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In vitro transcription termination activity of the *Drosophila* mitochondrial DNA-binding protein DmTTF

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Abstract

DmTTF is a *Drosophila melanogaster* mitochondrial DNA-binding protein which binds specifically to two homologous non-coding sequences located at the 3′ ends of blocks of genes encoded on opposite strands. In order to test whether this protein acts as transcription termination factor, we assayed the capacity of DmTTF to arrest in vitro the transcription catalyzed by mitochondrial and bacteriophage RNA polymerases. Experiments with human S-100 extracts showed that DmTTF is able to arrest the transcription catalyzed by human mitochondrial RNA polymerase bidirectionally, independently of the orientation of the protein–DNA complex. On the contrary when T3 or T7 RNA polymerases were used, we found that DmTTF prevalently arrests transcription when the DNA-binding site was placed in the reverse orientation with respect to the incoming enzymes. These results demonstrate that DmTTF is a transcription termination factor with a biased polarity and suggest that the DNA-bound protein is structurally asymmetrical, exposing two different faces to RNA polymerases travelling on opposite directions.

Keywords: *Drosophila*; Mitochondrial transcription; DNA-binding protein; Termination factor; Transcription termination assay

In mammals, mitochondrial DNA (mtDNA) transcription depends on an organelle-specific RNA polymerase and on accessory factors [1]. They include TFAM, TFB1M, and TFB2M, which are required for promoter recognition and transcription initiation [2], and mTERF, responsible for termination. mTERF is a polypeptide that binds DNA as a monomer and contains three leucine zippers probably establishing intramolecular interactions needed to expose the two basic binding domains to the target DNA sequence. It promotes specific termination of the H-strand ribosomal transcription unit at the 16S rRNA-tRNA$^{Leu(UUR)}$ boundary by binding a tridecameric sequence within the tRNA$^{Leu(UUR)}$ gene [3].

Although during animal evolution mtDNA gene organization has undergone profound variations, protein factors required for transcription seem to be quite conserved. Proteins showing significant similarity with mTERF have been described in sea urchin and in *Drosophila melanogaster*. mtDBP (348 amino acids) is a protein, identified and cloned from sea urchin *Paracentrotus lividus*, which binds specifically two homologous sequences: one is located in the main non-coding region in proximity of the H-strand replication origin, the other encompasses the adjacent 3′ ends of the oppositely transcribed ND5 and ND6 genes [4]. The protein contains two leucine zipper-like domains, and small N- and

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C-terminal basic domains; similar to mTERF, mtDBP binds DNA as a monomer. The termination activity of the protein has been demonstrated by means of an in vitro transcription assay [5].

DmTTF (366 amino acids) is the *Drosophila* homologue of mTERF and mtDBP [6]. It binds mtDNA in the boundary region between ND3 and ND5 and between cyt *b* and ND1 genes. In particular, the protein recognizes two short, homologous non-coding sequences located at the 3′ end of blocks of genes encoded on opposite strands. The location of DmTTF binding sites on mtDNA and its similarity with human mTERF and sea urchin mtDBP suggest a role in mitochondrial transcription termination for the *Drosophila* protein. To demonstrate this function, we assayed in vitro the capacity of DmTTF to arrest the transcription catalyzed by mitochondrial and bacteriophage RNA polymerases. The results here reported show that DmTTF functions as a bidirectional transcription termination factor with a biased polarity, according to the orientation of the protein with respect to the RNA polymerase transcription direction.

