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Two distinct, sequence-specific DNA-binding proteins interact independently with the major replication pause region of sea urchin mtDNA

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ABSTRACT

We have identified a second DNA-binding protein in sea urchin embryo mitochondria, which interacts with a binding site in the major replication pause region, at the junction of the genes for ATP synthase subunit 6 and cytochrome *c* oxidase subunit III (COIII). We provisionally designate this protein mtPBP2, to distinguish it from the previously characterized mitochondrial pause-region binding protein mtPBP1, whose properties and binding site are quite distinct. The high-affinity binding site for mtPBP2 lies at the 5′ end of the COIII gene, and exhibits partial dyad symmetry, although modification interference analysis indicates that recognition is complex. Binding of mtPBP2 to this site induces a bend of approximately 45° in the DNA. Southwestern blots show that mtPBP1 and 2 are both single polypeptides, of apparent molecular weights 25 kD and 18 kD respectively. *In vitro*, mtPBP1 and mtPBP2 bind independently to their high-affinity sites, which are separated by about 50 bp.

INTRODUCTION

Despite the wealth of sequence information on mtDNA from various taxa, as well as the detailed studies, dating from the 1970s, demonstrating that it replicates by a strand-asymmetric mechanism similar to that of some prokaryotic extrachromosomal elements (1), we remain largely ignorant of the biochemistry of metazoan mtDNA replication. Several aspects of the mtDNA replication cycle in animal cells remain particularly mysterious. These include the rationale and mechanisms responsible for the creation and maintenance of the D-loop intermediate, the remarkably slow overall speed of DNA synthesis, as compared with almost all other systems, the precise mechanism of lagging-strand priming, the systems for preventing or reversing the formation of genomic multimers, and the steps leading to the formation of deleted molecules in certain pathological states. In many of these phenomena, the likely involvement of protein(s) binding specifically to the DNA template can be or has been invoked (e.g. see Refs. 2, 3), especially by analogy with prokaryotic systems (4–6), but such proteins remain largely undocumented, or at best, poorly characterized.

In previous studies in the sea urchin, we have made observations relevant to some of the above issues. We have demonstrated the existence of a major pause site for leading-strand replication in sea urchin mtDNA (7), at the gene boundary between ATP synthase subunit 6 (A6), and cytochrome *c* oxidase subunit III (COIII). This site co-incides with a prominent origin of lagging-strand synthesis, suggesting that the two phenomena may be related. Protein binding studies, using as substrate a cloned fragment of sea urchin mtDNA spanning this junction, identified a protein from blastula stage mitochondria (8), which interacts specifically with a sequence of the A6 gene, located about 50 bp upstream from the gene junction. This protein, designated mtPBP1 (for mitochondrial pause-region binding protein), binds to the sequence AGCCTN$_7$AGCAT.

In this paper, we present data indicating the existence of a second sea urchin mitochondrial protein, which binds specifically to a distinct site in this region, in this case located exactly at the 5′ end of the COIII gene. This protein has properties which distinguish it clearly from mtPBP1, and the two proteins appear to interact independently with their binding sites. We provisionally designate the newly identified protein as mtPBP2, which should not be taken to imply any particular function. We suggest that the two proteins may perform distinct roles connected with replication pausing and lagging-strand initiation, although their exact functions remain to be determined by *in vitro* assays.

MATERIALS AND METHODS

Purification of mitochondria from blastulae

Mitochondria were prepared from 24-hour blastula stage embryos of the sea urchin *S.purpuratus* by Dr F. J. Calzone (University of California, Irvine) as described previously (8).

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Heparin–sepharose chromatography

Mitochondrial lysis and heparin–sepharose (Pharmacia) chromatography were performed as described previously (8, 9). After washing the column with 0.2 M KCl in buffer 'A' the proteins were eluted with successive steps of 0.4 M and 0.6 M KCl. Protein-containing fractions were pooled, concentrated by ultrafiltration (Millipore) or ammonium sulfate precipitation, dialysed against a buffer containing 10 mM Tris–HCl (pH 8.0), 0.1 M KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM EDTA and 50% glycerol, and stored at −20°C. Protein concentrations were determined by the Bradford assay (Biorad).

Southwestern blots

Proteins were electrophoresed on a 12% Laemmli gel (10) and electroblotted onto a 'Hybond-C super' nitrocellulose membrane (Amersham) in 20 mM Tris–HCl (pH 8.0), 150 mM glycine, 0.005% SDS and 20% methanol, over 5–6 hours at 4°C. All subsequent steps of denaturation and renaturation of immobilized proteins, as well as DNA-binding and washing, were carried out at 4°C according to Vinson *et al.* (11).

Protein elution and renaturation from SDS-polyacrylamide gels

Proteins (70 μg per lane) were separated on Laemmli gels (10) and 0.5 cm slices were cut out. Protein elution, precipitation and renaturation were essentially according to Hager and Burgess (12), with the exception that the renaturation buffer did not contain NaCl.

