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***Drosophila* nuclear factor DREF regulates the expression of the mitochondrial DNA helicase and mitochondrial transcription factor B2 but not the mitochondrial translation factor B1**

Miguel A. Fernández-Moreno^{a,b,*}, Rosana Hernández^{a,b}, Cristina Adán^{a,1}, Marina Roberti^c, Francesco Bruni^{c,2}, Paola Loguercio Polosa^c, Palmiro Cantatore^c, Yuichi Matsushima^{d,3}, Laurie S. Kaguni^d, and Rafael Garesse^{a,b,**}

^aDepartamento de Bioquímica, Instituto de Investigaciones Biomédicas “Alberto Sols” UAM-CSIC and Centro de Investigación Biomédica en Red (CIBERER), Facultad de Medicina, Universidad Autónoma de Madrid, Spain, c/ Arzobispo Morcillo 4, 28029 Madrid, Spain

^bInstituto de Investigación Sanitaria Hospital Universitario 12 de Octubre (i+12), Madrid, Spain

^cDipartimento di Bioscienze, Biotechnologie e Biofarmaceutica, Università di Bari “Aldo Moro”, Via Orabona 4, 70125 Bari, Italy

^dDepartment of Biochemistry and Molecular Biology and Center for Mitochondrial Science and Medicine, Michigan State University, East Lansing, MI 48823, USA

Abstract

DREF [DRE (DNA replication-related element)-binding factor] controls the transcription of numerous genes in *Drosophila*, many involved in nuclear DNA (nDNA) replication and cell proliferation, three in mitochondrial DNA (mtDNA) replication and two in mtDNA transcription termination. In this work, we have analysed the involvement of DREF in the expression of the known remaining genes engaged in the minimal mtDNA replication (*d-mtDNA helicase*) and transcription (the activator *d-mtTFB2*) machineries and of a gene involved in mitochondrial mRNA translation (*d-mtTFB1*). We have identified their transcriptional initiation sites and DRE sequences in their promoter regions. Gel-shift and chromatin immunoprecipitation assays demonstrate that DREF interacts *in vitro* and *in vivo* with the *d-mtDNA helicase* and *d-mtTFB2*, but not with the *d-mtTFB1* promoters. Transient transfection assays in *Drosophila* S2 cells with mutated DRE motifs and truncated promoter regions show that DREF controls the transcription of *d-mtDNA helicase* and *d-mtTFB2*, but not that of *d-mtTFB1*. RNA interference of DREF in S2 cells reinforces these results showing a decrease in the mRNA levels of *d-mtDNA helicase* and *d-*

*Correspondence to: M.A. Fernández-Moreno, Departamento de Bioquímica, Instituto de Investigaciones Biomédicas “Alberto Sols”, Lab B19, c/ Arzobispo Morcillo 4, 28029 Madrid, Spain. Tel.: +34 91 4973129; fax: +34 91 5854401. **Correspondence to: R. Garesse, Departamento de Bioquímica, Instituto de Investigaciones Biomédicas “Alberto Sols”, Lab B19, c/ Arzobispo Morcillo 4, 28029 Madrid, Spain. Tel.: +34 91 40975452; fax: +34 91 5854401.

¹Current address: Departamento de Ciencia e Ingeniería de Materiales e Ingeniería Química. Universidad Carlos III de Madrid. Avda. de la Universidad, 30. Leganés, 28911 Madrid, Spain.

²Current address: Wellcome Trust Centre for Mitochondrial Research, Institute for Ageing and Health, Newcastle University, Framlington Place, NE2 4HH, Newcastle upon Tyne, UK.

³Current address: Department of Clinical Chemistry and Laboratory Medicine, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.

Appendix A. Supplementary data

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mtTFB2 and no changes in those of the *d-mtTFB1*. These results link the genetic regulation of nuclear DNA replication with the genetic control of mtDNA replication and transcriptional activation in *Drosophila*.

Keywords

DREF; *Drosophila*; Nuclear DNA replication; Mitochondrial biogenesis; Transcriptional regulation

1. Introduction

Mitochondria are essential energy-producing organelles that contain multiple copies of a small circular genome. In spite of limited coding content, mtDNA is critical because the 13 polypeptides it encodes are essential components of the oxidative phosphorylation (OXPHOS) system. The remainder of the approximately 1500 mitochondrial proteins are nuclear encoded. Thus, mitochondrial biogenesis requires a strict control of mtDNA copy number, and a precise coordination of the expression of two genomes located in different cell compartments [1]. This coordination is achieved by nuclear-encoded mitochondrial proteins belonging to the mtDNA replication, transcription and translation machineries, which are induced in response to signals promoting mitochondrial biogenesis [1,2].

In mammals, transcription factors such as NRF-1 and NRF-2 (Nuclear Respiratory Factor-1 and -2) and co-activators of the PGC-1 (peroxisome proliferator-activated receptor γ co-activator-1) family such as PGC-1 α , PGC-1 β and PRC, have been demonstrated to integrate intra- and extracellular signals and to coordinate multiple DNA-binding factors that ultimately regulate the expression of many nuclear genes encoding proteins involved in mitochondrial biogenesis [2]. In *Drosophila*, several candidates have been identified as general regulators of mitochondrial biogenesis. The characterisation of the homologues of mammalian PGC-1 α and NRF-2 α , Spargel and Delg respectively [3,4], supports their central role in the proper expression of genes involved in mitochondrial biogenesis in *Drosophila*, although they do not represent precise functional counterparts of the mammalian proteins. For example, Spargel is not involved in some of the functions of mammalian PGC genes that appear to be vertebrate-specific, such as regulation of mitochondrial mass or thermogenesis [3]. A putative homologue of the mammalian transcription factor NRF-1 was described in *Drosophila*, and termed EWG (erect wing) [5]. Although the proteins are structurally and functionally related (EWG recognises the NRF-1 binding site) and many genes are inferred to be under EWG control, no direct target genes for EWG have been identified to date in *Drosophila*, and EWG has never been demonstrated to coordinate the expression of nuclear-encoded mitochondrial protein expression in flies [6,7]. A putative master key element for mitochondrial biogenesis in *Drosophila* was identified by *in silico* analysis of the *Drosophila melanogaster* genome. This element of ten nucleotides in length is called NRG (Nuclear Respiratory Gene) and is present in more than 50% of genes with mitochondrial function; it is conserved in the OXPHOS orthologs of all insect species examined [8]. However, to date no functional studies support its involvement in mitochondrial biogenesis in *Drosophila*.

