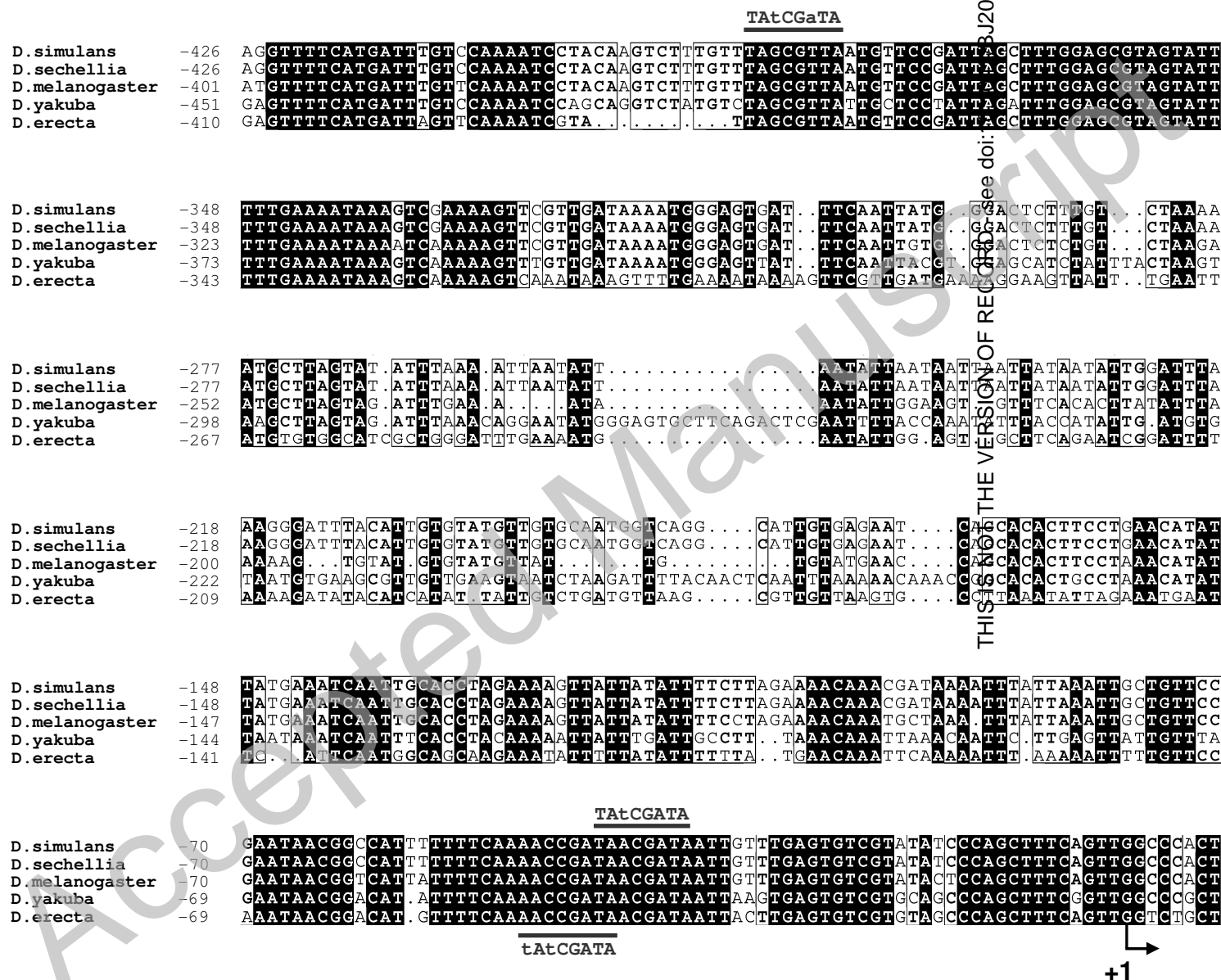


Fig.1A

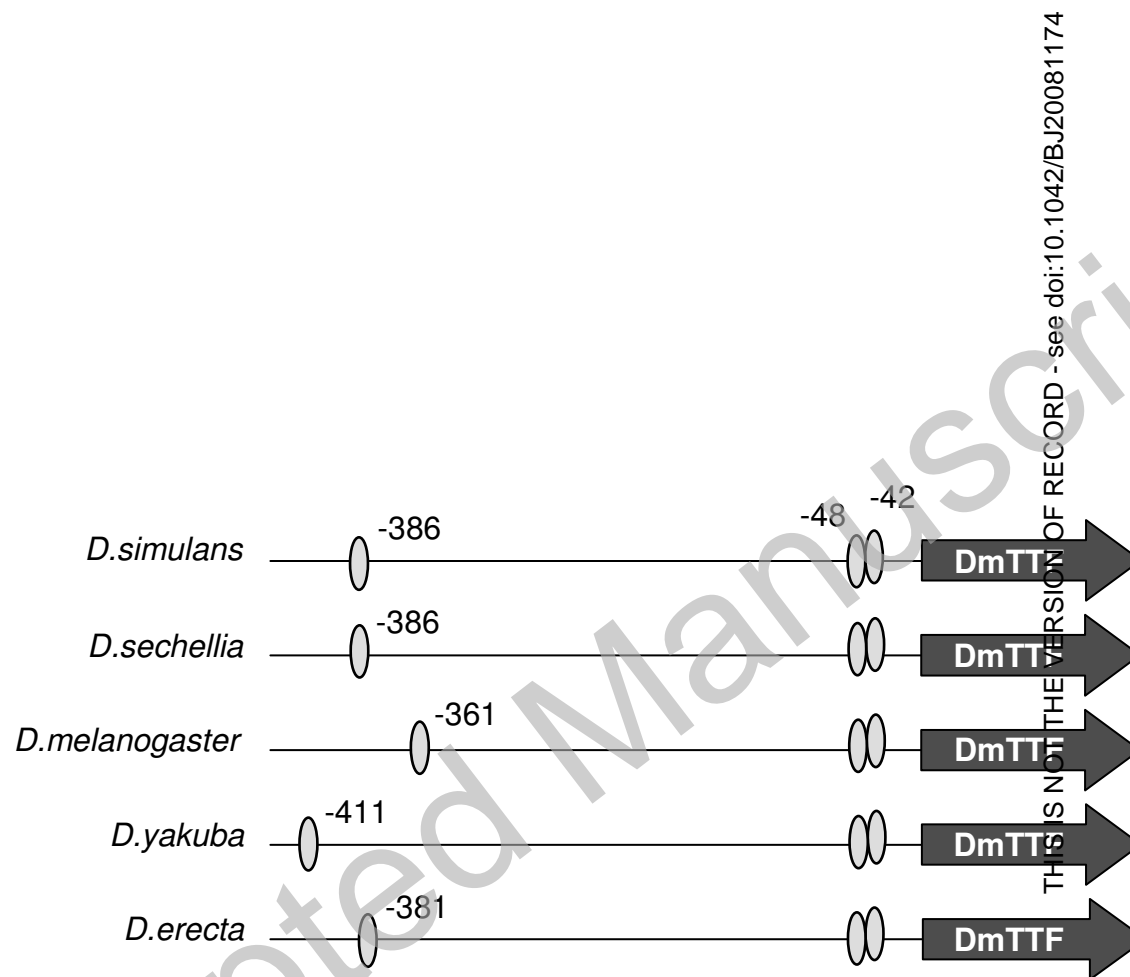


