Nucleic Acids Research, 1993, Vol. 21, No. 4 811–816

Characterization of a high-affinity binding site for a DNA-binding protein from sea urchin embryo mitochondria

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Received January 5, 1993; Accepted January 19, 1993

ABSTRACT

Based on electrophoretic mobility shift assays, DNase I footprinting and modification interference analyses we have identified a sequence-specific DNA-binding protein in blastula stage mitochondria of the sea urchin *Strongylocentrotus purpuratus*, which interacts with a binding site around the major pause site for DNA replication. This region straddles the boundary of the genes for ATP synthase subunit 6 and cytochrome *c* oxidase subunit III, and contains also a prominent origin of lagging-strand synthesis. The protein is thermostable, and its natural high-affinity binding site comprises the sequence 5′-AGCCT(N_7)AGCAT-3′. Binding studies have demonstrated that two copies of the imperfect repeat, as well as the 7 bp spacing between them, are essential for tight binding. Based on the location of its binding site, we tentatively designate the protein mitochondrial pause-region binding protein (mtPBP) 1.

INTRODUCTION

Sequence-specific DNA-binding proteins are known to be important regulators of mitochondrial transcription in mammals, and almost certainly play similar roles in plant and fungal mitochondria. These proteins include the enhancing factor for transcriptional initiation, designated mtTF1, which has recently been shown to be a protein of the HMG-1 family (1–6), and a transcriptional termination factor, which interacts with the attenuator sequence downstream of the rRNA genes in mammalian mtDNAs (7, 8), and is believed to regulate the relative synthetic rates of different classes of RNA. In yeast, at least one other protein, MTF1 (9, 10) seems to be involved in promoter discrimination, although it requires the presence of the core subunit of the mitochondrial RNA polymerase for sequence-specific DNA-binding (11).

Little is known, however, of the role of sequence-specific mtDNA-binding proteins in the regulation of DNA replication or other processes in animal cell mitochondria, other than the requirement for mtTF1 for replication primer synthesis at the heavy-strand origin (12). The known mechanics of metazoan mtDNA synthesis (13), involving D-loop formation, D-loop expansion, second-strand priming at a specific site on the displaced strand, specific termination (presumably close to the leading-strand origin) and daughter molecule segregation, suggest the likely involvement of other regulatory proteins, with specific template-binding properties.

We have become interested in the mechanism of mtDNA replication in early sea urchin development, which is developmentally regulated. Mitochondrial DNA is amplified during oogenesis, but does not replicate in the mature oocyte, egg or early embryo (14), where it is maintained in the D-loop form (15). In earlier studies, we exploited the observation that mtDNA replication may be precociously induced in enucleated sea urchin eggs by ammonia activation (16, 17), to study the structure of replication intermediates by gel electrophoretic techniques (18). This led us to identify replication pause sites, the most prominent of which maps close to a major site of lagging-strand initiation, near to the boundary of the genes for ATP synthase subunit 6 (A6) and cytochrome c oxidase subunit III (COIII).

In this paper, we report the identification and characterization of a high-affinity binding site in the A6/COIII gene boundary region of sea urchin mitochondrial DNA, for a protein found in blastula stage mitochondria. Because of the location of its binding site we suggest that the protein may play a role in mtDNA replication, and tentatively designate it mitochondrial pause-region binding protein (mtPBP) 1.

MATERIALS AND METHODS

Purification of mitochondria from blastulae

Twenty-four hour blastula embryos of the sea urchin *S.purpuratus* were prepared by Dr F.J.Calzone (University of California, Irvine) according to Calzone *et al.* (19), stored at −70°C and lysed by thawing. After two low-speed spins at 900 g_{max} and one spin at 2500 g_{max}, mitochondria were harvested by centrifugation of the supernatant at 10,000 g_{max} . The crude mitochondrial pellet was resuspended in 0.25 M sucrose in DEV buffer (30 mM Tris–HCl (pH 7.8), 0.25 M NaCl, 2 mM EDTA) and purified on a 0.6/1.2 M sucrose-DEV step gradient, centrifuged in a Beckman SW28.1 rotor at 25,000 rpm for 30 minutes at 4°C. Pure mitochondria were then resuspended in an

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equal volume of a buffer containing 10 mM Tris−HCl (pH 8.0), 0.5 mM EDTA, 1 mM DTT, 20% glycerol, and frozen at −70°C.

Heparin-sepharose chromatography

Mitochondrial lysis and heparin-sepharose (Pharmacia) chromatography were performed as described (20). After washing the column with 0.2 M KCl in buffer 'A' the proteins were eluted either with 1.0 M KCl or with successive steps of 0.4 M and 1.0 M KCl. Bradford (Biorad) positive fractions were pooled, concentrated by ultrafiltration (Millipore), 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.

Recombinant clones

AM65, a genomic subclone of the A6/COIII gene boundary region of *S.purpuratus* mtDNA (see Fig. 1) was provided by Dr A.G.Mayhook.

