Contrahelicase activity of the mitochondrial transcription termination factor mtDBP

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ABSTRACT

The sea urchin <u>m</u>itochondrial <u>D</u>-loop <u>b</u>inding <u>p</u>rotein (mtDBP) is a transcription termination factor that is able to arrest bidirectionally mitochondrial RNA chain elongation. The observation that the mtDBP binding site in the main non-coding region is located in correspondence of the 3′ end of the triplex structure, where the synthesis of heavy strand mitochondrial (mt) DNA is either prematurely terminated or allowed to continue, raised the question whether mtDBP could also regulate mtDNA replication. By using a helicase assay in the presence of the replicative helicase of SV40, we show that mtDBP is able to inhibit the enzyme thus acting as a contrahelicase. The impairing activity of mtDBP is bidirectional as it is independent of the orientation of the protein binding site. The inhibition is increased by the presence of the guanosine-rich sequence that flanks mtDBP binding site. Finally, a mechanism of abrogation of mtDBP contrahelicase activity is suggested that is based on the dissociation of mtDBP from DNA caused by the passage of the RNA polymerase through the protein–DNA complex. All these findings favour the view that mtDBP, besides serving as transcription termination factor, could also act as a negative regulator of mtDNA synthesis at the level of D-loop expansion.

INTRODUCTION

According to the traditional model, mammalian mitochondrial (mt) DNA replicates in a continuous, unidirectional and strand-asymmetric mode, starting from two specific origins of replication, O_H for the heavy (H) or leading strand, and O_L for the light (L) or lagging strand (1). Only a few initiation events at O_H result in the production of a complete genome; the nascent H-strand frequently terminates ∼500–1000 bp downstream of O_H (depending on the species) and, since the terminated strand remains annealed to the template, a typical triple-stranded structure is created that is called the displacement (D) loop. Recently, an additional replication model of coupled leading and lagging-strand mtDNA synthesis has been proposed (2,3).

In sea urchin, the present knowledge on mtDNA replication relies on early studies by Jacobs and coworkers who suggested the presence of an O_H in the main non-coding region (NCR) of *Strongylocentrotus purpuratus* mtDNA (4). The NCR (∼130 bp long) is much shorter than in vertebrates and includes a string of continuous guanosines and an AT-rich sequence that probably functions as transcriptional promoter. The D-loop structure associated with O_H is very short (∼80 bp) and the nascent strand seems to consist of an RNA primer of ∼60 nt joined to a small stretch of DNA chain (Figure 1, top panel), (5,6).

Regarding the molecular mechanisms that control the expansion of the D-loop structure, some DNA-binding activities with a putative role in this event have been identified in several species. In bovine Madsen *et al.* (7) characterized a *trans*-acting factor of ∼48 kDa, which binds a conserved sequence element (TAS, termination associated sequence) located upstream of the D-loop 3′ end. By means of *in vivo* footprinting, we detected in rat and human mitochondria two protein binding sites in the 3′ region of the D-loop (8).

In the Mediterranean species of sea urchin *Paracentrotus lividus* we identified the mitochondrial DNA-binding protein mtDBP (9). It binds two regions on sea urchin mtDNA, one located in the NCR, the other placed at the boundary of the oppositely transcribed genes for ND5 and ND6. Recently, we showed that mtDBP serves as bidirectional transcription termination factor, able to arrest RNA chain elongation on both strands (10). Interestingly, mtDBP binding site in the

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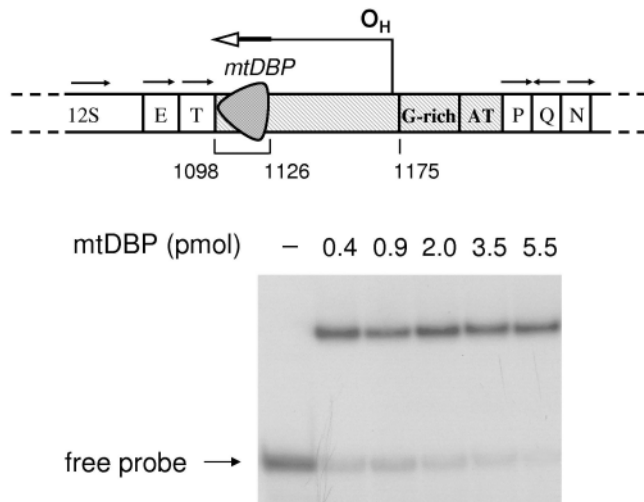
Figure 1. Gel mobility shift analysis of mtDBP to measure the protein:DNA ratio for the helicase assays. (Top) Schematic representation of the non-coding region, NCR, (grey bar) of *P.lividus* mtDNA displaying the mtDBP binding site occupied by the protein, the downstream G-stretch (G-rich) and the AT-rich sequence (AT). Numbers mark the position on mtDNA of the mtDBP binding site (square bracket) as from DNase I footprinting analysis (9) and the first guanosine of the G-stretch. Some genes flanking the NCR are shown. Horizontal black arrows indicate the direction of RNA transcription; O_H refers to the replication origin of the leading DNA strand; open arrow indicates the direction of DNA synthesis. The possible DNA portion of the D-strand is indicated as a bold line, the remaining RNA portion as a thin line. (Bottom) Gel shift analysis with increasing amounts of mtDBP. The assay was performed as described in Materials and Methods, in the presence of 5 fmol of a 46mer double-stranded oligonucleotide, containing the protein binding site, which was ^{32}P-labelled at its 5′ ends.