The DRE (DNA Replication-related Element) sequence (TATCGATA) and its specific recognition factor DREF (DRE-binding Factor) are associated with the transcriptional regulation of numerous genes controlling nuclear DNA replication and cell cycle progression [9]. Among the target genes are DNA polymerase α and PCNA (Proliferating Cell Nuclear Antigen) [10] that comprise part of the nuclear DNA replication machinery, ORC2 [11] and ORC5 [12] that form part of the DNA replication origin recognition complex, and E2F [13] and cyclinA [14] that are involved in cell cycle control. These observations and many additional data have led to the suggestion that DREF is a master key factor for nuclear DNA replication and cell proliferation in *Drosophila*. DREF is ~80 kD in size and binds DRE sequences as a homodimer. It can function as a traditional transcription factor, when DREs are located several hundred base pairs upstream of the transcriptional start site [10], or as part of the basal transcriptional machinery in which, together with TRF2 (TBP Related Factor 2 in *Drosophila*), it may function as a promoter selectivity factor [15]. Interestingly, DREF also controls the expression of several genes involved in mtDNA replication and transcription: *d-mtssb* (mitochondrial single-stranded DNA-binding protein) [16], *d-pol* γ - β (the accessory-subunit of the mitochondrial DNA polymerase) [12], *d-tfam* (mitochondrial transcription factor A) [17], *d-mTTF* (the mitochondrial transcription termination factor) [18] and *d-MTERF5* (an insect-specific factor also acting in transcription termination) [19], thus providing evidence for a coordinated genetic regulation of nuclear DNA replication, and therefore cell cycle progression and cell proliferation, and mitochondrial DNA metabolism.

In this report, using a combination of *in silico*, *in vitro* and *in vivo* approaches, we demonstrate that DREF binds to the promoter regions and modulates the transcription of the *d-mtDNA helicase* and *d-mtTFB2* but not the *d-mtTFB1* gene. Notably, although *mtTFB1* was originally described as a mtDNA transcription factor *in vitro* [20], that function has not been confirmed *in vivo* [21,22]. However, its involvement in mitochondrial mRNA translation, both in *Drosophila* [22] and in mammals [23] has been demonstrated. This study completes the DREF-dependence map of the known genes directly involved in mtDNA replication and transcription, and expands and strengthens the link between cell cycle regulation and mitochondrial biogenesis in *Drosophila*.

2. Materials and methods

2.1. RLM-5'-RACE (RNA ligase-mediated 5'-rapid amplification of cDNA ends)

To characterise the promoter region of the *d-mtDNA helicase*, *d-mtTFB2* and *d-mtTFB1* genes, we first identified their transcriptional start sites. We used the First Choice RLM-RACE kit (cat. #1700, Ambion Inc.) following the manufacturer's instructions. Total RNA was isolated using TRIzol Reagent, according to the manufacturer's instructions (Invitrogen). Identical results were obtained when total RNA from S2 cells or from *D. melanogaster* Oregon R embryos was used. For the *d-mtDNA helicase*, *d-mtTFB2* and *d-mtTFB1* genes, the outer and the inner primers were dmtHelic-outer/dmtHelic-inner, dmtTFB2-outer/dmtTFB2-inner and dmtTFB1-outer/dmtTFB1-inner respectively (oligonucleotides used in this work are shown in Table 1). Final PCR products were cloned

into the pGEM-T Easy vector (Promega) and at least ten clones from each gene were sequenced.

2.2. Promoter constructs

Two-kb fragments corresponding to the promoter regions of the *d-mtDNA helicase*, *d-mtTFB2* and *d-mtTFB1* genes were amplified by PCR using specific oligonucleotide primers. The resulting DNA fragments served as substrates for generating deletion constructs, either by new PCR reactions or by digestion with appropriate restriction endonucleases. DNA fragments were purified by agarose gel electrophoresis and inserted into the pXP2 vector, which contains the luciferase gene as a reporter. The nucleotide sequences of all promoter constructs were confirmed by sequencing analysis.

2.2.1. Construction of pH2000DD and derivatives of the d-mtDNA helicase promoter

—The $-2000/+1$ promoter construct of *d-mtDNA helicase* (considering as +1 the transcriptional start site located 16 nucleotides upstream of the translational start codon, see below) was obtained by PCR amplification using primers pH2000-fw and pH-rev incorporating BamHI and XhoI restriction sites respectively (Table 1). This fragment was used as a DNA source for the rest of the constructs. After digestion, the 2 kb BamHI/XhoI fragment was cloned into the pXP2 vector yielding plasmid pH2000DD (numbers in the plasmid names refer to the sizes of the cloned region). Deletion constructs were obtained as pH2000DD using the 2 kb promoter region as a template, the reverse primer pH-rev, and the forward primers pH1851-fw, pH1776-fw, pH313-fw and pH222-fw to generate plasmids pH1851, pH1776, pH313 and pH222.

Deletion constructs pH1651, pH924, pH601, and pH545 were obtained in a similar way: an initial endonuclease digestion of pH2000DD (NcoI, PstI, DraI, and HindIII, respectively), followed by blunting-end if required, and then XhoI digestion; the resulting fragments were cloned into the pXP2 vector cut with SmaI/XhoI. Construct p415 was obtained by BglII/XhoI digestion of pH2000DD and cloning the fragment into the pXP2 vector cut with BamHI/XhoI. Using this strategy, all constructs start at different 5'-ends but share the same 3'-end.

Mutagenesis of the double DRE element located near the transcriptional start site (proximal DRE sites) was performed using a strategy similar to that used for the construction of pH2000DD, but using 3'-primers containing mutated DRE sequences. Thus, three PCR reactions were carried out using pH2000dir as forward primer paired with different 3'-primers containing single- or double- DRE mutated sites. Thus, the reverse primers were: pH2000Dd-rev to obtain plasmid pH2000Dd (containing a mutated 3'-proximal DRE), pH2000dD-rev to obtain pH2000dD plasmid (containing a mutated 5' proximal DRE) and pH2000dd-rev to obtain pH2000dd plasmid (both proximal DREs mutated) (Table 1).

For mutagenesis of distal DRE elements (at -1830 and -1819) three different PCR reactions were made using pH1851 as a template, the reverse primer pH-rev, and the forward primers pHdD1851-fw, pHdD1851-fw and pHdd1851-fw (Table 1) to obtain, in a similar way to pH1851, plasmids pHdD1851, pHdD1851 and pHdd1851 respectively.

2.2.2. Construction of pB₂2007 and derivatives of the d-mtTFB2 promoter—

The –2007/+1 promoter region of *d-mtTFB2* (considering as +1 the transcriptional start site located at nucleotide 93 upstream of the translational start codon, see below) was amplified by PCR using the primers pB₂2007-fw (forward) and pB₂-rev (reverse), which contain SalI and BglII respectively (Table 1). After restriction enzyme digestion, the 2 kb promoter fragment was purified from an agarose gel and ligated into the pXP2 vector cut with SalI/BglII to generate pB₂2007. Deletion constructs were generated by digestion of pB₂2007 with different restriction enzymes and cloning the resulting fragments into the pXP2 vector. pB₂1519 was obtained by digestion with XbaI (and rendered blunt-ended)/BglII followed by ligation into pXP2 (SmaI/BglII). For pB₂840, digestion was with DraI/BglII followed by ligation into pXP2 (SmaI/BglII). pB₂363 was made by digestion with BamHI/BglII followed by ligation into pXP2 cut with the same enzymes, and pB₂236 was constructed by digestion with XhoI/BglII and ligation into pXP2 (SalI/BglII). pB₂167 and pB₂67D were obtained by PCR amplification using the BamHI recognition site containing forward primers pB₂167-fw and pB₂67-fw respectively, and pB₂-rev as the reverse primer. BamHI/BglII digested PCR products were cloned into pXP2 (BamHI/BglII).