Materials and methods

Plasmid constructs. The templates used for transcription termination assay were obtained by inserting a fragment containing *Drosophila* and human mtDNA sequences, between *Kpn*I and *Xba*I sites of pBluescript KS(+) vector. For template pDmTTF-term(A), a *Kpn*I–*Bam*HI fragment of 394 bp, comprising nucleotides 490–739 and 3063–3195 of human mtDNA [7] and obtained by PCR on pTER clone [8], was first ligated to a 57 bp *Bam*HI–*Xho*I fragment (nt 6298–6354 of *D. melanogaster* mtDNA, GenBank Accession No. NC_001709) prepared by PCR. The resulting *Kpn*I–*Xho*I fragment was further ligated to a 320 bp human mtDNA *Xho*I–*Xba*I fragment (nt 3275–3594). For template pDmTTF-term(B), the chimeric insert was obtained as for pDmTTF-term(A), except that the ends of the PCR amplified *Drosophila* mtDNA fragment were inverted (*Xho*I–*Bam*HI instead of *Bam*HI–*Xho*I). In this way, the DmTTF-binding sequence was placed in the reverse polarity with respect to HSP human promoter (see Fig. 2A).

Preparation of protein fractions and assay of DmTTF DNA-binding activity. HeLa cell S-100 mitochondrial fraction was prepared as described by Fernandez-Silva et al. [5]. DmTTF was expressed in *Escherichia coli* and affinity purified by heparin–Sepharose chromatography at 500 mM KCl as reported by Roberti et al. [6]. The DNA-binding activity of DmTTF was tested by means of gel mobility shift assay as previously reported [6]. In particular, the probe was a *D. melanogaster* mtDNA fragment, nt 6261–6350, obtained by PCR and labelled with T4 polynucleotide kinase and [γ-^{32}P]ATP. The DNA fragment was incubated with purified DmTTF at 25 °C for 10 min in 20 μl reaction mixture containing 20 mM Hepes–KOH, pH 7.9, 5 mM $MgCl_2$, 75 mM KCl, 1 mM DTT, 0.1 mM EDTA, 2 μg of BSA, and 0.2 μg of poly(dI–dC) · poly(dI–dC).

In vitro transcription termination assays. Standard reactions for termination of transcription by human mtRNA polymerase were carried out in 50 μl of a mixture containing 10 mM Tris–HCl, pH 8.0, 10% glycerol, 10 mM $MgCl_2$, 1 mM DTT, 100 μg/ml BSA, 1 mM ATP, 0.1 mM CTP and GTP, 0.01 mM UTP, 1 μl of [α-^{32}P]UTP (400 Ci/mmol, 10 mCi/ml), 5 μl of a HeLa S-100 mitochondrial fraction, the template DNA at a final concentration of 4 μg/ml, and the desired amount of recombinant purified DmTTF. Control reactions received always column elution buffer (10 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM EDTA, 500 mM KCl, and 20% glycerol) corresponding to the highest volume of eluted protein used in the assay. The mixture was incubated at 30 °C for 30 min. Termination of transcription by T3 or T7 RNA polymerases was performed in a final volume of 20 μl under the same reaction conditions except that the S-100 fraction was omitted, the nucleotide concentration was 0.5 mM for ATP, CTP, and GTP, and 0.05 mM for UTP, template DNA was 20 μg/ml, and 5 U of commercial (Invitrogen) RNA polymerase was added. Reactions were stopped by adding 150 μl of transcription stop buffer (10 mM Tris–HCl, pH 7.4, 0.5% SDS, 0.2 M NaCl, and 10 mM EDTA) containing 20 μg of yeast tRNA; nucleic acids were phenol extracted and ethanol precipitated. The pellet, dissolved in 150 μl of transcription stop buffer, was phenol extracted, ethanol precipitated, and finally resuspended in 15 μl of DEPC-treated water. Samples were mixed with 1 volume of urea-dye, denatured at 80 °C for 10 min, and run on 5% polyacrylamide/7 M urea gels in TBE. After running gels were dried and then exposed for autoradiography. Quantification of the amount of labelled RNA was carried out in the autoradiograms, after selecting the appropriate exposures, using a LKB Ultroscan XL laser densitometer and a GelScan XL software. Termination activity was calculated as the percentage of terminated transcripts relative to total transcripts after correction for the different content in uridine.