NCR is placed in correspondence of the 3′ end of the triplex structure, where H-strand DNA synthesis is either prematurely terminated or allowed to continue (Figure 1, top panel) (4,11). This observation raised the possibility that mtDBP may be involved in mtDNA replication; in particular, it could act as a negative regulator by antagonizing some enzymes of the replication machinery such as replicative DNA helicases.

In the present work, we wished to investigate whether the sea urchin DNA-binding protein mtDBP is able to exert a contrahelicase activity. Since an *in vitro* replicative system from sea urchin mitochondria is yet to be developed, we have used the helicase activity of the SV40 large T antigen (12), taking advantage of the fact that the viral enzyme has been used in several *in vitro* heterologous replication systems (13–15), and it has reproduced the properties of the specific helicases (16,17).

The data reported here demonstrate that mtDBP impairs the helicase activity of T antigen when bound to a template that is serving as helicase substrate. The contrahelicase activity is independent of the orientation of the protein binding site with respect to the direction of DNA unwinding; furthermore, the inhibitory effect of mtDBP is increased by the presence of the guanosine-rich sequence flanking mtDBP recognition site. In addition, we show that T7 RNA polymerase transcribing through DNA–mtDBP complex causes the dissociation of the protein from the complex, thus providing an interesting clue to the mechanism of contrahelicase activity abrogation.

MATERIALS AND METHODS

Proteins

SV40 T antigen, expressed in insect cells and purified, was a gift of Friedrich Grummt (13). To obtain the recombinant mtDBP, the cDNA coding for the mature version of the protein (9) was amplified by PCR and inserted into pBAD/His C expression vector (Stratagene) that had been digested with NcoI and EcoRI to eliminate the 6×His tag and the anti-Xpress Epitope. The recombinant protein was expressed in *Escherichia coli* TOP 10 cells by inducing a log-phase culture with 0.2% L(+)-arabinose for 3 h at 37°C. Bacteria were harvested by centrifugation and resuspended in 20 ml of lysis buffer (50 mM sodium phosphate buffer, pH 7.8, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, 2 mM EDTA and 1 mM DTT), containing 1 ml of Protease Inhibitor's Cocktail (Sigma). The suspension was sonicated on ice in short bursts and centrifuged at 130 000 *g* for 60 min at +4°C; the supernatant (S130) was diluted to 175 mM NaCl with a buffer containing 50 mM sodium phosphate buffer, pH 7.8, 10% glycerol, 2 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride and 0.5 µg/ml each of antipain, chymostatin, elastatinal, leupeptin and pepstatin A (Sigma). The diluted S130 was applied to Heparin-Sepharose CL-6B (Amersham Biosciences) equilibrated with a buffer containing 10 mM Tris–HCl, pH 8.0, 150 mM KCl, 10 mM MgCl₂, 1 mM EDTA, 10% glycerol, 1 mM DTT and protease inhibitors, as before. mtDBP was eluted with 500 mM KCl buffer; its buffer was changed into 100 mM KCl by Centricon 10 (Amicon). The purity of the protein was ∼80% as estimated by SDS–PAGE and Coomassie Brilliant blue staining.

Gel mobility shift assay

The reactions were performed in a 20 µl volume containing 50 mM Tris–HCl, pH 7.5, 8 mM MgCl₂, 0.1 mg/ml BSA, 0.5 mM DTT, 5 fmol of a double-stranded 46mer oligonucleotide probe containing the mtDBP binding site that was labelled at its 5′-termini with polynucleotide kinase (Roche) and [γ-32P]ATP, and varying amounts of mtDBP as indicated in the figure legend. The reactions were carried out at 25°C for 30 min and resolved on a 6% native polyacrylamide gel in 0.5× Tris-borate–EDTA (TBE) that was dried and analysed by phosphorimaging.