To generate pB₂67d (pB₂67D containing a mutated DRE construct), the –67/+1 promoter fragment was PCR amplified using pB₂67d-fw as forward primer (including a HindIII recognition site at its 5') and pB₂-rev as the reverse primer. HindIII/BglII digested PCR product was cloned into pXP2 (HindIII/BglII).

2.2.3. Construction of pB₁1964 and derivatives of the d-mtTFB1 promoter—

The –1964/+1 promoter region of *d-mtTFB1* (considering as +1 the transcriptional start site located at nucleotide 507 upstream of the translational start codon, see below) was amplified by PCR using the pair of oligonucleotides pB₁1964-fw as forward and pB₁-rev as reverse, which contain SalI and BglII recognition sites respectively. After SalI/BglII digestion, the approximately 2 kb promoter fragment was purified by agarose gel electrophoresis and ligated into the pXP2 vector previously treated with SalI/BglII to generate pB₁1964. pB₁1158 was made by digestion of pB₁1964 with DraI/BglII, followed by agarose gel purified of the 1158 bp fragment, and ligation into the pXP2 vector treated with SmaI/BglII. pB₁731 was obtained by treatment of pB₁1964 with NcoI (and rendered blunt-ended)/BglII and cloning the resulting fragment into pXP2 treated with SmaI/BglII. pB₁497 was made in the same manner by cloning a XhoI/BglII fragment into pXP2 (SalI/BglII). To generate pB₁329, we PCR amplified the corresponding 329 bp fragment using pB₁1964 as template, pB₁329-fw as forward primer and pB₁-rev as the reverse primer. The SalI/BglII digested PCR product was cloned into pXP2 (SalI/BglII). Finally, pB₁105 was constructed by digestion of pB₁1964 with HincII/BglII, agarose gel purification of the fragment, and then ligation into the pXP2 vector treated with SmaI/BglII.

2.3. Schneider S2 cell culture

Schneider S2 cells were maintained in *Schneider Drosophila Medium* (Invitrogen Corp.) supplemented with 10% Fetal Bovine Serum (Invitrogen Corp.), penicillin G (100 IU/ml) and streptomycin (100 µg/ml) at 25 °C. Cells were diluted 1:4 twice a week.

2.4. Transient transfection and luciferase reporter assays

Transient transfection assays in *Drosophila* S2 cells were carried out according to [24] using 3 μ g of the luciferase gene-containing plasmids. 48 h after transfection, cells were harvested by centrifugation and washed with PBS. Cell lysis and luciferase measurements were performed using the Luciferase Assay System (Promega) according to the manufacturer's instructions. Promoter activities were considered as the ratio luciferase activities vs. β -galactosidase activity from plasmid pAc5.1/V5-His/LacZ (Invitrogen), 1 μ g of which was co-transfected. β -Galactosidase activity was measured using the Beta-Glo Assay System (Promega) according to the manufacturer's recommendations.

2.5. Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assays were carried out using nuclear protein extracts prepared from S2 cells as described [18]. Probes, binding reactions and electrophoretic conditions were carried out according to [12]. In brief, 10 μ g of nuclear proteins were incubated with 50,000 cpm (0.05 pmol) of a probe containing DRE motifs or their mutated versions in the presence of 1 μ g of poly(dI)-poly(dC). In super shift assays, rabbit antiserum against recombinant DREF protein (1 μ l of a 1:100 dilution) was added after 15 min of incubation and the samples were incubated for an additional 15 min. Pre-immune serum was used at the same dilution as a negative control.

The *d-mtDNA helicase* probes consisted of two 40-mer double-stranded DNA fragments including either the proximal or the distal DRE sites under study. Thus, the probe containing distal *d-mtDNA helicase* DREs was the oligonucleotide Dhelic-probe, annealed to its antiparallel primer, and the probe containing proximal *d-mtDNA helicase* DREs was Pxhelic-probe, annealed to its antiparallel primer (Table 1). Competitor oligonucleotides were the non-radioactive version of the probes, Dhelic-compet, the corresponding double-stranded oligonucleotide containing 3-base pair substitutions within each DRE element for distal DREs, or Pxhelic-compet for proximal DREs (Table 1). Competitors were included in the reaction mixtures at 200-fold molar excess.

For *d-mtTFB2* promoter bandshifts, the 40-mer oligonucleotide B₂-probe annealed to its corresponding antiparallel primer was used as a probe. Both, the wild type double-stranded oligonucleotide and the version with a modified DRE B₂-compet (Table 1) were used as competitors in a 200-fold molar excess when indicated.

DRE mutations in unlabelled competitor oligonucleotides have the same nucleotide changes as the constructs used for *in vivo* transfection and luciferase assays.

2.6. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were carried out with DNA obtained from 0.45 g of *D. melanogaster* embryos collected overnight as described previously [18]. Homogenised embryos were sonicated, yielding DNA fragments between 200 and 600 bp on average with a median size of ~400 bp. For immunoprecipitation, rabbit polyclonal anti-DREF, its respective pre-immune sera and guinea-pig polyclonal anti-Eyegone were used. Chromatin extracts were immunoprecipitated initially with preimmune serum for 6 h (IgG

control to remove nonspecific interactions) and then overnight at 4 °C with polyclonal antiserum. Immunoprecipitated DNA was used for PCR amplification of DRE sites using specific primers. For the distal DREs in the *d-mtDNA helicase* promoter, primers were Dhelic-chipfw and Dhelic-chiprev, amplifying a 258 bp fragment. Primers for amplifying the *d-mtDNA helicase* promoter proximal DREs were Pxhelic-chipfw and Pxhelic-chiprev, yielding a 168 bp fragment. For the DRE in the *d-mtTFB2* promoter, primers used were B₂-chipfw and B₂-chiprev, amplifying a 225 bp DNA fragment. As a negative control, we amplified in all experiments a 299 bp off target region corresponding to the Rh2 gene (Gene ID: CG16740; FlyBase ID: FBgn0003248), using primers Rh2-chipfw and Rh2-chiprev. The PCR products (obtained after 25 cycles within the linear range of amplification) were resolved by 2.5% agarose gel electrophoresis and visualised by ethidium bromide staining.

2.7. DREF mRNA interference assay

Knock-down of DREF was carried out in S2 cells according to [18] with no modifications. 15 µg of dsRNAs internal to DREF and to lacZ mRNAs were incubated independently with 10⁶ S2 cells, and the level of DREF was analysed after 72 h by immunoblot. Membranes were incubated with rabbit anti-DREF and rabbit anti-β-actin antibodies (Sigma), followed by incubation with anti-rabbit IgG (Santa Cruz Biotechnology). Protein bands were visualised using the ECL® Plus Western Blotting Detection System (GE Healthcare).