Oligonucleotides and DNA probes

Oligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer, using phosphorimidite chemistry, and subsequently purified on 20% denaturing polyacrylamide gels. Complementary single-stranded oligonucleotides 5′-AAATAG-GAGTAGCCTGCATCCAAGCATAT-3′ (top) and 5′-AATA-CATATGCTTGGATGCAGGCTACTCC-3′ (bottom) were annealed by incubation 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. For modification interference experiments, the double-stranded oligonucleotide was cloned into the *Sma*I site of pSP6/T7 (BRL) using standard techniques (22). Restriction fragment and double-stranded oligonucleotide probes were labelled by filling in the 3′ recessed ends with appropriate radionucleotides (NEN, 3000 Ci/mmol), using Klenow enzyme (Boehringer Mannheim).

Construction of binding site variants

To engineer mutations in the binding site a bottom-strand oligonucleotide containing degeneracies at three positions was synthesized [5′-AATACATA(T/G)GCTTGGAT(G/C)CA (G/T)GCTACTCC-3′] and annealed to the top strand of the oligonucleotide described above. After filling in the 3′ protruding ends the oligonucleotide mixture was cloned into the *Sma*I site of a modified plasmid pSP6-T7, containing an inactivated *Bam*HI site. Recombinant plasmids were identified and sequenced. For the construction of spacing variants the *Bam*HI site in variant D (see Table 1) was cleaved, and partially or completely filled in using Klenow and various combinations of dNTPs. Where necessary, the remaining single-stranded DNA tails were digested with mung bean nuclease (Boehringer Mannheim) prior to ligation. For use in electrophoretic mobility shift assays, the inserts of all variants were excised by digestion with *Eco*RI and *Xba*I, gel-purified, and radiolabelled as described above.

Electrophoretic mobility shift assays (EMSA) and DNase I footprinting

EMSAs were carried out in a total volume of 15 μl, containing 1 μg of poly(dA-dT).poly(dA-dT) as nonspecific competitor DNA, 0.2 ng of end-labelled DNA probe and 4 μg of the 0.2−0.4 M KCl heparin-sepharose protein fraction in a reaction consisting of 20 mM HEPES-KOH (pH 7.9), 5 mM $MgCl_2$, 0.5 mM EDTA, 50 mM KCl, 1 mM DTT and 7.5% glycerol. The binding reactions were incubated at 20°C for 30 minutes and then loaded directly onto a 5% polyacrylamide (30:0.8) gel prepared in 0.5×TBE (pH 8.3), which had been pre-cooled at 4°C and prerun for 30 minutes at 200 V. Following electrophoresis the gels were dried and exposed to X-ray film (Kodak). For DNase I footprinting assays the complexes were digested with 25 ng of DNase I (Worthington) in 35 μl of 5 mM $MgCl_2$ and 5 mM $CaCl_2$ solution for 15 seconds at 20°C. DNase I was inactivated by phenol. Reactions were extracted once with phenol-chloroform (5:1) and ethanol precipitated. The DNA was dissolved in 3 μl formamide loading buffer and electrophoresed on an 8% sequencing gel. Maxam & Gilbert chemistry (23) was performed on Hybond M&G paper, according to the instructions provided by Amersham.

Protein−DNA contact analysis

Thymine- and guanine-specific modifications were carried out as described by Truss *et al.* (24) and Maxam and Gilbert (23), respectively, with minor modifications. Both $KMnO_4$ and DMS reactions were stopped by chromatography on Sephadex G-50 spun columns equiliberated with TEN (10 mM Tris−HCl (pH 8.0), 1 mM EDTA, 0.1 M NaCl). The modified DNA was purified and used in a five-fold scale-up of EMSA as described above.

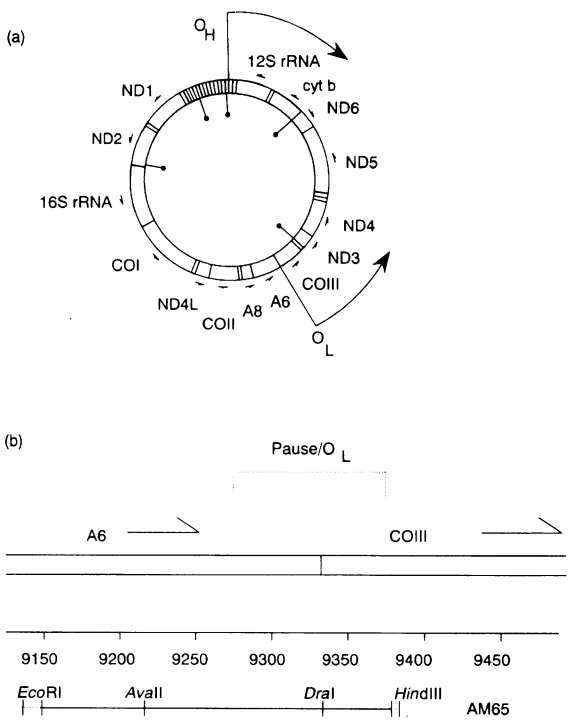

Figure 1. Organization of the *S. purpuratus* mitochondrial genome. **(a)** Schematic physical map of sea urchin mtDNA showing the rRNA and protein coding genes with their transcriptional orientations indicated by half-arrows. The replication origins are denoted using the mammalian nomenclature: the 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 AM65, used in this study, with the genes indicated as A6 (ATP synthase subunit 6) and COIII (cytochrome *c* oxidase subunit III), and restriction sites indicated. The remains of the polylinker of the pSP64 vector are shown as a broken line. The map co-ordinates of the region are as shown (based on ref. 21).