Results

To test whether DmTTF is able to terminate mitochondrial transcription, we set up an in vitro transcription assay including a human HeLa cell S-100 submitochondrial lysate, as a source of mitochondrial RNA polymerase activity, recombinant purified DmTTF and chimeric human/*Drosophila* mtDNA templates. Templates contained the DmTTF-binding site at the ND3/ND5 boundary (nt 6314–6341 of *Drosophila* mtDNA) downstream the human promoter HSP. The choice of ND3/ND5-binding site was justified by the observation that in vitro this sequence forms with DmTTF a complex twice more stable than that formed by cyt *b*/ND1 target site [6].

Recombinant DmTTF was purified from *E. coli* lysate by means of heparin–Sepharose chromatography; the SDS–PAGE analysis of the eluted protein (Fig. 1A) demonstrates the high purity of DmTTF (about 80%). To test the activity of the DNA-binding protein and to set up the conditions for the termination assay, we performed a gel shift experiment using as a probe a *Drosophila* mtDNA fragment containing ND3/ND5-binding site of DmTTF. Fig. 1B (lane 3) shows that the DNA probe is fully saturated by an about 7-fold molar excess of protein.

For the termination assay we prepared two constructs, pDmTTF-term(A) and pDmTTF-term(B) (Fig. 2A), containing the DmTTF-binding site in either orientation with respect to the HSP promoter. When template term(A), bearing DmTTF-binding site in the ‘forward orientation’ (that is, increasing numbering of *Drosophila* mtDNA) with respect to the HSP promoter, was used in the assay, transcripts of 717 nt were obtained (Fig. 2B, lane 1). This size corresponds to that of RNA molecules initiating at HSP and terminating in correspondence of *Xba*I site.

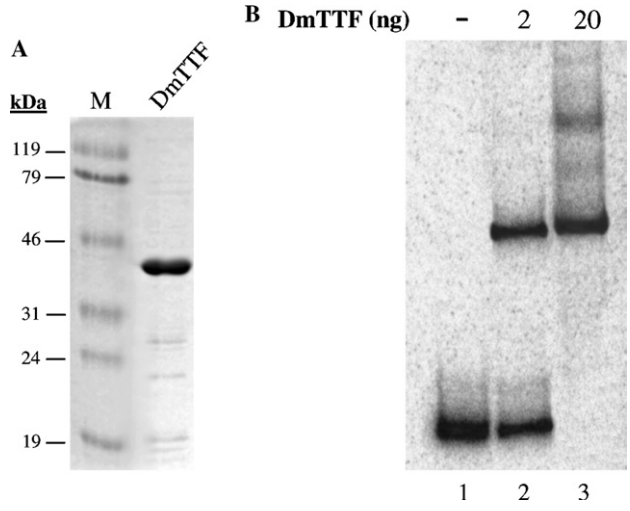

Fig. 1. DNA-binding activity assay of DmTTF. (A) SDS–PAGE of the protein fraction eluted at 500 mM KCl from the heparin–Sepharose column. M, molecular weight marker. (B) Mobility shift assay performed using 60 fmol of DNA probe and the indicated amounts of recombinant purified DmTTF.

Addition to the assay of increasing amounts of DmTTF (up to a protein/DNA molar ratio of 6) caused the progressive and almost complete disappearance of these transcripts that were converted into shorter molecules of about 340 nt (Fig. 2B, lanes 2, 3). This size corresponds to that of molecules starting at HSP and ending at the DmTTF-binding site. This result indicates that DNA-bound DmTTF is able to stop the progression of the human mitochondrial RNA polymerase when it is reached by the transcribing enzyme. In order to test the polarity of the termination activity, we performed the experiment using template term(B), bearing DmTTF-binding site in the 'reverse orientation' (that means decreasing numbering of *Drosophila* mtDNA) with respect to the the HSP promoter. Also in this case (Fig. 2B, lanes 4–6) the addition of increasing amounts of DmTTF caused the almost complete conversion of the run-off transcripts into terminated molecules. These experiments show that DmTTF is able to arrest bidirectionally the transcription catalyzed by the human mitochondrial RNA polymerase.