2.8. Real-time reverse transcription-PCR assay

Quantification of mRNAs was carried out by reverse transcription-PCR (RT-PCR) according to [18] without modifications. Primers for qPCR were designed using Primer Express 2.0 software (Applied Biosystems), and the resulting sequence positions were as follows: nucleotides (nts) 453–472 (forward), nts 563–543 (reverse) for *d-tfam* (accession NM_079691); nts 257–277 (forward), nts 377–358 (reverse) for *d-mtssb* (accession NM_080496); nts 2619–2638 (forward), nts 2749–2730 (reverse) for *d-pol γ-a* (accession NM_057473); nts 629–649 (forward), nts 749–729 (reverse) for *d-pol γ-β* (accession NM_001032091); nts 1295–1315 (forward), nts 1425–1406 (reverse) for *d-mtDNA helicase* (accession NM_135474); nts 1275–1296 (forward), nts 1375–1356 (reverse) for *d-mtTFB1* (accession NM_140242); nts 641–662 (forward), nts 761–742 (reverse) for *d-mtTFB2* (accession NM_141714); nts 583–602 (forward), nts 683–663 (reverse) for *d-mTTF* (accession AY196479); nts 2352–2371 (forward), nts 2452–2434 (reverse) for *d-mtRNApol* (accession NM_134721); nts 189–208 (forward), nts 319–301 (reverse) for Rp49 (accession NM_170461), which was used as a reference gene.

Each qPCR 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) together with 200 nM primers in a final reaction volume of 30 µl. Amplification conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. To ensure that only a single product was amplified, 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. The analysis of the results was performed as described previously [25].

2.9. Sequence and statistical analysis

Sequence analysis was performed using Serial Cloner 2.6 software. Statistical analysis was performed using R statistical software package.

3. Results

3.1. Identification of the transcriptional start sites (tss) in the d-mtDNA helicase, d-mtTFB2 and d-mtTFB1 genes

After confirming the mitochondrial location of the *d-mtDNA helicase*, *d-mtTFB2* and *d-mtTFB1* proteins (Supplementary material, Fig. S1), we performed 5'-RACE to determine precisely the transcriptional initiation sites in their respective genes. We used RNA from S2 cells and *D. melanogaster* Oregon R embryos with identical results (Supplementary material, Fig. S2).

For *d-mtDNA helicase* we identified two transcriptional start sites: two adenines located 16 and 10 nucleotides upstream from the first nucleotide of the translational start codon. Because the former was observed in 8 out of 10 sequenced clones, we consider the adenine located 16 nucleotides upstream from the translational start codon as the reference transcriptional initiation site for the *d-mtDNA helicase* gene.

We identified three transcriptional start sites in the *d-mtTFB2* gene: two adenines and a guanine located 93, 82 and 62 nucleotides upstream from the translational start codon, respectively. Six out of ten sequenced clones represent the first of these, and two of ten clones identify each of the others, so that we consider the adenine located 93 nucleotides upstream from the translational start codon as the reference transcriptional initiation site of the *d-mtTFB2* gene.

We identified multiple transcriptional start sites in the *d-mtTFB1* gene that mapped between nucleotides 441 and 518 upstream from its translational start codon, each represented equally. We never observed the predicted start site assigned in the databases (569 nts upstream from the ATG) in any of the 23 sequenced clones. We consider as the reference transcriptional initiation site of the *d-mtTFB1* gene the adenine located 507 nucleotides upstream from its translational start codon, because it is included in a nearly perfect Inr (Initiator) element, a sequence that is located around the transcriptional start sites of many genes in *Drosophila* (TCA₊₁G/TTC/T, being A₊₁ the transcription start point) [26]. Thus, our 5'-RACE data show heterogeneous transcriptional start sites in the *d-mtDNA helicase*, *d-mtTFB2* and *d-mtTFB1* genes, and only those for *d-mtTFB2* coincide partially with predicted sites in the databases.

3.2. DRE sequences are present in the d-mtDNA helicase, d-mtTFB2 and d-mtTFB1 promoters

To expand the knowledge on the participation of DREF in controlling the transcription of genes involved in mitochondrial replication, transcription and translation, we searched for the DRE consensus sequence TATCGATA in an approximately 2 kb DNA region upstream and around of the *d-mtDNA helicase*, *d-mtTFB2* and *d-mtTFB1* transcriptional initiation

sites according to those identified in Section 3.1. We considered as putative DRE elements those that retain at least 6 out of 8 nts of the DRE consensus sequence. We found 10 putative DREs in the *d-mtDNA helicase* promoter, whose 3'-ends mapped at positions + 7 (8/8 matched nts), +1 (7/8), -236 (6/8), -240 (7/8), -407 (6/8), -1259 (6/8), -1626 (7/8), -1661 (6/8), -1819 (7/8) and -1830 (6/8). The analysis of the *d-mtTFB2* and *d-mtTFB1* promoter regions revealed a single putative DRE at position -32 (8/8) in the former and two putative DREs at positions -203 (7/8) and -1164 (6/8) in the latter.

A comparison of DREs present in the *d-mtDNA helicase* and *d-mtTFB2* promoter regions of different *Drosophila* species, particularly those belonging to the *melanogaster* subgroup, showed a high degree of conservation (Supplementary material, Fig. S3). The DRE element in *d-mtTFB2* shows a perfect match with the consensus sequence in the five species of the subgroup except for that in *Drosophila erecta*, where one nucleotide is not conserved (Supplementary material, Fig. S3A). The proximal DRE at +7 in the *d-mtDNA helicase* promoter also matches perfectly with the consensus sequence and is fully conserved in all species of the *melanogaster* subgroup. The proximal element at +1 in *d-mtDNA helicase* promoter is conserved in *D. melanogaster*, *Drosophila simulans* and *Drosophila sechellia* (7/8 nts matching the consensus sequence) but contains a mismatch in the first nucleotide in *Drosophila yakuba* and *D. erecta* (Supplementary material, Fig. S3B). The distal DRE elements (-1830 and -1819) in the *d-mtDNA helicase* promoter also show a high level of conservation among the different species and with the consensus sequence, 7/8 in the vast majority of cases, (Supplementary material, Fig. S3C). The conservation of the relative positions and sequence of the DREs in the *melanogaster* subgroup is not as high in other species belonging to different *Drosophila* subgroups, although it is significant; in comparison with *melanogaster*, the species *annasae*, *willistoni*, *persimilis*, *mojavensis* and *pseudoobscura* show putative DREs in the *d-mtDNA helicase* and *d-mtTFB2* promoters in similar or close relative positions, but they contain 0–2 mismatches depending on the DRE and the species (data not shown). The functional relevance of these DREs must be examined further.

Finally, the two DREs within the *D. melanogaster d-mtTFB1* promoter region seem not to be conserved at the same relative positions among *melanogaster* species (not shown).