Oligonucleotides

Equal amounts of the two complementary, HPLC-purified, single-stranded, mtPBP2 binding site oligonucleotides 5'-CCCAAATTATGGCTCATCAACACCCATATCAT-3' (sense) and 5'- AAATGATATGGGTGTTGATGAGCCATAATTTG-3' (antisense; Oswell DNA Service, Edinburgh), were annealed by heating at 65°C with gradual lowering of the temperature to 20°C in a buffer containing 20 mM Tris–HCl (pH 7.6), 5 mM $MgCl_2$, 0.1 mM DTT and 0.01 mM EDTA. The two mtPBP1 binding-site oligonucleotides described previously (8), 5'-AAATAGGAGTAGCCTGCATCCAAGCATAT-3' (sense) and 5'-AATACATATGCTTGGATGCAGGCTACTCC-3' (antisense), were annealed under identical conditions.

Recombinant clones

Plasmid AM62, containing nt 9148–9401 of *S.purpuratus* mtDNA (13), across the A6-COIII gene boundary (see Fig. 1), was provided by Dr A. G. Mayhook. The *Dra*I-*Hin*dIII fragment from AM62 was subcloned into *Sma*I/*Hin*dIII-digested pSP6/T7 (BRL). Cloning of the double-stranded oligonucleotide containing the mtPBP2 binding site was carried out by filling in the 3'-recessed ends using Klenow enzyme (Boehringer Mannheim), and ligation into the *Sal*I site of pBEND2 (14), which had also been made blunt by end-filling. Recombinant plasmids were identified, and sequenced by the dideoxy chain termination method (15). Several errors in the published sequence came to light and have been reported to the EMBL data library.

Polymerase chain reaction

Primers for the polymerase chain reaction consisted of the following sequences: 5'-AAATAGGAGTAGCCTGCATCCAAGCATAT-3' (nt 9256–9284, mtPBP1 sense strand, as above) and 5'-GGGTCATGGGCTTTGGTCTACTAAA-3' (nt 9380–9356). PCR was carried out in a 50 μl reaction, which, in addition to the *Taq* polymerase (Promega) and its buffer, contained dNTPs, 1 μg of each of the primers and 1 ng of AM62 DNA. The product, after 30 cycles of amplification, was analyzed on a 5% (non-denaturing) polyacrylamide gel.

DNA probes

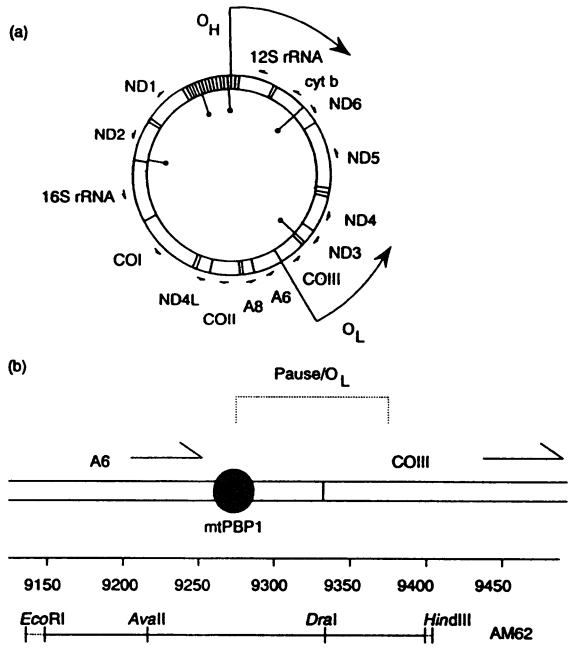
The double-stranded oligonucleotides and the DNA restriction fragments were labelled by filling in the 3' recessed ends with the appropriate ^{32}P-deoxynucleotides (NEN, 3000 Ci/mmol) in the presence of Klenow DNA polymerase. Labelling of the 125 bp PCR fragment was carried out by taking approximately 10 ng of the amplified DNA and putting it through a single cycle of PCR in a 10 μl reaction containing dATP, dCTP, dGTP and 30 μCi of ^{32}P-TTP (3000 Ci/mmol). After extension at 72°C for 5 minutes, 10 μl of a mixture containing cold TTP in *Taq* DNA polymerase buffer was added, and the reaction incubated for a further 10 minutes at 72°C.

Electrophoretic mobility shift assays (EMSAs) and DNase I footprinting

EMSAs were carried out as described previously (8), using 0.1–0.2 ng of labelled DNA, in the presence of various protein fractions and nonspecific competitors (see Figure legends). Binding reactions were carried out for 30 minutes at 4°C and loaded directly onto a 5% polyacrylamide gel prepared in 0.5×TBE (pH 8.3) and precooled at 4°C. EMSAs using fragments derived from recombinant pBEND2 were electrophoresed on 8% polyacrylamide gels under the same conditions. DNase I footprinting on the *Dra*I-*Hin*dIII fragment of AM62, recloned in pSP6/T7 (see above), was carried out as described earlier (8), with the exception that the products were analyzed on a 12% sequencing gel. Maxam–Gilbert chemistry (16) was carried out on Hybond M&G paper (Amersham) according to the manufacturer's instructions.