Fig.1B

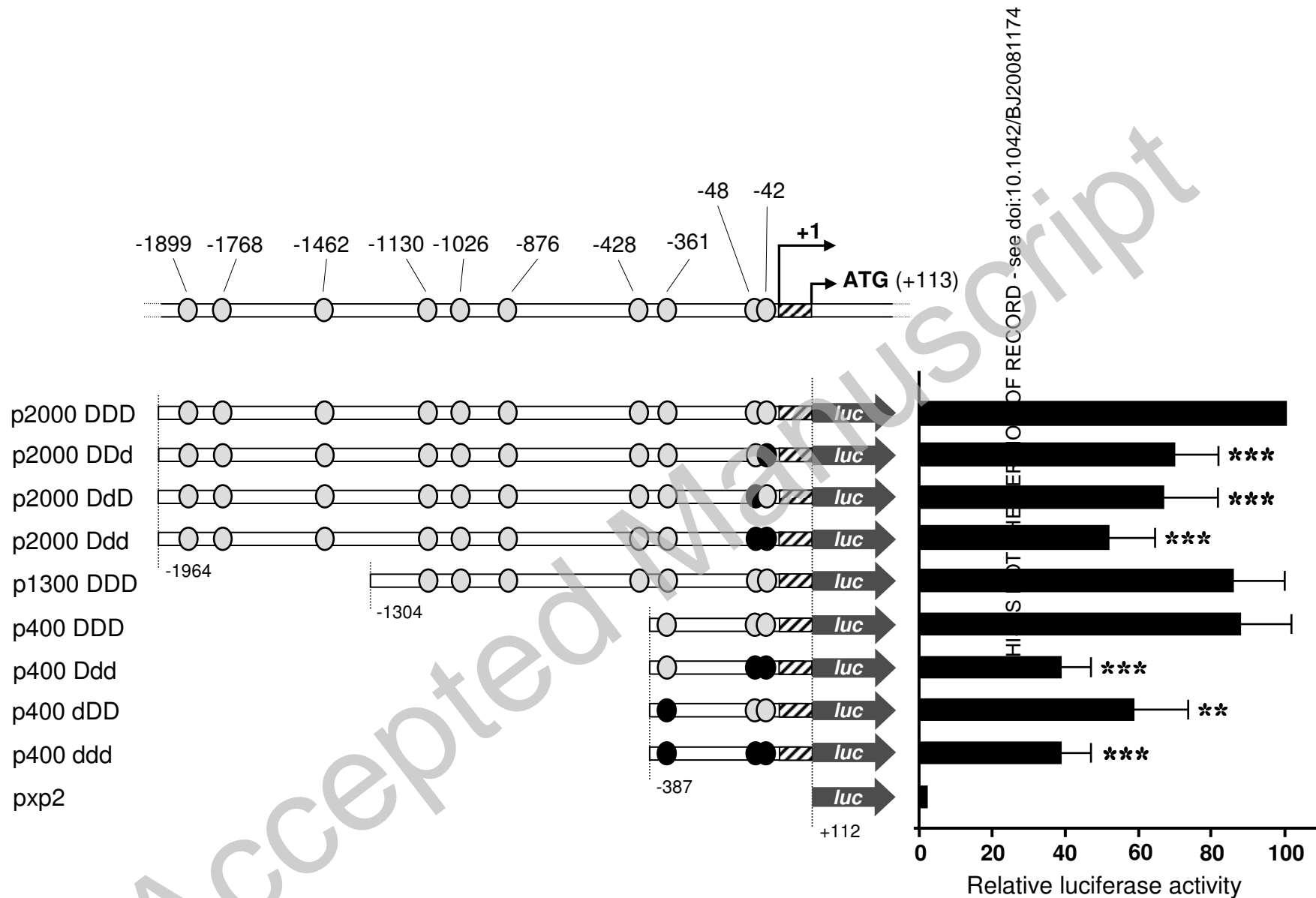


Fig.2