Helicase substrates

The single-stranded DNAs used in the helicase assays were prepared from recombinant pBluescript II KS(+) plasmids constructed as follows. Plasmid For was made by inserting into BamHI–XhoI digested plasmid a 157 bp fragment [nt 1090–1246, according to Cantatore *et al.* (6)] prepared by PCR on *P.lividus* mtDNA. Plasmid Rev was obtained as for plasmid For, except that the ends of the PCR-amplified DNA fragment were inverted. Single-stranded recombinant pBluescript was rescued by using M13K07 Helper Phage (Invitrogen) according to the manufacturer's instructions. Partial duplex DNAs for the helicase assays were prepared as follows. Oligonucleotides designed on *P.lividus* mtDNA and consisting of 46 nt (O-DBP.for, nt 1090–1135; O-DBP.rev, nt 1135–1090) and

110 nt (O-NCR.for, nt 1090–1199; O-NCR.rev, nt 1199–1090) were labelled with ^{32}P at their 5′-termini with polynucleotide kinase (Roche) and [γ-^{32}P]ATP; a 3-fold excess of each oligonucleotide was annealed to single-stranded DNA in a 10 µl reaction containing 50 mM NaCl, 6.6 mM MgCl$_2$, 6.6 mM Tris–HCl, pH 7.6. This procedure gave rise to substrates DBP.For, DBP.Rev, NCR.For and NCR.Rev. The helicase substrate that does not contain the G-rich stretch in the heteroduplex region (NCR-NoGs.For) was prepared by annealing, as described before, the 110 nt oligonucleotide O-NCR-NoGs.for (see Figure 3E), labelled with ^{32}P at its 5′-termini, to single-stranded DNA obtained from the recombinant plasmid pDBP.term2(R) (10). The unspecific substrate was prepared by annealing a 5′ end-labelled 20mer oligonucleotide [nt 6288–6307 of M13mp18(+)] to M13mp18(+) single-stranded DNA (Amersham Biosciences). The annealed DNAs were purified from unannealed oligonucleotides by Centricon 100 (Amicon) using the annealing buffer, according to the manufacturer's instructions.

DNA helicase assay

The reaction mixture (20 µl) contained 50 mM Tris–HCl, pH 7.5, 8 mM MgCl$_2$, 6 mM ATP, 0.1 mg/ml BSA, 50 mM K-Glutamate, 0.5 mM DTT, 13 pmol of SV 40 T antigen, 5 fmol of the DNA substrate and increasing amounts of mtDBP as indicated in figure legends. mtDBP-containing reactions were preincubated for 30 min at 25°C before adding SV40 T antigen. Reactions were then incubated for 30 min at 37°C and stopped by addition of 2 µl of stop solution (3.3% SDS, 50 mM EDTA, pH 8.0, 30% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol). The samples with no mtDBP always received the protein buffer corresponding to the highest volume of mtDBP used in the assay. Reaction products were separated by electrophoresis through a 10% non-denaturing polyacrylamide gel that was dried and analysed by phosphorimaging. The intensities of the bands were quantified by densitometry using the program ImageQuant (Molecular Dynamics).

Transcription-gel mobility shift assay

The template was an 895 bp fragment obtained by PCR on plasmid pDBP-term2(R) (10) using as primers the T7 promoter and A.For (nt 490–508) sequences. Template DNA (100 fmol) was incubated with recombinant mtDBP at 25°C for 15 min in a 20 µl mixture containing T7 RNA polymerase buffer [40 mM Tris–HCl, pH 8.0, 25 mM NaCl, 8 mM MgCl$_2$ and 2 mM spermidine-(HCl)$_3$], 28 U of RNaseOUT (Invitrogen), 0.5 mM each of ATP, CTP and GTP, and 0.05 mM UTP. Five units of T7 RNA polymerase (Invitrogen) were added and incubated for 1 min, following which 12 fmol of a [γ-^{32}P]-labelled 46mer double-stranded oligonucleotide containing the mtDBP binding site was added and incubation was continued for 30 min at 32°C. All the reactions, including those in the absence of transcription, contained T7 RNA polymerase buffer and nucleotides unless specified. The reactions were resolved in a 6% native polyacrylamide gel in 0.5× TBE that was dried and analysed by phosphorimaging.

RESULTS

The transcription termination factor mtDBP inhibits the activity of the replicative SV40 helicase in a bidirectional mode

In this work, we investigated whether the sea urchin protein mtDBP bound to its cognate site in the NCR of mtDNA is able to exert a contrahelicase activity. To assay this function, we used the replicative helicase of the virus SV40 (large T antigen). This is an ATP-dependent DNA helicase that translocates along a single-stranded template in 3′ to 5′ direction and unwinds DNA hybrids when it encounters a duplex region (12).

First, we wished to determine the protein:DNA ratio that yielded a complete saturation of the DNA probe by mtDBP. Therefore, we performed a gel mobility shift assay with different amounts of the recombinant protein varying from 0.4 to 5.5 pmol, in the presence of 5 fmol of the labelled probe containing mtDBP binding site. As reported in Figure 1, we observed that, beginning from 2 to 3 pmol up to 5.5 pmol of mtDBP, the DNA was almost completely bound, as very little free DNA was present; therefore we chose 3.2 pmol as the maximum amount of mtDBP for the contrahelicase assays described below.