Protein–DNA contact analysis

All modification interference experiments were carried out on the synthetic double-stranded oligonucleotide harboring the mtPBP2 binding site. Methylation of DNA with dimethylsulfate (Aldrich) was performed essentially as described by Maxam and Gilbert (16), with the reactions incubated at room temperature for seven minutes and stopped by chromatography on a Sephadex G-50 (Pharmacia) spun column equiliberated with TEN buffer [10 mM Tris–HCl (pH 8.0), 1 mM EDTA and 0.1 M NaCl]. For thymine-specific modifications each of the strands was treated with $KMnO_4$ (Sigma) according to Truss *et al.* (17) for 5 minutes, before stopping the reaction by chromatography on a spun column as described above. The two modified strands were then annealed in TEN. Ethylation of DNA was carried out with ethylnitrosourea (ENU, Sigma) for 20 minutes under the conditions described by Siebenlist and Gilbert (18). Modified labelled DNAs were precipitated, and used in a ten-fold scale-up of the EMSAs as described above. Following electrophoresis the free and the bound DNAs were detected by autoradiography, excised, eluted and precipitated. ENU-modified DNA was cleaved in a solution containing 10 mM phosphate buffer (pH 6.8), 1 mM EDTA and 150 mM NaOH at 90°C for 30 minutes, whereas DMS- and $KMnO_4$-modified DNAs were cleaved in 10% piperidine at 90°C for 30 minutes, before electrophoresis on a 20% sequencing gel.



(c)

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                    mtPBP1 binding site
9251 GCTAGAAATAGGAGTAGCCTGCATCCAAGCATATGTATTCACTGCTCTAA

                          DraI   COIII start
9301 TTCATTTCTACCTACAACAGAACATTTAAATTATGGCTCATCAACACCCA

                                                HindIII
9351 TATCATTTAGTAGACCAAAGCCCATGACCCTTGACAGG....(AAGCTT)
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Figure 1. Organization of the *S.purpuratus* mitochondrial genome. (**a**) Schematic physical map of sea urchin mtDNA showing the rRNA and protein coding genes with their transcriptional orientations indicated by half-arrows. The replication origins are denoted using the mammalian nomenclature: the leading-strand origin as O_H, and the prominent origin of lagging-strand synthesis identified in earlier studies, as O_L, with the direction of synthesis in both cases shown by bold arrows. (**b**) Map of clone AM62, used in this study, with the genes indicated as A6 (ATP synthase subunit 6) and COIII (cytochrome *c* oxidase subunit III), and the mtPBP1 binding-site shown as a shaded circle. Restriction sites are as indicated, with the remains of the polylinker of the pSP64 vector shown as a broken line. Map co-ordinates are based on Ref. 13. (**c**) Corrected sequence of the A6/COIII gene junction region of *S.purpuratus* mtDNA, showing the mtPBP1 binding site (shaded box), the COIII start codon (underlined) and the *Dra*I restriction site of AM62.

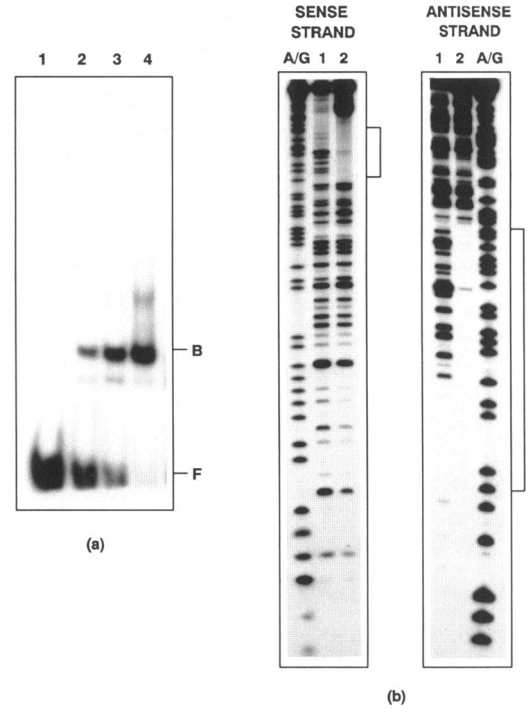


Figure 2. Localization of the mtPBP2 binding site. (**a**) EMSAs using the 78 bp radiolabelled *Dra*I-*Hin*dIII fragment of AM62 (see Fig. 1). Proteins eluted from the heparin−sepharose column in the 0.4−0.6 M KCl step were used as follows: (lane 1) no protein, (lane 2) 1 μg, (lane 3) 2 μg and (lane 4) 3 μg. The bound (B) and free (F) DNAs are shown. (**b**) DNase I footprinting of the sense and the antisense strands of the *Dra*I-*Hin*dIII fragment of AM62, recloned in pSP6/T7. The two strands were labelled, respectively, at the *Hin*dIII site and at the *Eco*RI site of the vector polylinker. Marker tracks are Maxam−Gilbert (A/G) sequencing ladders (16) of the strands indicated. Footprinting reactions contained: (lane 1) no protein and (lane 2) 3 μg of the 0.4−0.6 M protein fraction. The protected regions are shown by brackets. All reactions contained 1 μg of double-stranded poly(dG-dC) as the nonspecific competitor.