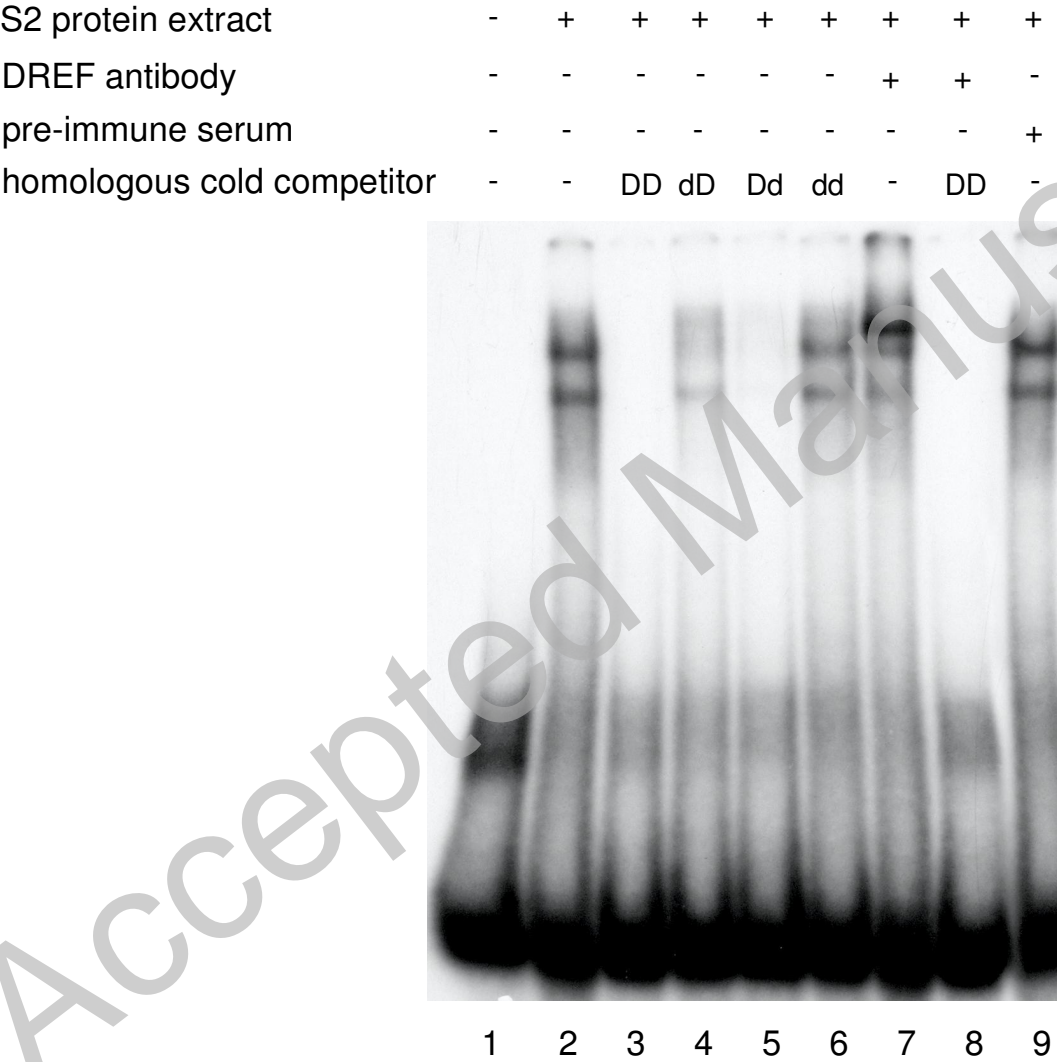


Fig.3