To prepare the specific helicase substrates, we employed a circular single-stranded recombinant pBluescript bearing, in either orientation, the entire NCR of *P.lividus* mtDNA that includes mtDBP binding site and its flanking sequence. For the first helicase assay, the partial duplex was formed by annealing the 5′ end-labelled oligonucleotides containing the mtDBP binding site in both orientations to the complementary sequence present on the single-stranded DNA. This produced two partial duplex substrates, named DBP.For and DBP.Rev, each containing the protein binding site in one possible orientation (Figure 2A and C). Helicase assays were performed by incubating the substrates with the SV40 helicase. As reported in the autoradiograms shown in Figure 2B and D, lane 3, addition of 13 pmol of T antigen resulted, with both substrates, in the release of ∼60% of the annealed oligonucleotide. When substrate DBP.For was preincubated with increasing amounts of recombinant mtDBP and then exposed to the enzyme, we observed a progressive impairment of the helicase activity in a dose-dependent manner (panel B, lanes 4–7). When the substrate was reversed, mtDBP inhibited the helicase activity approximately at the same extent (panel D, lanes 4–7). In both cases, the DNA unwinding activity was almost completely abolished by 3.2 pmol of protein. These findings indicate that mtDBP possesses a bidirectional contrahelicase activity.

To rule out the possibility that the T antigen helicase activity could be inhibited in a non-specific manner by the free mtDBP rather than by the DNA-bound protein, we performed a helicase assay with a control non-specific substrate whose duplex region did not contain mtDBP binding site. No inhibitory effect was observed upon addition to the assay of 3.5 pmol of mtDBP (panel E, lanes 3 and 4). This indicates that the DNA unwinding activity of large T antigen is not inhibited either by the unbound mtDBP or by additional components present in the mtDBP-containing chromatographic fraction.
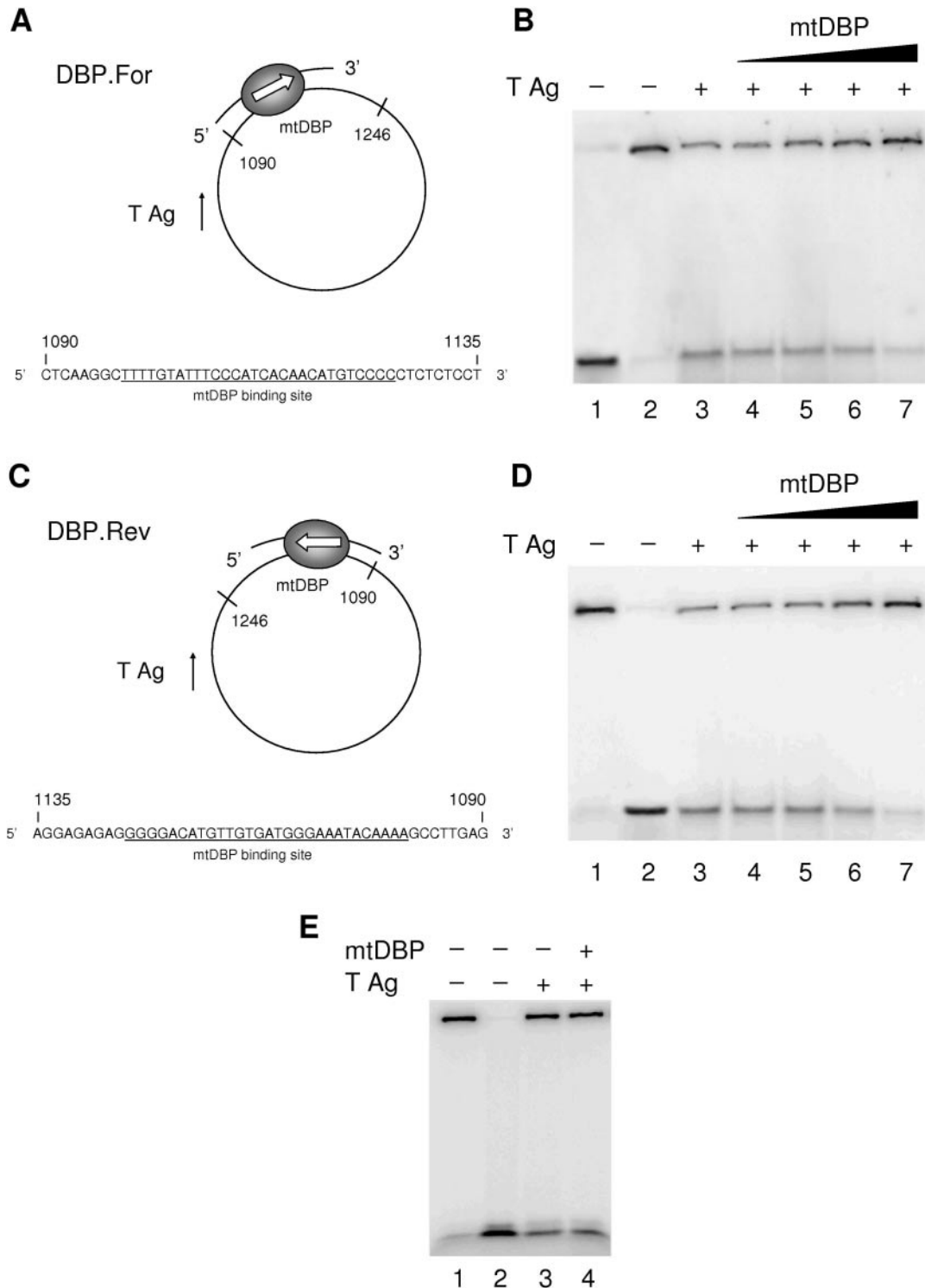
A