RESULTS

Mapping of the mtPBP2 binding site

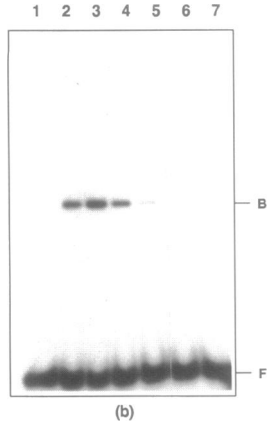
Preliminary EMSA experiments (not shown) detected a blastula-stage sea urchin mitochondrial protein, eluting from heparin−sepharose between 0.4−0.6 M KCl, and distinct from the previously characterized mtPBP1 (Ref. 8), that bound specifically to a sequence within the pause region. To locate the binding site for this protein, EMSA was carried out on the *Dra*I-*Hin*dIII fragment of plasmid AM62 (see Fig. 1), which contains the 5′ end of the COIII gene, in the presence of increasing amounts of the 0.4−0.6 M heparin−sepharose protein fraction, and a constant, large excess of double-stranded poly(dG-dC) as a nonspecific competitor. This experiment revealed the presence of a single major complex, the amount of which increased quantitatively with increasing protein concentration (Fig. 2a), as well as two very minor complexes (which were not investgated further). To map the binding site for the protein more precisely, DNase I footprinting was carried out on this fragment, recloned into pSP6/T7, under conditions in which all of the substrate DNA (as judged by EMSA) was bound. This experiment identified a 30 bp region on both strands, which was resistant to cleavage by DNase I (Fig. 2b). The protected region maps precisely to the 5′ end of the COIII gene. Its location right at the end of the fragment makes it rather difficult to see the end of the antisense-strand footprint, which upon longer exposure is more prominent (data not shown). The binding site for this protein, which we designate mtPBP2, is centred on the imperfect palindrome TATGGCTN$_7$ACCCATA.

Binding studies using the mtPBP2 binding-site oligonucleotide

Based on the footprinted region, a 34 bp double-stranded oligonucleotide was synthesized (Fig. 3a), and used in EMSAs in the presence of various protein fractions and nonspecific competitor DNAs. The results (Fig. 3b) showed that complexes were formed in the presence of various nonspecific DNAs, and that double-stranded poly(dA-dT) was the least and the plasmid pSP6/T7 the most effective nonspecific competitors. EMSAs using the boiled 0.2−0.4 M KCl heparin−sepharose fraction, which contains active mtPBP1, did not result in any complex formation under the conditions used. The thermostability of

5'-CCCAAATTAT GGCTCATCAA CACCCATATC ATTT-3'
3'-GGGTTTAATA CCGAGTAGTT GTGGGTATAG TAAA-5'

(a)



(b)

Figure 3. EMSAs with the mtPBP2 binding site oligonucleotide. (**a**) Sequence of the 34 bp oligonucleotide (oligo-PBP2), derived from the DNase I footprint. (**b**) EMSAs using the radiolabelled oligo-PBP2 in the presence of various nonspecific DNA competitors and protein fractions. Lane 1: no protein; lanes 2–5: 1 μg 0.4–0.6 M protein fraction plus 1 μg, respectively, of double-stranded poly(dG-dC), poly(dA-dT), poly(dI-dC) and the plasmid pSP6/T7 as nonspecific DNA competitors; lanes 6 & 7: 1 μg heat-treated (100°C for 2 minutes) 0.4–0.6 M and 0.2–0.4 M protein fractions, respectively, along with 1 μg double-stranded poly(dG-dC).

mtPBP2 was also tested by boiling the 0.4–0.6 M fraction for 2 minutes prior to using it in EMSA. This procedure resulted in complete loss of DNA-binding activity: therefore, in contrast to mtPBP1, mtPBP2 is thermolabile.

Characterization of the mtPBP2 binding site by chemical modification interference

To identify nucleotides which serve as critical contact points for sequence-specific DNA binding by mtPBP2 modification interference studies were carried out using the 34 bp oligonucleotide. DMS methylation of the two guanine residues on the sense strand reproducibly had no significant effect on binding (data not shown). Modification of the guanines on the antisense strand, however, yielded a wealth of information (Fig. 4a), identifying a total of six G residues, methylation of which impeded protein binding.

Thymine C-5/C-6 oxidation interference proved problematic, due to the hyporeactivity of many of the T residues in the binding site. This was not surprising, considering that the binding site contains a substantial region of dyad symmetry. This would presumably allow each of the strands to assume a secondary structure, rendering some of the thymine residues unavailable for oxidation by $KMnO_4$. Consequently, these experiments resulted in the identification of only a single T residue of the antisense strand which was found to interfere strongly with binding after modification (Fig. 4b).

Ethylation modification interference studies were used to identify phosphates in the binding site which are critical for binding. The resulting footprints (Fig. 4c) indicate that the principal contacts with phosphates from the two strands are made in successive turns (rather than the same turn) of the DNA double helix, corresponding approximately with the two copies of the inverted repeat. The findings of the modification interference experiments are summarized in Figure 4d.

DNA bending by mtPBP2

Since the structure of the mtPBP2 binding site resembles that of many other proteins which have been implicated in bending the DNA upon binding (e.g. see Ref. 19), we carried out experiments to investigate whether mtPBP2 also bends DNA. For this the 34 bp oligonucleotide containing the mtPBP2 binding site was cloned into the *Sal*I restriction site of pBEND2 (14). The recombinant was digested separately with each of four restriction enzymes (*Bgl*II, *Xho*I, *Xma*I and *Bam*HI), in order to produce DNA fragments which were identical in length but which differed from one another with respect to the location of the mtPBP2 binding site. Figure 5a shows the position of the binding sites in the four fragments. Each fragment was labelled and used in EMSA with the 0.4–0.6 M heparin–sepharose protein fraction, the products being analysed on an 8% polyacrylamide gel. The result of this experiment (Fig. 5b) showed that in the bound form the *Bgl*II fragment (where the binding site is near one end) had the fastest, whereas the *Xma*I fragment (where the binding site is closest to the center) had the slowest mobilities on the gel. The *Xho*I fragment, where the binding site is about 20 bp from the center, migrated in the bound form slightly faster than the bound *Xma*I fragment. These data are consistent with the observation that DNA molecules which are bent in the middle have a tendency to move comparatively slower in a polyacrylamide gel than those in which the bend is further away from the center. It may be noted that although the free form of the *Xma*I fragment cannot be seen in the exposure shown, all four free fragments migrated identically, indicating that the mtPBP2 binding site is not intrinsically bent.