DmTTF could stop the progression of the human mitochondrial RNA polymerase either by establishing specific interactions with the human enzyme or by imposing a physical blockage to the moving RNA polymerase. To investigate this point, we analyzed the ability of DmTTF to arrest RNA chain elongation catalyzed by the bacteriophage T3 and T7 RNA polymerases, whose

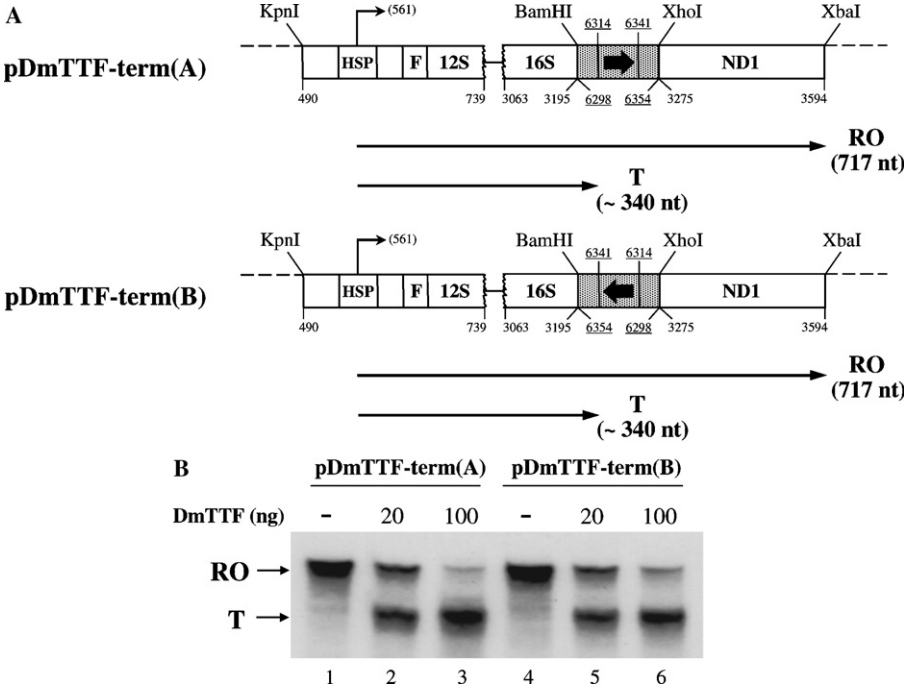

Fig. 2. DmTTF promotes termination of transcription by human mtRNA polymerase. (A) Schematic diagram of the DNA templates, pDmTTF-term(A) and pDmTTF-term(B), used in the assays. Open and filled boxes indicate the human and *Drosophila* mtDNA portions, respectively. Numbers give the nucleotide position on human mtDNA sequence (regular numbers) [7] and *Drosophila* mtDNA sequence (underlined numbers). HSP, H-strand promoter. The major transcription initiation site is indicated at nucleotide 561. The enclosed arrows in *Drosophila* mtDNA portion mark the orientation of DmTTF target site with respect to HSP. RO, run-off transcripts proceeding from HSP; T, terminated transcripts. Transcripts are indicated by arrowed lines; sizes are in brackets. (B) Autoradiogram of a 5% polyacrylamide–7 M urea gel showing products of transcription assays (indicated by arrows) obtained in the absence and presence of purified recombinant DmTTF. Plasmid constructs were double-digested with *Kpn*I and *Xba*I, and the inserted chimeric templates were purified by agarose gel electrophoresis and elution. Reactions contained 4 μg/ml of the indicated template, 5 μl of S-100 mitochondrial extract, and either no DmTTF (−) or the indicated amount of protein. Reactions with no DmTTF contained always column elution buffer corresponding to the highest volume of eluted protein used in the assay.