DBP.For

1090
|
5' CTCAAGGCTTTTGTATTTCCCATCACAACATGTCCCCCTCTCTCCT 3'
1135
mtDBP binding site

B

C

DBP.Rev

1135
|
5' AGGAGAGAGGGGGGACATGTTGTGATGGGAAATACAAAAGCCTTGAG 3'
1090
mtDBP binding site

D

E

Figure 2. Inhibition of SV40 T antigen helicase by DNA-bound mtDBP. (**A** and **C**) Schematic representation of DBP.For and DBP.Rev partial duplex substrates used in the unwinding reactions. The 46mer oligonucleotides O-DBP.for and O-DBP.rev containing the mtDBP binding sequence (underlined) were end-radiolabelled and annealed to the recombinant single-stranded DNA to construct the double-stranded region of DBP.For and DBP.Rev substrates, respectively. Numbers give the position of sea urchin mtDNA nucleotides; open arrow marks the orientation of the protein target site with respect to T antigen unwinding direction (black arrow). (**B** and **D**) Autoradiograms of representative displacement analyses showing the annealed partial duplex substrate (upper band) and the released oligonucleotide (lower band). Assays were performed as described in Materials and Methods, in the presence of 5 fmol of helicase substrate and, where indicated, 13 pmol of T antigen helicase. Lane 1 (B) and lane 2 (D), substrate heated to 100°C before loading; lane 2 (B) and lane 1 (D), substrate untreated; lanes 3–7, substrate incubated with either no mtDBP (lane 3) or increasing amounts of mtDBP (0.20, 0.60, 1.20 and 3.20 pmol in lanes 4–7, respectively). (**E**) Displacement analysis in the presence of unbound mtDBP, performed with the substrate lacking the protein binding site. Reactions contained 5 fmol of partial duplex M13mp18 obtained by annealing M13mp18 single-stranded DNA to a complementary 5′ end radiolabelled 20mer oligonucleotide: 13 pmol of T antigen and 3.5 pmol of mtDBP were used where indicated. Lane 1, substrate untreated; lane 2, substrate heated to 100°C before loading.

Having established that mtDBP possesses a contrahelicase activity, we asked whether such capacity was affected by the sequence flanking the protein binding site, in particular the 25-guanosine rich sequence that is placed ~50 bp downstream of mtDBP target site (Figure 1, top panel). To address this point, we constructed two partial duplex substrates (NCR.For and NCR.Rev) by annealing the single-stranded recombinant pBluescript to two 5′ end-labelled oligonucleotides spanning from mtDBP binding site to the stretch of guanosine residues, with both orientations (Figure 3A and C). The results of the experiments are reported in panels B and D. In the absence of mtDBP, the helicase caused DNA unwinding that released ~50% of the annealed oligonucleotide with both substrates (panels B and D: lane 3). Since the extent of release was similar to that observed with the short oligonucleotides, it results that neither the length of the heteroduplex region nor the presence of the G-rich element affected substantially the helicase activity. Addition of increasing amounts of mtDBP caused, with both substrates NCR.For and NCR.Rev, a progressive impairment of the helicase activity that was almost completely abolished already by 1.2 pmol of protein in the assay (panels B and D, lanes 4–7). The inhibitory effect of mtDBP did not remarkably change with the orientation of the binding site, thus confirming that mtDBP contrahelicase activity is orientation-independent. At this point, we endeavoured to test whether such increased helicase inhibition by mtDBP was caused by the G-rich element or rather by the fact that the heteroduplex region was longer (110 bp as compared with 46 bp). We constructed a helicase substrate lacking the G-string, whose partial duplex region consisted of a 75 bp region of sea urchin mtDNA bearing the mtDBP binding site in the forward orientation, flanked by additional sequences so as to yield a double-stranded region of 110 bp (Figure 3E). The autoradiogram displayed in panel F shows that the extent of helicase arrest by mtDBP on this substrate was substantially similar to that observed with the 46 bp duplex substrate (Figure 2B and D). A similar result was obtained with the substrate in the opposite orientation (data not shown). These data indicate that the ability of mtDBP to impede T antigen helicase activity was not substantially affected by the length of the heteroduplex region in the substrate but, rather, by the presence of the uninterrupted 25 bp guanosine sequence. Figure 4 summarizes the results of the helicase assays. It shows that, with the two long substrates containing mtDBP binding site and the G-rich stretch (NCR.For and NCR.Rev), 50% inhibition of the helicase activity was achieved with ~0.3 pmol of mtDBP. Almost a 4-fold excess of mtDBP was however needed to reach the same level of helicase impairment on the short substrates DBP.For and DBP.Rev as well as on the long heteroduplex substrate lacking the G-rich stretch (NCR-NoGs.For, top panel). Thus, we conclude that the presence of a G-rich element in the substrate reinforces the contrahelicase effect of mtDBP.