To translate electrophoretic data numerically, the relative mobility, R_L, was estimated by dividing the distance travelled by the slowest complex by the distance travelled by the fastest complex, giving a value of 0.92. Substituting this value into the equation $R_L=\cos(\alpha/2)$ (where α is the bend angle; Ref. 20), which has been optimized for R_L values derived from an 8% polyacrylamide gel, we calculate α to be 46°. The resolution of the bending experiment is insufficient to delineate the exact location of the bend within the 34 bp sequence. However, careful inspection of the DNAse I footprint indicates that the bend probably occurs in the middle of the imperfect palindrome.

mtPBP1 and mtPBP2 interact independently with their binding sites

Previous studies (8) defined the mtPBP1 binding site as the sequence $AGCCTN_7AGCAT$, located about 50 bp from the newly characterized binding site for mtPBP2. To test whether the two proteins would bind independently to their respective sites or whether some long-range effect of the binding of one of the factors would influence (positively or negatively) that of the other, PCR was used to amplify the 125 bp region spanning the pause region and containing the binding sites for both proteins. This fragment was radioactively labelled and used in EMSA in the presence of the boiled 0.2–0.4 M protein fraction, which contains mtPBP1 activity, or the 0.4–0.6 M fraction, or a combination of both. The result is shown in Figure 6, and illustrates that, in the presence of both factors, a ternary complex consisting of both proteins bound to the DNA does form. The fact that it is a ternary complex can be demonstrated by gradually