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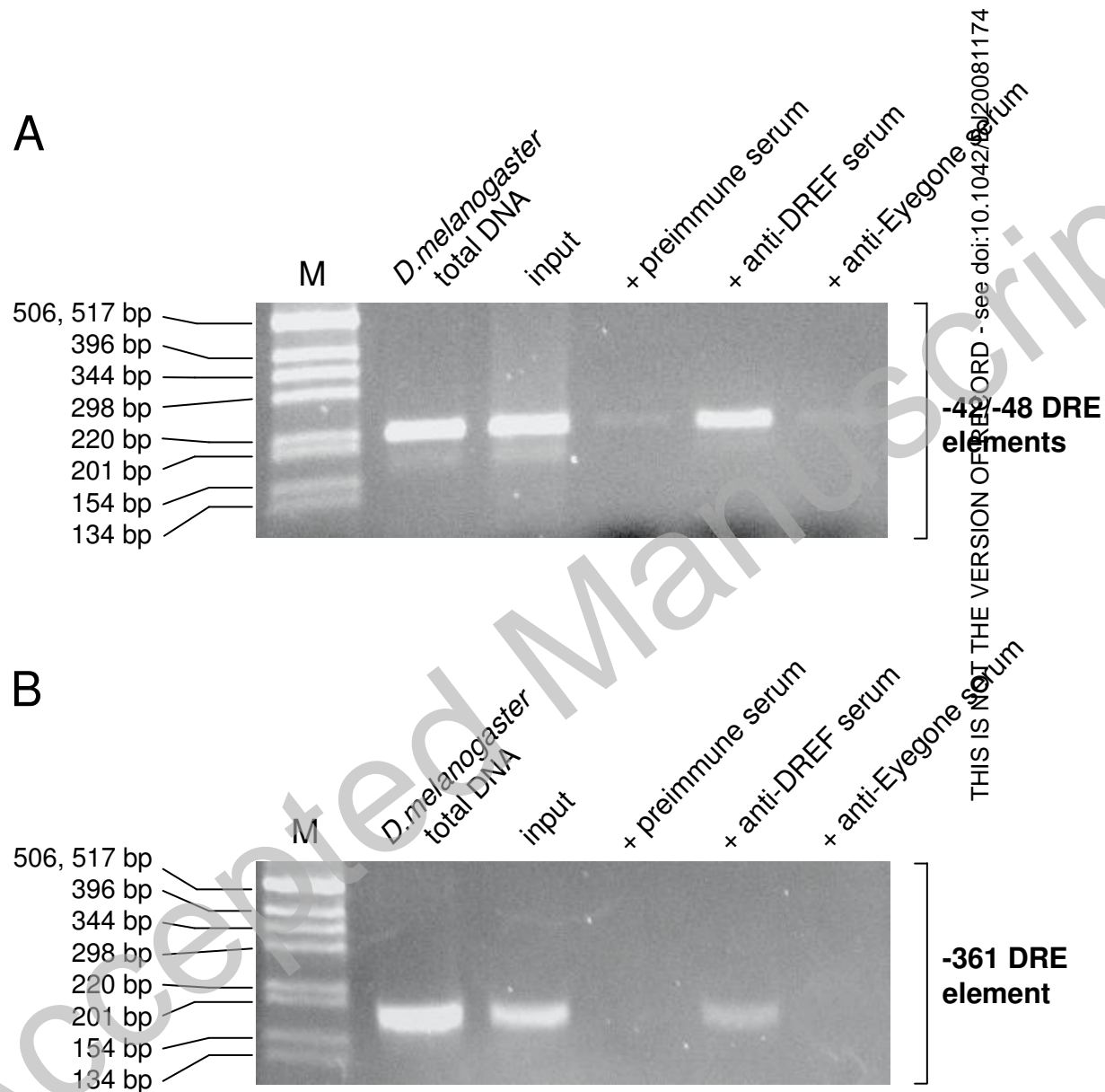