The DNA-bound mtDBP is dislodged by an invading RNA transcript

It has been demonstrated that in bacteria, when T7 RNA polymerase transcribes through the complex formed by the contrahelicase Tus and its cognate site on DNA, dislodging of the

protein from DNA occurs, that is followed by abrogation of helicase impediment (18). As a first step towards investigating whether a similar mechanism may take place also in sea urchin mitochondria, we analysed the capability of T7 RNA polymerase to dislodge bound mtDBP from its cognate DNA site when the enzyme is allowed to pass through the DNA–mtDBP complex. T7 RNA polymerase was chosen since we had previously shown that the enzyme is not arrested by the DNA-bound mtDBP when it travels in the direction opposite to that of DNA replication (10). We performed an experiment whose strategy is depicted in Figure 5, top panel. Briefly, a transcription assay was carried out on a template containing the T7 promoter upstream of the mtDBP binding site. A ^{32}P-labelled, double-stranded 46mer oligonucleotide containing the mtDBP site was added to the mixture to trap the protein that might be dislodged from the template during transcription. The values of DNA template–protein ratios used in the assay were such that there was undetectable or little free protein available for gel shift. The results of the experiment are shown in Figure 5, bottom panel. Whereas in the absence of transcription, due to the lack of RNA polymerase or NTPs in the assay, very little mobility shift was obtained (lanes 1, 3 and 4), transcription of the template produced a retarded band whose intensity was much stronger (compare lane 2 with 1 and lane 5 with 4). The retarded band had the same size as that produced by the purified mtDBP (compare lanes 2 and 5 with lane 7). These results can be explained by assuming that the passage of T7 RNA polymerase through the DNA–mtDBP complex caused the dissociation of mtDBP that was trapped by the labelled probe.

DISCUSSION

We have previously inferred that the DNA-binding protein mtDBP could play a complex role in regulating sea urchin mtDNA expression (10). The ability of mtDBP to bidirectionally arrest the progression of mtRNA polymerase would mainly serve two functions: to generate the 3′ end of H- and L-strand transcripts and, at the same time, to control the passage of the transcribing enzymes that are moving in opposite directions. Furthermore, since the sea urchin main NCR is a condensed version of the vertebrate regulatory region, with transcriptional and replicative elements being co-localized in a much shorter space (4), we proposed that mtDBP could also participate in regulating the mtDNA replicative process (10). In particular, mtDBP, bound to the target sequence placed near the 3′ end of the D-loop structure, could counteract enzyme(s) of the replicative machinery and determine the formation of the triple-stranded structure, thus acting as negative regulator of mtDNA replication. The results presented in this paper strongly support this view. We show that the mitochondrial transcription termination factor mtDBP possesses a contrahelicase activity. In fact, using partial duplex substrates that contain the protein binding site and its flanking region, we find that DNA-bound mtDBP inhibits bidirectionally the helicase activity of SV40 large T antigen. The sea urchin factor is to our knowledge the first documented example of a mitochondrial DNA-binding protein that, besides arresting RNA transcription, is also able to impede the DNA unwinding activity of a replicative helicase.
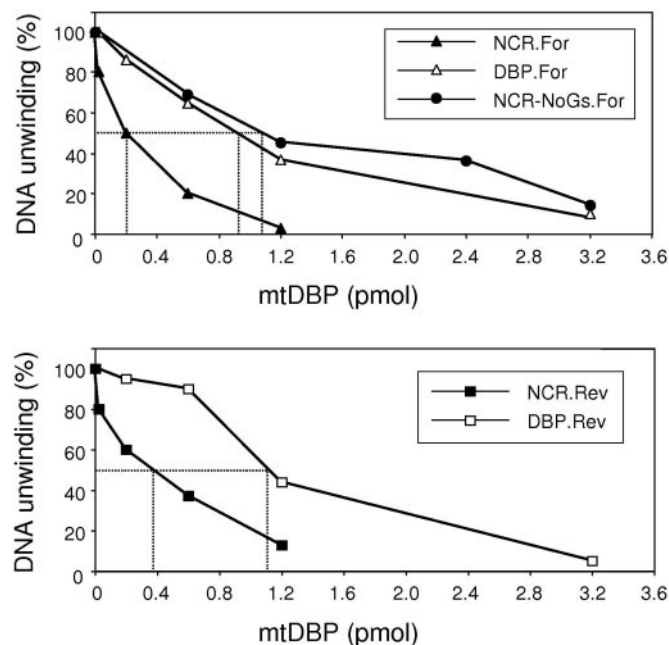
Figure 3. Effect of the G-rich element on the contrahelicase activity by DNA-bound mtDBP. (**A** and **C**) Schematic representation of NCR.For and NCR.Rev helicase substrates used in the unwinding reactions. The 110mer oligonucleotides O-NCR.for and O-NCR.rev, containing the mtDBP binding sequence and the G-stretch (underlined), were end-radiolabelled and annealed to single-stranded DNA to construct the double-stranded region of NCR.For and NCR.Rev substrates, respectively. Numbers give the position of sea urchin mtDNA nucleotides; open arrow marks the orientation of the protein target site with respect to T antigen unwinding direction (black arrow). (**B** and **D**) Autoradiograms of representative displacement analyses showing the annealed partial duplex substrate (upper band) and the released oligonucleotide (lower band). Assays contained 5 fmol of substrate and, where indicated, 13 pmol of T antigen. Lane 1, substrate untreated; lane 2, substrate heated to 100°C before loading; lanes 3–7, substrate incubated with either no mtDBP (lane 3) or increasing amounts of mtDBP (0.02, 0.20, 0.60 and 1.20 pmol in lanes 4–7, respectively). (**E**) Schematic representation of the partial duplex substrate lacking the G-rich stretch, used in the unwinding reactions. The 110mer oligonucleotide O-NCR-NoGs.for, containing the mtDBP binding sequence (underlined), was 5′ end radiolabelled and annealed to the recombinant single-stranded DNA prepared from plasmid pDBP-term2(R) (10). This procedure yielded the partial duplex NCR-NoGs.For substrate. (**F**) Autoradiogram of the gel showing a representative displacement experiment. Assays contained 5 fmol of substrate and, where indicated, 13 pmol of T antigen. Lane 1, substrate heated to 100°C before loading; lane 2, substrate untreated; lanes 3–7, substrate incubated with either no mtDBP (lane 3) or increasing amounts of mtDBP (0.60, 1.20, 2.4 and 3.2 pmol in lanes 4–7, respectively).