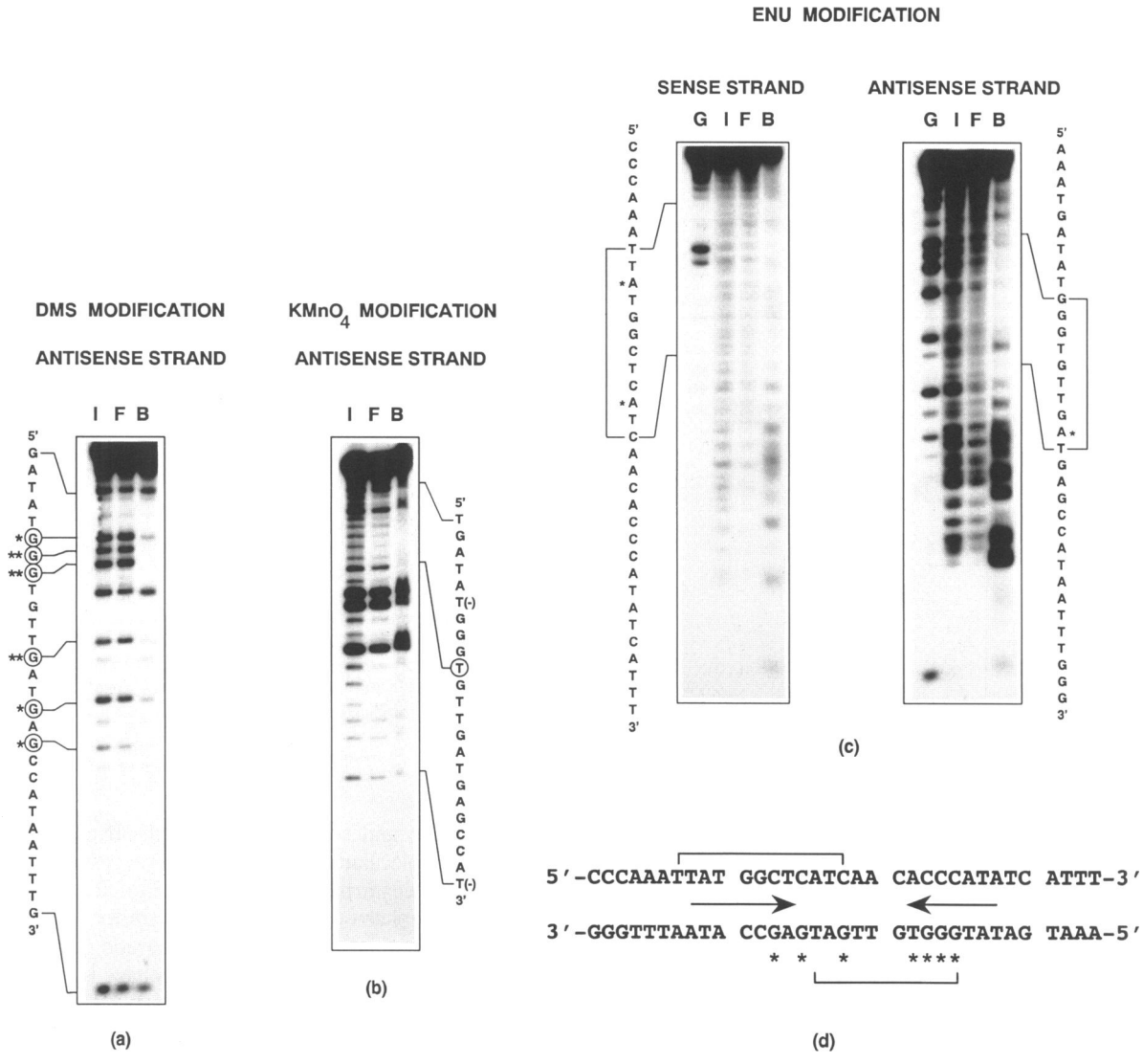


Figure 4. Probing of the mtPBP2 contact points by DMS, $KMnO_4$ and ENU modification of the 34 bp oligonucleotide. (**a**) Methylation and (**b**) oxidation interference analysis of the antisense strand; (**c**) ethylation interference analysis of the sense and antisense strands. After modification with the various reagents the DNA was used in a preparative EMSA. The bound (B) and free (F) DNAs were recovered, and together with the input (I) DNAs, cleaved under appropriate conditions and electrophoresed on a 20% sequencing gel. Marker tracks in (c) are Maxam–Gilbert (G) sequencing ladders (16). Residues whose modification interfered with mtPBP2 binding are circled, in (a) and (b), and shown by brackets in (c). T residues hyporeactrive to oxidation are denoted in (b) as T(−). Asterisks are used in (a) to distinguish strong and weak interference, and in (c) to denote residues within the ENU footprints whose modification did not appear to interfere with binding. Note that as a result of ENU modification, bands in (c) migrate as doublets. (**d**) Summary of the modification interference data: modified bases which interfere with mtPBP2 binding are asterisked, the ENU footprints are shown as brackets, and the partial dyad symmetry of the binding site is denoted by arrowheads.

reducing the amount of mtPBP2 from the binding reaction and monitoring the proportional decrease in its intensity. Confusingly, the complexes formed by this fragment individually with either mtPBP1 or mtPBP2 comigrate under these gel conditions. However, competition experiments (not shown) using the respective binding-site oligonucleotides showed clearly that the proteins binding to the PCR fragment in these complexes are mtPBP1 and 2, respectively. Furthermore, the two protein fractions tested in this experiment are devoid of the reciprocal activity under the conditions used. Although the amount of binding in this experiment has not been quantitated precisely, the data of Figure 6 indicate clearly that the two proteins do not bind co-operatively, nor do they interfere appreciably with each other's binding.

Southwestern blots

In order to determine whether mtPBP1 and 2 are single polypeptides, and if so, of what molecular weights, mitochondrial proteins, partially purified on heparin–sepharose columns, were electrophoresed and transferred to nitrocellulose filters. After renaturation, the immobilized proteins were reacted separately with oligonucleotides containing each of the binding sites. These Southwestern blots identified a single prominent polypeptide of an apparent molecular weight of about 25 kD, binding strongly to the mtPBP1 oligonucleotide, in addition to three other minor bands (Fig. 7a). The identity of the 25 kD band as mtPBP1 was confirmed by EMSA, using renatured proteins recovered from gel slices (Fig. 7b). Similarly, a single polypeptide of 18 kD was