Fig.4

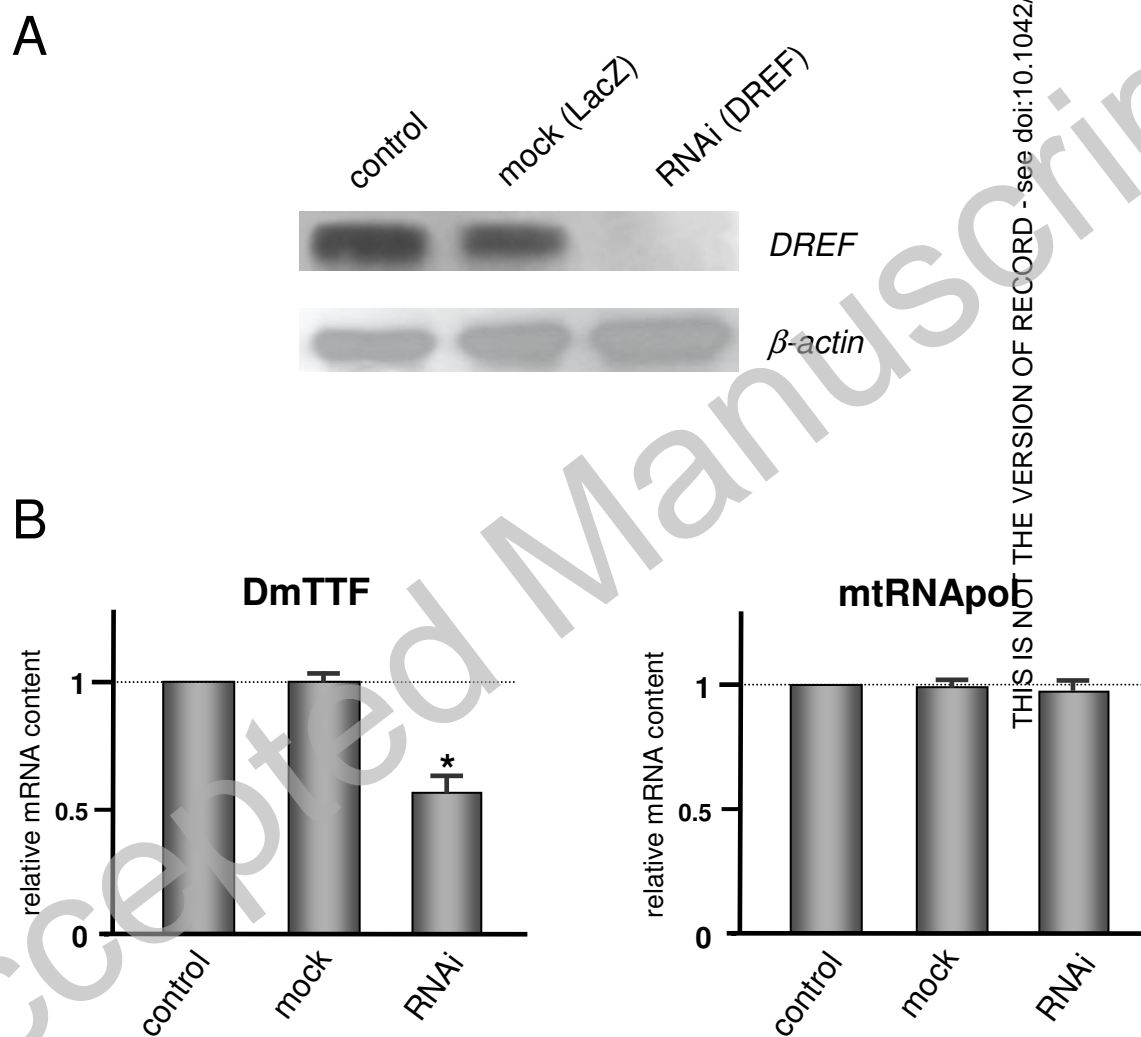
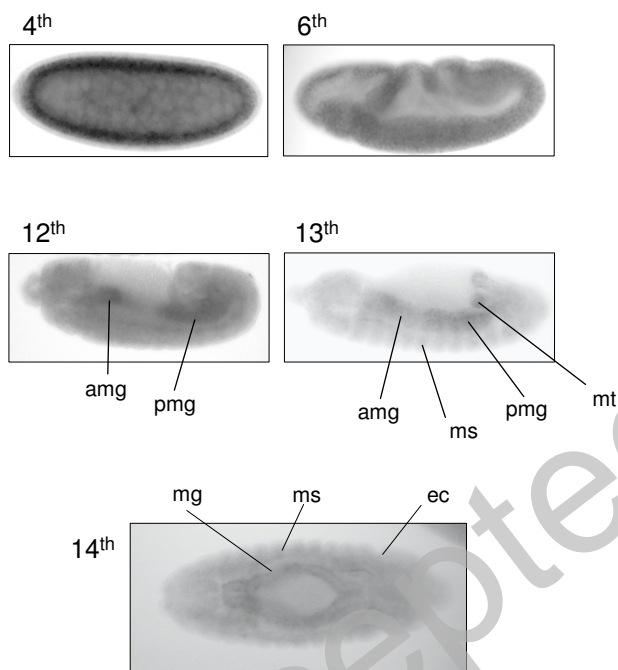