Figure 4. Quantitative analyses of the displacement reactions. Representative standard curves of the percentage of DNA unwinding by SV40 T antigen on substrates DBP.For, NCR.For and NCR-NoGs.For (Top) and DBP.Rev, NCR.Rev (Bottom) are shown. The percentage of DNA unwinding was plotted against the amount of mtDBP; dotted lines indicate the amount of protein needed to obtain 50% inhibition of the helicase activity. Extent of release of labelled oligonucleotides in the absence of mtDBP was set as 100%.

Figure 5. Fate of mtDBP when T7 RNA polymerase transcribes through mtDBP–DNA complex, as determined by combining RNA transcription with gel mobility shift assays. (Top) Diagrammatic representation of the experimental strategy. (Bottom) Autoradiogram of a 6% polyacrylamide gel showing the results of gel shift assay with the ^{32}P-labelled 46mer. Reactions were performed as described in Materials and Methods. DNA template: protein ratios were 1:6 and 1:13 in lanes 1–3 and 4–7, respectively. A transcription assay in the presence of [α-^{32}P]UTP was performed in parallel (data not shown).

Proteins acting as transcription terminators and displaying a contrahelicase activity have been described in prokaryotic as well as in eukaryotic systems. The replication termination factor Tus in *E.coli* binds to its cognate terminator sequence and arrests the replication fork in a polar manner by establishing specific protein–protein contacts with the replicative helicase (19). The same factor is also able to exert a polar block to RNA chain elongation (20). In eukaryotes, the DNA-bound transcription terminator factor TTF-I, which promotes arrest of RNA polymerase I, was also shown to terminate replication of the rDNA replicon by impeding in a polar manner the helicase activity of SV40 large T antigen (13). Interestingly, we found that mtDBP stops the T antigen helicase independently on the orientation of its binding site. The bipolar functionality of mtDBP agrees with its ability to arrest bidirectionally the elongating mitochondrial RNA polymerase. Similarly, the prokaryotic replication terminator protein RTP, when bound to the bipolar replication terminus of the plasmid pLS20, was shown to arrest both DNA helicase and RNA polymerase in a bidirectional mode (21). This evidence indicates that the polarity of transcriptional arrest is generally consistent with that of helicase impairment. The bidirectionality of mtDBP contrahelicase activity might be used to block helicases that unwind DNA from both directions, as it would occur if mtDNA replicates by the bidirectional strand-coupled mechanism (3).