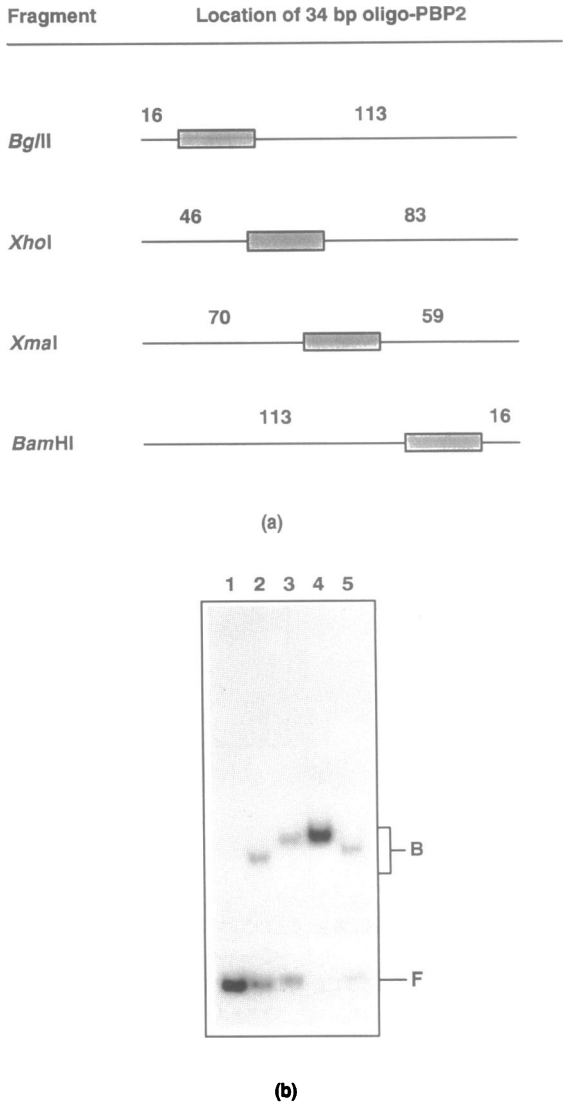


Figure 5. DNA bending by mtPBP2. (**a**) Location of the 34 bp oligo-PBP2 (shaded box) within the 163 bp *Bgl*II, *Xho*I, *Xma*I and *Bam*HI fragments. Note that the mtPBP2 binding site as defined by the above experiments is slightly displaced from the centre of the oligonucleotide. (**b**) EMSAs using the four radiolabelled fragments described above, on an 8% polyacrylamide gel. Lanes 2–5 contained *Bgl*II, *Xho*I, *Xma*I and *Bam*HI fragments, respectively. With the exception of lane 1 (*Bgl*II fragment, no protein), all reactions contained 1 μg of the 0.4–0.6 M protein fraction and 1 μg double-stranded poly(dA-dT). Bound (B) and free (F) DNA fragments are indicated.

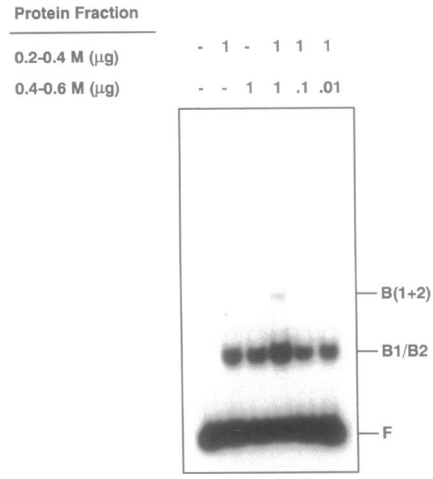


Figure 6. EMSA using the radiolabelled 125 bp PCR fragment (see text) and the various protein fractions containing mtPBP1 and mtPBP2. The amount of protein used in each of the binding reactions, which contained 1 μg double-stranded poly(dA-dT) as non-specific competitor, is indicated. The 0.2–0.4 M fraction was boiled before use. The various bands are denoted as follows: F—free DNA; B1, B2—complexes formed, respectively, with mtPBP1 or mtPBP2 alone; B(1+2)—ternary complex containing both mtPBP1 and mtPBP2. The proportion of probe bound by mtPBP1 in the presence of different amounts of mtPBP2 appears to be invariant, indicating that the two proteins interact independently with their binding sites.

found to interact with the mtPBP2 oligonucleotide (Fig. 7c). In both cases, the reactive polypeptide was detected only weakly, if at all, by labelled 'non-specific' control DNA, which nevertheless reacted strongly with other polypeptides.

DISCUSSION

In this study we have identified a second protein which binds specifically to a DNA sequence at the A6/COIII gene boundary of sea urchin mtDNA, adjacent to the previously characterized (8) binding site for mtPBP1 (mitochondrial pause-region binding protein 1). We propose to name this protein mtPBP2, since it is clearly distinguishable from mtPBP1 by the following criteria: the location and sequence of its high-affinity binding site, its apparent molecular weight on SDS polyacrylamide gels, as judged by Southwestern blots, its fractionation properties on heparin–sepharose and its thermo-lability.

DNase I footprinting, electrophoretic mobility shift and modification interference experiments have identified the binding site for mtPBP2 in *S.purpuratus* mtDNA as the sequence 5′-TATGGCTCATCAACACCCATA-3', which is located between nt 9332–9352 of the corrected sequence, at the start of the COIII gene (the ATG start codon for which begins at nt 9333). As shown in Fig. 4d, this sequence contains an imperfect palindrome, although modification interference experiments indicate that the repeated residues in the two halves of the binding site do not interact with the protein in an identical manner, and implicate in binding some residues which are not repeated. However, these results must be viewed cautiously, as only a small number of all the possible modifications have been tested, and information about many T residues was unobtainable, because of their hyporeactivity to $KMnO_4$ oxidation.

Nevertheless, the symmetry of the binding site, taken together with the methylation and ethylation interference data, suggests that contacts are made in the major groove of two successive turns of the double helix, consistent with the idea that mtPBP2 may bind as a dimer. mtPBP2 binding also induces a bend in the DNA of about 45°. These properties resemble those of DNA-binding proteins of the helix-turn-helix class (19).

The high-affinity binding sites for mtPBP1 and 2 are separated by about 50 bp, but the two proteins appear to bind independently to these sites *in vitro*, i.e. without co-operativity or mutual interference. Close inspection of the mtPBP2 binding site reveals, in addition, an overlapping sequence, with a weak homology to a half-site for mtPBP1 binding. In very large protein excess, we