3.3. DREF modulates the activity of the d-mtDNA helicase and d-mtTFB2 promoters, but not of the d-mtTFB1 promoter

To analyse the functional role of DRE sequences in regulating the transcriptional efficiency of the *d-mtDNA helicase*, *d-mtTFB2* and *d-mtTFB1* promoters, we performed transient transfection experiments in S2 cells. For this purpose, different deletion and/or nucleotide substitution mutants of their promoter regions were linked to a *luciferase* reporter gene in the pXP2 vector. The luciferase activity promoted by the 1851 bp *d-mtDNA helicase* promoter region (pH1851) was arbitrarily set at 100 (Fig. 1A). Removing the 5'-75 nts containing the two distal DREs from pH1851 to yield pH1776 reduces transcriptional activity by more than 50%. *In silico* analysis using the TRANSFAC® database and the programs package from Biobase Gene-Regulation (<http://www.gene-regulation.com/pub/programs.html>) did not reveal putative binding sites for other relevant transcription factors

in addition to DREF in this region, suggesting the involvement of these DRE sequences in regulating luciferase expression. Deletion of nts –1776 to –1651, which includes one DRE (6/8), results in a marked loss of promoter activity, suggesting again the involvement of this DRE and/or other DNA sequences in the regulation of expression of the *d-mtDNA helicase* gene. Further deletions of the *d-mtDNA helicase* promoter had no effect on *luciferase* transcription, suggesting these sequences are not relevant for transcription in cultured S2 cells.

Because the two most proximal DREs overlap the transcriptional initiation site, deletion analysis involving these DREs could not be performed. To study their putative role in *d-mtDNA helicase* transcription we analysed the transcriptional activity of the 2 kb promoter region with either or both of these DREs mutated. The transcriptional activity of the *d-mtDNA helicase* promoter is reduced to approximately 50% when the 5'-DRE of the proximal pair is mutated (pH2000dD), and is abolished when the 3'-DRE is mutated (pH2000Dd) (Fig. 1B). As expected, mutagenesis of both DREs results in no promoter activity (pH2000dd). These data demonstrate that both DREs participate in *d-mtDNA helicase* transcription, with the proximal DRE being essential for transcription.

In order to test the putative role of the two distal DRE sequences and to study their relative contribution to *d-mtDNA helicase* expression, we analysed the activity of single and double DRE mutations on pH1851. The pHdD1851 (mutant DRE at –1830) retains 90% of wild type pH1851 activity, suggesting it plays only a minor role in *d-mtDNA helicase* promoter transcription (Fig. 1C). The pHdd1851 (mutant DRE at –1819) promoter activity is reduced to 70%, indicating a more substantial role, although not essential, in *d-mtDNA helicase* expression (Fig. 1C). Finally, the double DRE mutant (at –1830 and at –1819; pHdd1851) exhibits 34% of wild type pH1851 promoter activity, showing a synergistic effect of both distal DRE mutations and suggesting strongly the involvement of both DRE in *d-mtDNA helicase* expression. Notably, this is the first promoter described in which DREF functions both as a traditional transcription factor and as part of the basal transcription machinery.

We followed a similar strategy for functional analysis of DREs in the promoter of the *d-mtTFB2* gene (Fig. 2A). The luciferase activity promoted by the 2007 bp fragment (pB₂2007) was set at 100. Progressive deletions from the 5'-end of *d-mtTFB2* promote resulted largely in retention of full activity, or even increased activity. Removing the region from –167 to –67 reduces the transcriptional activity to ~40%, suggesting the presence of activator DNA elements in this region, although pB₂67D still shows notable luciferase activity. Mutation of the DRE sequence in pB₂67D yields the plasmid pB₂67d, which shows levels of luciferase activity similar to those of the empty vector pXP2, and therefore lacks any transcriptional activity. These data demonstrate that DREF regulates the transcription of the *d-mtTFB2* gene in cultured S2 cells.

Several deletions covering the entire 1964 bp cloned on pB₁1964 plasmid were made in the *d-mtTFB1* promoter. As shown in Fig. 2B, every sequential deletion from the pB₁1158 reduces progressively the promoter activity, suggesting that these regions contain relevant sequences for transcriptional regulation. However, constructs lacking DRE sequences show the same luciferase activity as the constructs including it, strongly suggesting that DREF is

not involved directly in the regulation of transcription of the *d-mtTFB1* gene in cultured S2 cells.

3.4. DREF binds in vitro to DRE sequences in the d-mtDNA helicase and d-mtTFB2 promoters

To test whether DREF binds to DRE sequences in the *d-mtDNA helicase* and *d-mtTFB2* promoters *in vitro* and *in vivo*, we carried out gel mobility shift and chromatin immunoprecipitation assays. Electrophoretic mobility shift assays (EMSA) were performed using nuclear extracts from S2 cells and 5'-³²P-labeled 40-mer double-stranded oligonucleotides including the proximal (+1 and +7) and distal (−1830 and −1819) DREs from the *d-mtDNA helicase* promoter, and the −32 site in the *d-mtTFB2* promoter. Identical results were obtained when *D. melanogaster* embryo extracts were used as the protein source. EMSAs showed similar results for the three DRE-containing regions in both promoters analysed. Fig. 3 shows evidence of protein–DNA complexes (lanes 2, black arrows) that are abolished completely by an excess of homologous unlabeled competitor oligonucleotide (lanes 3). Moreover, when we used oligonucleotides containing a mutated version of the DRE sites as unlabeled competitor (lanes 4), we found that they are unable to compete, confirming the involvement of the DRE sequences in the binding, and suggesting that DREF is the protein bound. When anti-DREF serum was included in the incubation mix (lanes 5, white arrows), we observed a super-shift. The super-shift is not produced in the presence of excess of homologous unlabeled competitor (lanes 6) or in the sample containing the pre-immune serum (data not shown), but it remains unaffected when mutated DRE versions are used as competitors (lanes 7; d-mthelicase distal DREs data not shown). These results demonstrate that DREF binds physically to the DRE in the *d-mtTFB2* promoter, and to both the proximal and distal DREs in the *d-mtDNA helicase* promoter.

In addition to these DREF–DNA complexes a second shifted band, absent in the probe lane and with slightly slower mobility than the probe, is observed in *d-mtTFB2* promoter. This band disappears completely in the presence of unlabeled competitor, almost completely with its mutated version and it is not supershifted by anti-DREF serum, arguing the possibility that a protein, perhaps a member of the basal transcription machinery, could bind, to the probe in addition to DREF, although not within the DRE.

3.5. DREF binds in vivo to DRE sequences in the d-mtDNA helicase and d-mtTFB2 promoters

To analyse binding of DREF *in vivo* to the *d-mtDNA helicase* and *d-mtTFB2* DRE elements, we performed ChIP assays using *D. melanogaster* embryos. Sonicated chromatin was immunoprecipitated with anti-DREF antibodies and the bound DNA was identified by PCR amplification with specific primers. As negative controls, PCR amplifications were also performed using chromatin immunoprecipitated with rabbit pre-immune serum or anti-Eyegone serum [27]. Although there is a hypothetical Eyegone recognition element around nucleotide −1366 in the *d-mtTFB2* promoter (6 out of 7 conserved nucleotides), this is far removed from the DRE site, located at −32, while the median size of chromatin fragments after sonication is 400 bp. Thus, even in the case that *d-mtTFB2* is regulated by Eyegone through binding to this element, it is a good choice as a negative control for anti-DREF

immunoprecipitation at position –32 in *d-mtTFB2* promoter. The *d-mtDNA helicase* promoter region lacks any sequence resembling the Eyegone binding element. As shown in Fig. 4, PCR reactions amplify single bands for each of the three DRE elements tested when the chromatin template is immunoprecipitated with anti-DREF, but no amplification is observed when the chromatin template is immunoprecipitated with preimmune or anti-Eyegone serum. As an off-target region, we amplified a region 299 nts in size that corresponds to the Rh2 gene (see Section 2.6.). PCR reactions gave no detectable signal in any immunoprecipitation reactions but did on input material (Fig. 4A).