promoters are contained in the vector on both sides of the chimeric constructs. Plasmids pDmTTF-term(A) and pDmTTF-term(B), carrying DmTTF binding site in either orientation relative to the bacteriophage promoters, were linearized downstream of the insert and used as templates for the proper RNA polymerase, in the absence and in the presence of increasing amounts of DmTTF. When DmTTF binding site was placed in the 'forward orientation' with respect to T3 promoter [template pDmTTF-term(A)], transcription by T3 phage RNA polymerase yielded mostly run-off transcripts, even in the presence of the highest amount of DmTTF, corresponding to a protein–DNA molar ratio of 13.5 (Fig. 3A, top, lane 5). The amount of terminated mole-

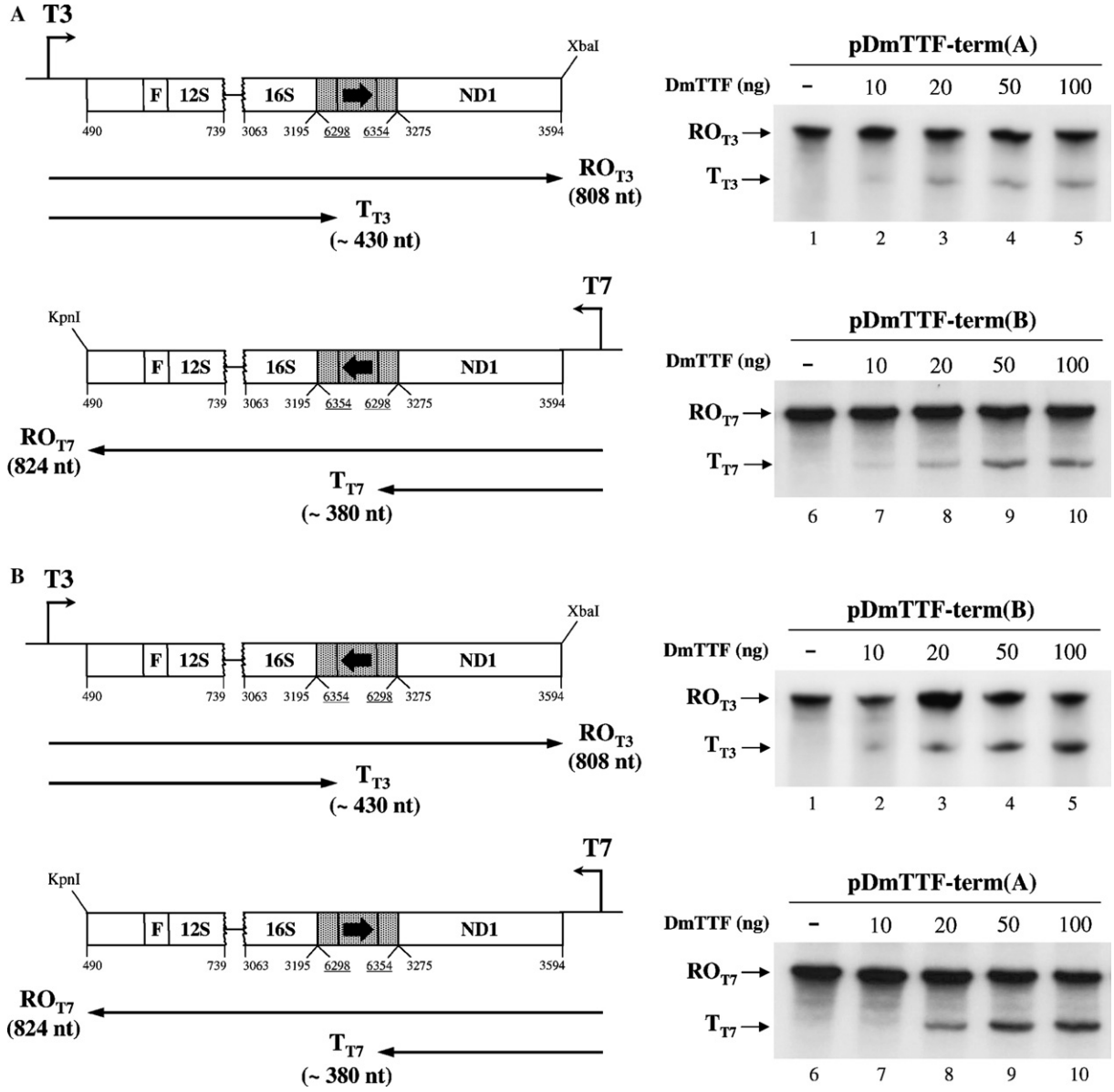

Fig. 3. Transcription termination activity of DmTTF toward bacteriophage RNA polymerases. (A) Transcription assay performed with T3 and T7 RNA polymerases moving in the direction of the 'forward orientation' of DmTTF-binding site. Left: schematic diagram of the DNA templates pDmTTF-term(A) and pDmTTF-term(B) (see legend of Fig. 2 for description) showing the position of T3 and T7 promoters, and DmTTF-binding site (filled box with enclosed arrow). pDmTTF-term(A) (top) and pDmTTF-term(B) (bottom) were linearized with *Xba*I and *Kpn*I, respectively. Run-off (RO_{T3}, RO_{T7}) and terminated (T_{T3}, T_{T7}) transcripts are indicated by arrowed lines. Right: autoradiogram of 5% polyacrylamide–7 M urea gels showing the run-off and terminated transcripts obtained with T3 (top) and T7 (bottom) RNA polymerases in the presence of the indicated template. (B) Transcription assay performed with T3 and T7 RNA polymerases moving in the direction of the 'reverse orientation' of DmTTF-binding site. Left: schematic diagram of the plasmid templates