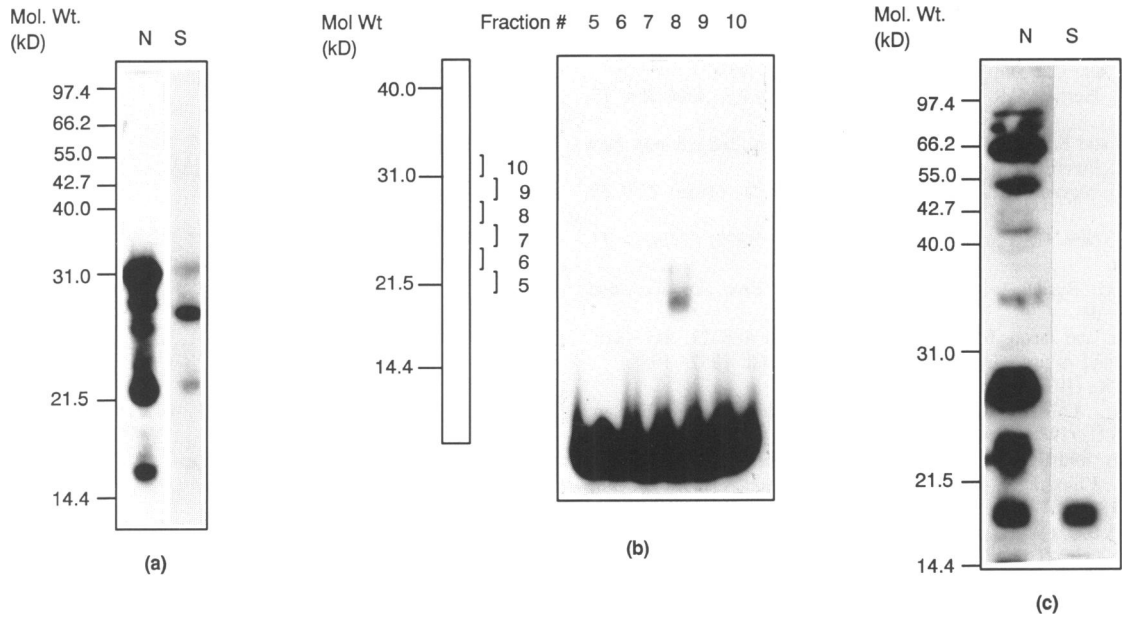


Figure 7. Determination of the molecular weights of mtPBP1 and mtPBP2. (**a**), (**c**) Southwestern blots and (**b**) EMSA using renatured protein fractions eluted from SDS-polyacrylamide gel slices. Assays were carried out using (a, b) the 0.2−0.4 M and (c) the 0.4−0.6 M protein fractions. For Southwestern blots, N denotes blot-strips probed (non-specifically) with the radiolabelled BRL 1 kb marker ladder, and S denotes those probed (specifically) with the radiolabelled oligonucleotides (a) oligo-PBP1, and (c) oligo-PBP2. In both cases, 0.5 μg/ml of double-stranded poly (dA-dT) was present in the binding reactions, as nonspecific competitor. The EMSA in (b) used radiolabelled oligo-PBP1, plus 1 μg double-stranded poly(dA-dT) as nonspecific competitor.

have indeed been able to detect very weak mtPBP1 binding to this site, although it is unlikely to be of physiological significance, unless the activity of mtPBP1 *in vivo* is many orders of magnitude greater than that of mtPBP2. This does not appear to be the case from the relative representation of the two proteins we have observed in mitochondrial extracts.

The binding site for mtPBP2 is completely conserved in the related sea urchin species *Paracentrotus lividus* (21), diverged from *S.purpuratus* by at least 40 Myr (22), suggesting that it is of functional significance. It is not conserved, however, in human mtDNA, although there are several copies of a related sequence in which most of the core nucleotides of the binding site, but not the dyad symmetry, are retained.

The relationship between mtPBP2 and DNA-binding proteins studied in other systems remains to be determined. Its apparent molecular weight, lack of thermostability, and binding site characteristics all distinguish it from mammalian mtTF1 (Refs. 23−25) or the 40 kD mitochondrial protein from *P.lividus*, studied by Roberti *et al.* (26). It is also unlikely to be related to the ~100 kD protein detected in human mitochondria by Welter *et al.* (27), which was found to bind to an intrinsically bent DNA fragment, 310 bp upstream of O_L. In contrast, mtPBP2 appears to induce bending in an otherwise unbent DNA sequence. Conceivably, DNA bending in the region of the lagging-strand origin, although brought about differently in the two systems, may function analogously in mtDNA replication in humans and sea urchins.

The possible functions for mtPBP2 remain a matter for speculation. The location of its binding site immediately at the 5′ end of the COIII gene suggests the possibility that it might function as a transcriptional terminator, to create the 3′ end of A6 mRNA. However, RNase-protection experiments have shown unequivocally that the 3′ end of this message is precise rather than ragged (28), disfavouring the kind of attenuation mechanism inferred for mammalian mtDNA at the 16S rRNA/tRNA-leu(UUR) gene boundary, although it cannot be ruled out that the precise 3′ end is created by a different termination mechanism, or by subsequent trimming.

Nevertheless, we favour a role for mtPBP2 in DNA replication. One interesting possibility is that it might be involved in priming of the lagging-strand. The partially symmetric site at the start of the COIII gene has the potential to form a short stem-loop structure in the ssDNA of the displaced strand, which elsewhere has been hypothesized as being capable of functioning as a lagging-strand origin by analogy with the mammalian O_L (Ref. 29). Interestingly, the residues identified by modification interference analysis as being critical for mtPBP2 binding are all on the leading (antisense) strand synthesized during the mtDNA replication cycle. This might indicate that mtPBP2 could remain bound to the stem-loop structure on the displaced strand, after passage of the replication fork, where it could function in lagging-strand priming.

To test these ideas we are attempting to purify mtPBP2 to homogeneity, in order to assay its various possible activities *in vitro*, and clone the gene encoding it from cDNA.

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