These results suggest that these are *bona fide* complexes formed *in vivo*, and that DREF binds *in vivo* to both proximal and distal DREs in the *d-mtDNA helicase* promoter region, and to the single DRE in the *d-mtTFB2* promoter.

3.6. DREF knock-down affects the mtDNA replication and transcription machinery in cultured S2 cells

To obtain further information regarding regulation of *d-mtDNA helicase* and *d-mtTFB2* gene expression by DREF, we analysed the effect of DREF knock-down in cultured *Drosophila* S2 cells. In addition, to develop a comprehensive evaluation of the control of genes involved in mtDNA replication and transcription by DREF, we analysed in parallel the effect of DREF knock-down on the expression of the *d-mtssb*, *d-pol* γ - α , *d-pol* γ - β , *d-tfam*, and *d-mtTFB1* genes. Previous data showed that *d-mtRNA polymerase* expression is not DREF-dependent, whereas *d-mTTF* expression is regulated by DREF and its steady-state mRNA level is reduced to ~50% upon DREF knock-down [18].

DREF depletion in S2 cells was effected by RNAi-mediated knockdown, and after treating cells for 72 h with a dsRNA internal to the DREF coding sequence, the level of the DREF polypeptide assessed by immunoblotting was reduced to a nearly undetectable level (Fig. 5A). An identical interference protocol using a non-related dsRNA (internal to bacterial *lacZ* gene) was carried out as a control. The steady-state levels of the various mRNAs measured by real-time RT-PCR are shown in Fig. 5B. The results obtained from three independent RNAi experiments indicate that in DREF-depleted S2 cells no changes were observed in the level of *d-mtRNApol* mRNA as shown earlier [18], thus serving as an internal negative control. The remainder of the genes showed variable quantitative changes. *d-mtTFB1* and *d-pol* γ - α retain mRNA levels very similar to controls and their small reductions were not statistically significant, suggesting that they are not under genetic control of DREF. In contrast, the *d-mtssb*, *d-tfam*, *d-mtDNA helicase*, *d-pol* γ - β and *d-mtTFB2* mRNA levels were reduced, in particular those of *d-mtDNA helicase* and the previously described *d-mTTF*. The degree of this reduction suggests strongly that other transcription factor/s participate in the expression of this group of genes. In composite, these results demonstrate clearly the involvement of DREF in the regulation of transcription of *d-mtDNA helicase* and *d-mtTFB2*, but not of *d-mtTFB1*, and provide new evidence demonstrating DREF control of *d-mtssb*, *d-pol* γ - β , *d-mTTF* and *d-tfam*, but not of *d-pol* γ - α and *d-mtRNApol* expression (Supplementary Material, Fig. S4).

4. Discussion

DREF is described as a master regulator of the expression of a group of key genes involved in nuclear DNA replication and thus cell cycle progression and cell proliferation [9–11,13,14]. In addition, transcriptional control by DREF has also been demonstrated for some nuclear genes encoding mitochondrial proteins involved in mtDNA replication/maintenance and transcription [1,6], establishing a genetic coordination between essential aspects of nuclear and mitochondrial DNA metabolism. Moreover, the essential role of DREF has been shown in controlling the expression of many ribosome biogenesis genes [28,29] and in the nutrient-dependent growth during development in *Drosophila* [29]. Thus, transcriptional regulation of mtDNA metabolism genes by DREF provides a genetic link between mitochondria biogenesis, nutrient-dependent animal growth and the biogenesis of ribosomes, ribonucleoparticles involved in the most energy consuming process in the cell.

The present study has focused on analysing DREF control of the *d-mtDNA helicase*, *d-mtTFB2* and *d-mtTFB1* genes, the former two being essential for mtDNA replication and transcription, respectively, in both *Drosophila* and mammals, and the latter involved in mitochondrial translation and biogenesis [20–23]. This study also completes the map of known genes encoding proteins involved in the initial steps in mtDNA metabolism that are regulated by DREF.

First, we mapped precisely the 5'-ends of their mRNAs in S2 cells and *Drosophila* embryos, and found discrepancies in almost all cases with the predictions described in databases. The transcriptional initiation sites were well defined (very few and closely situated) for *d-mtDNA helicase* and *d-mtTFB2* but not for *d-mtTFB1*, whose transcription starts in a 77 bp region without a preferred position. *In silico* analysis of the most proximal ~2 kb of their promoter regions revealed putative DRE elements in all of them. By a combination of *in vitro* and *in vivo* experiments, we demonstrated the physical interactions and functional involvement of DREF in the expression of the *d-mtDNA helicase* and *d-mtTFB2* genes, but not of the *d-mtTFB1* gene.

The *d-mtTFB2* and *d-mtDNA helicase* promoters contain DREF binding sites around position +1, showing 100% homology to the canonical DRE sequence (TATCGATA). Mutagenesis of these sites provokes the inability of the promoter to direct transcription, suggesting a role for DREF in the *Drosophila* basal transcription machinery. This function is supported by several approaches that strongly suggest DREF works as a part of a multisubunit complex together with TBP-related factor 2 (dTRF2) and other factors. This complex interacts with polII basal transcriptional machinery elements such as TFIIA and TFIIB to recognize core promoters and therefore to control the start of transcription of a specific group of genes [15,30,31]. In addition, the *d-mtDNA helicase* promoter region contains two elements (positions –1830/–1819) where DREF acts as a *bona fide* transcription factor. Thus, this is the first report of a case in which DREF functions as a transcription factor and as a core promoter recognition element for the same gene. The latter function may be responsible for the well-defined transcriptional initiation sites in the TATA-less promoters of the *d-mtTFB2* and *d-mtDNA helicase* genes. The role of DREF as a transcription factor appears to be important but not essential for *d-mtDNA helicase*

expression, because mutagenesis of its binding site leads to a reduction up to 34% of transcriptional activity in S2 cells but does not abolish it. This is consistent with previous results obtained with other DREF-regulated genes involved in different processes [11,32,33] including mtDNA metabolism [12,16,18], suggesting the involvement and coordination of other regulatory signals with DREF to yield the final transcriptional activity of the corresponding promoters.

Our RNA interference experiments are consistent with results obtained by other approaches, but are not as clear-cut; the essential role of the DRE for expression of the *d-mtDNA helicase* and *d-mtTFB2* genes in S2 cells is not reflected entirely upon interference of DREF expression, because the steady-state levels of their mRNAs are reduced but not abolished. The reduction observed would likely be higher in a DREF knock-out as compared to knock-down assays, because the very low levels of regulatory proteins usually required are sufficient to retain regulatory properties. It is possible that our knock-down cells contain trace levels of DREF below the threshold of detection by immunoblot analysis.