The presence of the uninterrupted 25-guanosine stretch downstream of the mtDBP binding site reinforces the inhibitory effect of the protein, since such enhancement is not observed when a substrate of the same length but lacking the G-string is used in the contrahelicase assay. It is well
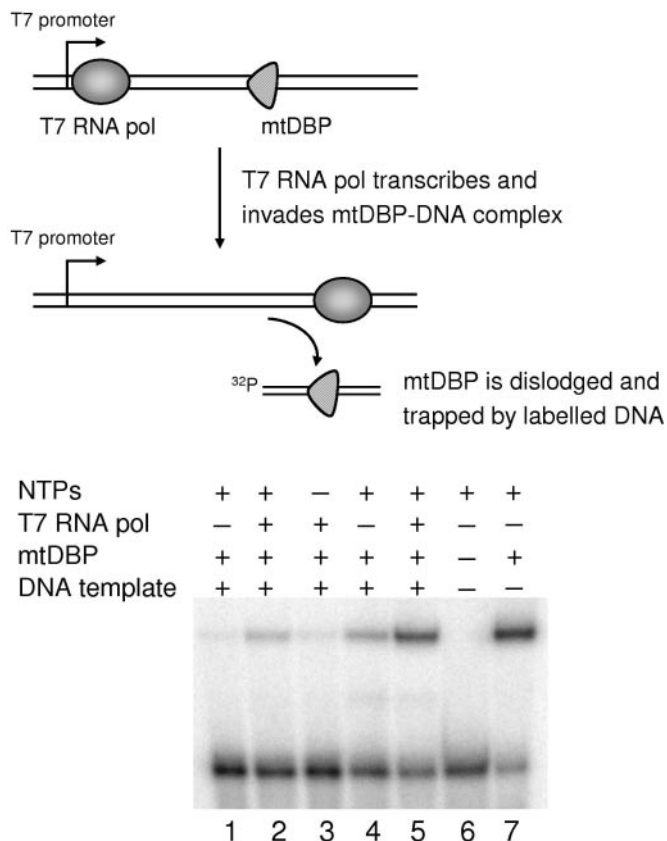
known that G-rich sequences can form higher order structures, such as a triple helix consisting of poly (dG-dG-dC) strands (22,23). However, the G-string doesn't seem to possess an intrinsic contrahelicase activity since we found that the annealed oligonucleotides containing such element are extensively released by the viral helicase. Thus, our findings point to a functional cooperativity between the DNA-bound protein and its flanking region in determining the arrest of the helicase. The role of G-rich elements in mediating the contrahelicase activity of a replication terminator has been reported also for TTF-I; in this case, the G-stretch is indispensable for the protein to exert its inhibitory activity (13).

The capability of mtDBP to arrest both the replicative helicase and the elongating mtRNA polymerase suggests that mtDNA replication and transcription are mutually interacting in the main NCR of sea urchin mitochondria. Some clues to a possible mechanism could be surmised from the mechanism of replication termination in prokaryotes. It has been reported that transcription invasion of the replication terminus abrogates the contrahelicase activity of Tus, as a result of its dislocation from DNA (18). We have investigated the impact of transcription on the DNA-bound mtDBP and have noted that the passage of the RNA polymerase through the protein–DNA
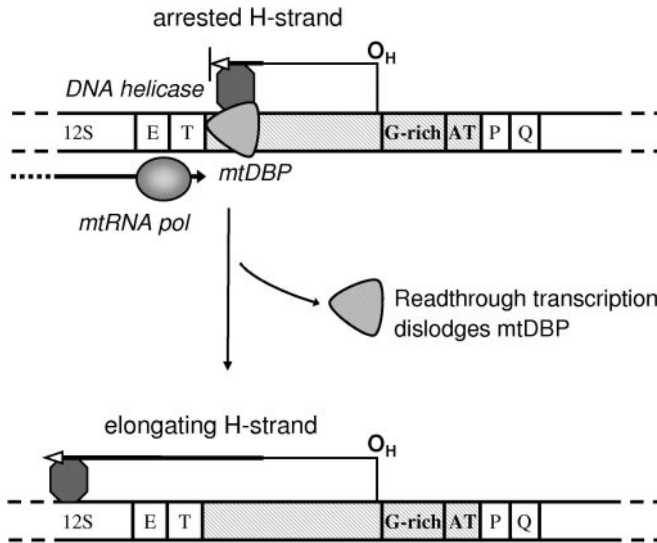
Figure 6. Diagram showing the suggested mechanism of abrogation of mtDBP contrahelicase activity by transcriptional invasion of the replication origin. The NCR of *P.lividus* mtDNA, including mtDBP binding site and the downstream regulatory elements (G-rich and AT-rich regions), is shown. Open arrow indicates the direction of H-strand DNA synthesis; filled arrow refers to the direction of RNA polymerase transcription. See legend to Figure 1 for description.

complex causes the dissociation of mtDBP from the DNA. On this basis, we present in Figure 6 a hypothetical model for mtDBP as the device that regulates the interplay between mtDNA transcription and replication. A possible change in the conformational state of bound mtDBP and/or a leakage of transcription termination could allow the movement through the complex of the mitochondrial RNA polymerase that is travelling oppositely to the direction of replication. The transcriptional passage would cause the dissociation of mtDBP from its target site and the subsequent abrogation of the helicase impairment. The existence of rare transcripts going beyond the transcription termination site and mapping in the main regulatory region (24) is consistent with the readthrough of the RNA polymerase. Taken together, these data invite speculation that permissive transcription through the DNA–protein complex might provide a mechanism for regulation of mtDNA synthesis. Our data should be a productive avenue for further investigations aimed at shedding light on the interplay between replication and transcription in animal mitochondria.

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Conflict of interest statement. None declared.

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