used. pDmTTF-term(B) (top) was linearized with *Xba*I, pDmTTF-term(A) (bottom) was linearized with *Kpn*I. Run-off (RO_{T3}, RO_{T7}) and terminated (T_{T3}, T_{T7}) transcripts are indicated by arrowed line. Right: autoradiogram of 5% polyacrylamide–7 M urea gels showing the run-off and terminated transcripts obtained with T3 (top) and T7 (bottom) RNA polymerases in the presence of the indicated template. Reactions (A,B) contained 20 μg/ml of properly linearized template DNA, 5 U of the bacteriophage polymerase, and either no DmTTF (–) or the indicated amount of protein.

cules was around 20% of the total transcripts, thus indicating that DmTTF is almost completely bypassed by the transcribing enzyme. A similar behavior was observed for T7 RNA polymerase transcribing the template pDmTTF-term(B) where the termination site was placed again in the 'forward orientation' with respect to the polymerase promoter (Fig. 3A, bottom, lane 10). Fig. 3B shows the results of the transcription assay on templates containing DmTTF binding site in the 'reverse orientation' with respect to the polymerase promoter. In this case, contrary to what was observed with the 'forward oriented' templates, the elongation of the RNA chain by both bacteriophage enzymes was considerably arrested by the presence of DmTTF in the assay, as the terminated molecules reached about 60% of the total transcripts (Fig. 3B, lanes 5, 10). The differences in the extent of termination were reproducibly observed in at least three independent experiments; they indicate that DmTTF termination activity on both T3 and T7 RNA polymerases is affected by the orientation of the protein–DNA complex, thus implying that the protein can arrest polymerases not simply by a physical blockage mechanism but by means of specific interactions with the transcribing enzyme.