Previous studies have shown that DREF controls the expression of *d-mtssb* [16], *d-pol* γ - β [12], *d-tfam* [17], *d-mTTF* [18] and *d-MTERF5* [19], but not that of *d-pol* γ - α [12] or *d-mtRNA polymerase* [18]. In this study, we conclude that every known component of the *Drosophila* mtDNA replication and transcription machinery is regulated by DREF, except for the catalytic core of the mitochondrial DNA polymerase (*pol* γ - α) and *mtRNA polymerase*; this likely results in regulation of their enzymatic activities solely at the level of nucleotide polymerization. At the same time, it is likely that DREF regulation of additional genes related to mitochondrial DNA metabolism beyond those involved in mtDNA replication and transcription will be described in the future. These may include genes involved in the translational machinery. One of them is *d-GatC*, a putative member of the mitochondrial trans-amidation machinery involved in glutamyl-*mt* tRNA^{Gln} synthesis [34], because *d-GatC* is encoded in a bicistronic mRNA together with *d-pol* γ - β [Clemente, P., manuscript in preparation].

Doubtless, DREF links cell cycle progression and cell proliferation with mitochondrial biogenesis. This orchestration may provide a genetic mechanism to guarantee the availability of energy for the G1-S transition checkpoint. Several lines of evidence support mitochondrial involvement in the cell cycle and in particular, the transition into S phase in *Drosophila*. For example, the *tenured* (cytochrome oxidase subunit Va) mutation decreases intracellular ATP to levels that are able to sustain cell survival, growth and differentiation, but insufficient to allow progression through the G1-S cell cycle checkpoint [35]. In addition, interference with *d-mtTFB2* and its associated mitochondrial dysfunction provokes serious defects in *Drosophila* cell proliferation [36]. Finally, it is noteworthy that specific interference in DREF function in *Drosophila* imaginal discs blocks the G1-S transition in this tissue [37]. DREF appears to be conserved evolutionarily, and a potential human orthologue, hDREF, has been identified [38]. As in *Drosophila*, hDRE sequences were found in the promoter regions of human genes involved in cell proliferation and cell cycle regulation. Interestingly, cell culture studies suggest that DREF regulates gene expression during the G1-to-S cell cycle progression in humans [39], and it will be important to determine if human mitochondrial genes are also under its control.

5. Conclusions

Drosophila transcription factor DREF regulates the expression of numerous genes controlling nuclear DNA replication, nutritional dependent growth and ribosome biogenesis. DREF also regulates the transcription of several nuclear genes encoding mitochondrial proteins involved in mtDNA replication and two in termination of transcription. No previous data were available regarding dependence on DREF regulation for *d-mtDNA helicase* or for the unique *bona fide* mtDNA transcriptional activator, *d-mtTFB2*. The current study allows the conclusion that the actual transcription start points, which differ from those described in databases, have been identified for these genes and for *d-mtTFB1*, a putative mitochondrial transcriptional factor and a documented regulator of mitochondrial mRNA translation. We can also conclude that DREF participates, albeit not exclusively, in the control of *d-mtDNA helicase* and *d-mtTFB2* transcription, through direct binding to their promoters, both *in vivo* and *in vitro*. Finally, we also conclude that *d-mtTFB1* gene expression is not regulated by DREF. In sum, we identify two new genes involved in mtDNA metabolism, namely replication and transcriptional activation that are regulated by DREF, reinforcing the link between nuclear DNA replication and cell cycle progression with mitochondrial biogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DREF	DRE (DNA replication-related element)-binding factor
<i>d-mtTFB2</i>	<i>Drosophila</i> mitochondrial transcription factor B2
<i>d-mtTFB1</i>	<i>Drosophila</i> mitochondrial transcription factor B1
tss	transcription start site
nts	nucleotides
mtmRNA	mitochondrial mRNAs

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Fig. 1. Functional analysis of the *d*-mtDNA helicase promoter region. (A) Schematic representation of the 1851 bp *d*-mtDNA helicase promoter region and deletion constructs; numbers in the names of the plasmids refer to the number of nucleotides 5' of the transcriptional start site cloned upstream of the luciferase reporter gene (grey arrow). The relative positions of ten putative DRE sites are shown. Grey bars represent relative luciferase activities of the constructs shown on the left. Luciferase activity was normalised to β -galactosidase activity for each transfection, and the activity of pH1851 was arbitrarily set at 100. Results are

means $\pm$ S.D. (n = 6). Statistical analysis was carried out using one-way ANOVA and the Bonferroni post-test ($***P < 0.001$). (B) Schematic representation of the 2 kb *d-mtDNA helicase* promoter region highlighting the proximal (+7 and +1) DRE sequences (open circles) and mutated DREs (filled circles). Grey bars represent relative luciferase activities of the constructs shown on the left. Luciferase activity was normalised to β -galactosidase activity for each transfection, and the activity of pH2000DD was arbitrarily set at 100. Results are means $\pm$ S.D. (n = 6). Statistical analysis was carried out using one-way ANOVA and the Bonferroni post-test ($***P < 0.001$). (C) Schematic representation of the 1851 bp *d-mtDNA helicase* promoter region highlighting the distal (–1830 and –1819) DRE sequences (open circles) and mutated DREs (filled circles). Grey bars represent relative luciferase activities of the constructs shown on the left. Luciferase activity was normalised to β -galactosidase activity for each transfection, and the activity of pH1851 was arbitrarily set at 100. Results are means $\pm$ S.D. (n = 6). Statistical analysis was carried out using one-way ANOVA and the Bonferroni post-test ($***P < 0.001$).

Fig. 2.

Functional analysis of the *d-mtTFB2* and *d-mtTFB1* promoter regions. Schematic representations of the *d-mtTFB2* (A) and *d-mtTFB1* (B) 2 kb promoter regions and deletion constructs are shown. Open circles represent wild type DRE sequences and the filled circle represents the mutated form of the DRE. The numbers in the names of the plasmids refer to the number of nucleotides 5' of the transcriptional start site cloned upstream of the luciferase reporter gene (grey arrow). Grey bars represent relative luciferase activities of the constructs shown on the left. Luciferase activity was normalised to β -galactosidase activity for each transfection, and the activity of the respective 2 kb parent constructs was arbitrarily set at 100. Results are means $\pm$ S.D. (n = 6). Statistical comparison of the deleted and mutated constructs versus their respective 2 kb parent constructs was carried out using one-way ANOVA and the Bonferroni post-test (***) $P < 0.001$.