Fig.5

A



B

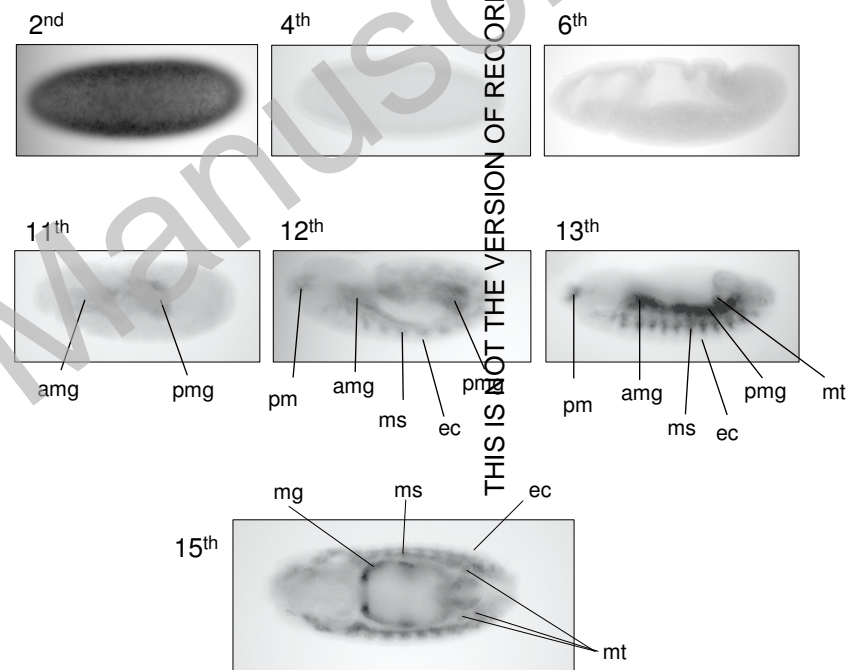


Fig.6