Discussion

Mitochondrial transcription termination factors are sequence-specific DNA-binding proteins able to arrest progression of mitochondrial RNA polymerases. Two animal termination factors have been well characterized to date: mTERF in mammals [3,9] and mtDBP in sea urchin [5]. Although they share a common evolutionary origin and the same biochemical activity, the reshuffling of mitochondrial genes occurred during evolution, and the consequent variations of transcription mechanisms led those proteins to select different target sites on mtDNA. Recently, in *D. melanogaster*, where mitochondrial genes form four clusters alternatively distributed on the two strands [10], we have identified DmTTF, a third member of this protein family. This protein recognizes two target sites on mtDNA, both placed at the two boundaries between the 3′ ends of clusters of genes transcribed in opposite directions [6].

By using an in vitro transcription system, based on the capacity of a S-100 human mitochondrial extract to transcribe exogenous templates bearing a human mtDNA promoter and the DmTTF-binding site, we demonstrated that the protein is able to terminate RNA synthesis catalyzed by the mitochondrial RNA polymerase bidirectionally and with high efficiency, independently of the orientation of the protein–DNA complex. DmTTF was also able to terminate transcription, albeit with a lower efficiency, when RNA synthesis was catalyzed by T3 and T7 polymerases, but in such a case it exhibits a biased polarity. In particular, the protein–DNA complex is almost completely bypassed by the phage enzymes in the 'forward' orientation, whereas the protein is more active in terminating transcription in the 'reverse' orientation. The biased polarity exhibited by DmTTF suggests that the DNA-bound protein is structurally asymmetrical and exposes two different faces to RNA polymerases travelling on opposite directions. The 'forward side' of bound DmTTF appears to establish more specific protein–protein interactions with the enzyme, since it arrests almost exclusively transcription catalyzed by the mitochondrial RNA polymerase. On the contrary, the 'reverse side' of DmTTF is able to stop both phage and mitochondrial RNA polymerases, thus indicating that the enzyme-factor interactions are less specific. A similar behavior was described for sea urchin mtDBP; this protein specifically halted RNA polymerase that is transcribing the H-strand, whereas it imposes a possible road block to enzymes moving on the opposite direction [5].

The bidirectional termination activity of DmTTF towards mitochondrial RNA polymerase is consistent with the previous hypothesis of the existence of transcription termination sites in correspondence of both DmTTF target sequences, which are placed at the 3′ ends of clusters of genes transcribed in opposite directions [11]. At these locations the protein could not only serve to stop transcription but also to avoid the interference between the transcription machineries moving on the two strands.

The physiological significance of the asymmetry of DmTTF-DNA complex still remains to be clarified. It could be related to other additional functions of the protein, such as a possible regulation of mtDNA replication. This is suggested by recent observations indicating that sea urchin homologue mtDBP could regulate the D-loop expansion via a contrahelicase activity [P. Loguercio Polosa, personal communication]. In this regard, we cannot exclude the possibility of the existence of DmTTF-binding sites in the so-called A + T region; this is the very large non-coding region of *D. melanogaster* mtDNA where the existence of a replication origin was postulated [12].

The capacity of both sea urchin and *Drosophila* factor to terminate in vitro the transcription catalyzed by the human mitochondrial RNA polymerase implies that, despite the low conservation of their primary structure (19% of amino acid identity between DmTTF and mTERF, and 22% between mtDBP and mTERF), the recognition mechanisms between the enzyme and the termination factor have remained quite conserved during evolution. Moreover, it is interesting to observe that these three factors bind at least two sequences each; in fact, very recently it has been demonstrated that mTERF binds mtDNA not only at the boundary between 16S rRNA and $tRNA^{Leu(UUR)}$ genes but also in the D-loop region [13]. The existence of two protein target sites might be required to control the large number

of transcripts originated by the multiple transcription units of sea urchin and *Drosophila* mtDNA or to regulate the two H-strand transcription units operating in mammalian mitochondria. This can take place either by an independent binding at the two sites or by an interaction between the two protein factors bound to their distinct target sites.

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