Fig. 3.
In vitro binding of DREF to DRE sites in the *d-mtDNA helicase* and *d-mtTFB2* promoter regions. DREF binding to DRE sites was evaluated in the *d-mtDNA helicase* (A) and *d-mtTFB2* (B) promoter regions by gel-shift analysis using 10 μ g of nuclear protein extract from S2 cells and a 32 P-radiolabeled 40-mer double-stranded oligonucleotides containing the different DRE sequences. Competitors were the unlabeled mutated (mut) and unmutated (wt) versions of the 40-mer oligonucleotide used as a probe (Table 1) added to the assay in 200-fold molar excess. For distal (panel A, left) and proximal (panel A, right) *d-mtDNA*

helicase DREs, mutated competitors contain the mutant versions of both DRE sequences in each element (–1830/–1819 for distal and +1/+7 for proximal; see 2.5). Filled arrows indicate DREF–DNA complexes and open arrows indicate the super-shifted band produced by adding anti-DREF antiserum to the binding reaction.

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Fig. 4.

In vivo binding of DREF to proximal and distal DRE sites in the *d-mtDNA helicase* and *d-mtTFB2* promoter regions. (A) *D.melanogaster* embryos were fixed to cross-link proteins to DNA. Next, they were homogenised, cells were lysed, and their DNA fragmented by sonication. Chromatin was immunoprecipitated with control IgG (preimmune serum) or polyclonal antiserum raised against DREF or against Eyegone. Immunoprecipitated DNAs were subjected to semiquantitative PCR with specific primers for each examined DRE, which are indicated to the right. DNA templates are indicated above each lane. Input shows PCRs containing as template 0.3% of the total amount of chromatin used in

immunoprecipitations. As a negative control, a 299 bp off-target region corresponding to the Rh2 gene was amplified. Representative PCR products are shown. (B) Boxplot showing the signal of the PCR amplification of immunoprecipitated material in three independent experiments. Differences between the signal obtained with the specific anti-DREF antibody and controls (IgG and anti-EyeGone) were statistically significant in all three cases (d-mtDNA helicase distal DRE, one-way ANOVA, $F_{2,6} = 72.02$, $P = 6.4e-05$; d-mtDNA helicase proximal DRE, one-way ANOVA, $F_{2,6} = 2054$, $P = 3.1e-09$; d-mtTFB2 DRE, one-way ANOVA, $F_{2,6} = 109$, $P = 1.92e-05$). Asterisks indicate significant differences in pairwise comparisons (Tukey's multiple comparison test; $P < 0.001$).

Fig. 5. Effect of DREF knock-down on the mRNA levels of mitochondrial DNA replication and transcription genes. (A) DREF protein was knocked down in S2 cells by means of RNAi. Cells treated with LacZ or DREF dsRNA were harvested and proteins were subjected to SDS-PAGE and immunoblotting analysis with polyclonal antibodies against DREF and β -actin. (B) Total RNA was extracted and relative quantification of mRNAs was carried out by Real-Time RT-PCR. Bars indicate the relative content of transcripts, normalised to 28S rRNA (endogenous control), in dsRNAi-DREF-treated cells (black bars) with respect to control dsRNAi-LacZ-treated cells (grey bars); RNA levels in control cells were arbitrarily set at 1. Relative quantification was performed according to the Pfaffl equation [40]. Data represent the mean ratios $\pm$ S.D. of three independent experiments. Statistical analysis was performed using Student's paired two-tailed *t*-test (* $P < 0.01$; *** $P < 0.001$).

Table 1

Oligonucleotides used in this work. Every oligonucleotides are shown 5' → 3'. Italic and boxed sequences indicate DRE. Base substitutions in DRE elements are shown as lowercase. BamHI (GGATCC), XhoI (CTCGAG), SalI (GTCGAC), BglIII (AGATCT) and HindIII (AAGCTT) recognition sites are underline.

RLM-5'-RACE	
dmtHelic-outer	CACCCGTCCGCTTGTTCACGT
dmtHelic-inner	CCAGCAGCCCATGATCGGT
dmtTFB2-outer	CCTCCGAATTTGCAATGTAC
dmtTFB2-inner	TGTGAGTGGGAACCTTTTGC
dmtTFB1-outer	GCCGTAGATGGCCAAACGG
dmtTFB2-inner	CGGAGACACCAAAGCGCC
<i>d-mt helicase constructs</i>	
pH2000-fw	CCGGATCCTGATGATGTTAGGTCGGGT
pH-rev	CCCTCGAGGT TATCGATA TCGAAAGTGA
pH1851-fw	CCGGATCCTAGATCACCGAAGACAGCGATA
pH1776-fw	CCGGATCCGTATTTTATGAATGATTTAATGA
pH313-fw	CCGGATCCTGGTGGAAGAGCCGCA
pH222-fw	CCGGATCCTGAATTTACCGTGCTTAAC
pH2000Dd-rev	CCCTCGAGGT TATtagTA TCGAAAGTGA
pH2000dD-rev	CCCTCGAGGT TATCGATA TtagAAGTGA
pH2000dd-rev	CCCTCGAGGT TATtagTA TtagAAGTGA
pHd1851-fw	CCGGATCCTAGATCACCGAAGA CAGtagTA GGC AATCGATA CATCG
pHd1851-fw	CCGGATCCTAGATCACCGAAGA CAGCGATA GGC AATtagTA CATCG
pHd1851-fw	CCGGATCCTAGATCACCGAAGA CAGtagTA GGC AATtagTA CATCG
<i>dmtTFB2 constructs</i>	
pB22007-fw	CCGTCGACCCGGCGGACTGGATGA
pB2-rev	CCAGATCTTCTTAAGATTTTAAACAAATTGA
pB2167-fw	CCGGATCCTGACCCATGCCAAAATATAT
pB267-fw	CCGGATCCTATGTTTTCGAATCAGCTGTT
pB267d-fw	CCAAGCTTTATGTTTTCGAATCAGCTGTTTCGTTATGC TATtagTA AGT
<i>dmtTFB1 constructs</i>	
pB11964-fw	CCGTCGACGAAAGTGCAGTGTCTATCG
pB1-rev	CCAGATCTGCAAGCGTATTATATAAAAG
pB1329-fw	CGTCGACCGAAATGATGATGATTGAAG
EMSA assays	
Dhelic-probe	TAGATCACCGAAGA CAGCGATA GGC AATCGATA CATCGAA
Pxhelic-probe	TTTCCACCCCTCAC TTTCGA TATCGATA ACATGGAAAATG
Dhelic-compet	TAGATCACCGAAGA CAGtagTA GGC AATtagTA CATCGAA
Pxhelic-compet	TTTCCACCCCTCAC TTTtag TATtagTA ACATGGAAAATG
B2-probe	CAGCTGTTTCGTTATGC TATCGATA AGTTATTGGGTTTTCG
B2-compet	CAGCTGTTTCGTTATGC TATtagTA AGTTATTGGGTTTTCG
ChIP assays	
Dhelic-chipfw	TAAAAAGTGGCACACGTGTG
Dhelic-chiprev	ACCATGGTAGTATCGTATCG
Pxhelic-chipfw	AGCACTGAGAGAGTACAAAG
Pxhelic-chiprev	GTTATTTATACGCAACAGCGG
B2-chipfw	AACCATTTGAAACCACTGA
B2-chiprev	CAAGCATCAGCTGTGTATTG
Rh2-chipfw	GCTGCGGCAACGGAGTGGTGG
Rh2-chiprev	CTTGATGGTCATGGGTGTGCC