The bound and free DNAs were localized by autoradiography, excised, and eluted from the gel slices. Both $KMnO_4$- and DMS-modified DNAs were cleaved with 10% piperdine at 90°C (23) and electrophoresed on a 20% sequencing gel.

RESULTS

Localization of the mtPBP1 binding site

In order to investigate whether the major replication pause region of sea urchin mtDNA contains binding-sites for sequence-specific DNA-binding proteins, the entire *Eco*RI-*Hin*dIII fragment encompassing the A6/COIII junction region, from clone AM65 (Fig. 1) was tested in EMSA, in the presence of a large excess of poly(dA-dT).poly(dA-dT), using the blastula stage mitochondrial lysate fractionated on heparin-sepharose. This resulted in the formation of a single complex whose specificity was established by using increasing amounts of cold self-competitor, and monitoring the proportional loss in the intensity of the retarded band (data not shown). The binding site was further investigated using shorter restriction fragments derived from AM65. From the three fragments tested (i.e., *Eco*RI- *Ava*II, *Ava*II-*Dra*I and *Dra*I-*Hin*dIII), only the *Ava*II-*Dra*I fragment produced a band shift in the presence of a large quantity of the nonspecific competitor (Fig. 2a). Finer fractionation of the extracts on heparin-sepharose columns showed that this factor elutes in the 0.2−0.4 M KCl step.

To localize the protein-binding site DNase-I footprinting was carried out using the *Eco*RI-*Hin*dIII fragment from AM 65, labelled on either strand. This experiment (Fig. 2b) gave a footprint on both strands, near to the 3′ end of the A6 gene (nt 9259−9284), which was resistant to competition from poly(dA-dT).poly(dA-dT).

EMSAs with the binding-site oligonucleotides

To confirm the location of the protein binding site inferred from DNase I footprinting, and to characterize further the protein(s) involved, a double-stranded oligonucleotide (oligo-PBP1) containing the sequence of the footprinted region in AM65 (nt 9255−9289) was synthesized (Fig. 3a). After radiolabelling, oligo-PBP1 was used in EMSAs in the presence of various protein fractions. These experiments (Fig.3b, lanes 1−3) showed clearly that a protein in the 0.2−0.4 M fraction binds to oligo-PBP1 in the presence of a large excess of poly(dA-dT).poly(dA-dT). Although the binding reactions for EMSAs were carried out in 50 mM KCl, increasing the salt concentration to 1.0 M did not have a significant effect on the affinity of the protein for its site (data not shown).

The thermostability of mtPBP1 was also studied by treating aliquots of the 0.2−0.4 M fraction at 20, 37, 50 and 100°C prior to using them in EMSA with oligo-PBP1. This experiment showed mtPBP1 to be completely resistant even to boiling, as

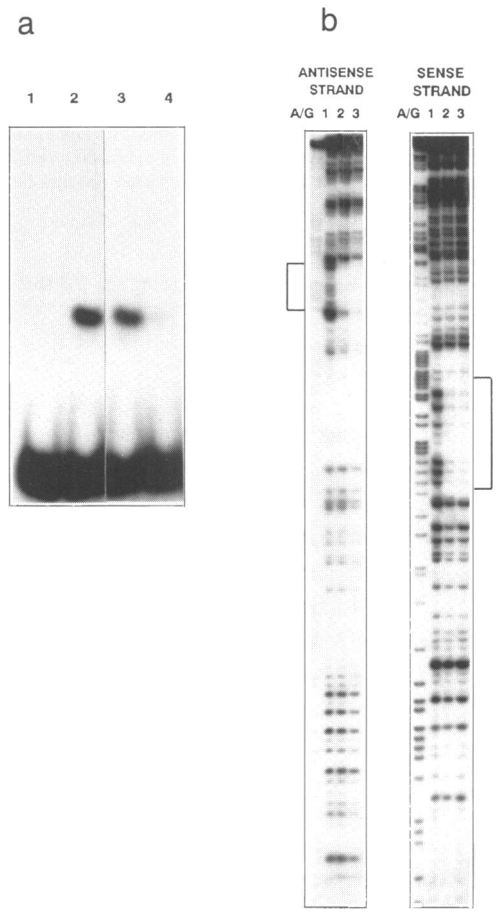

Figure 2. Determination of the protein binding site for mtPBP1. **(a)** EMSAs using the 112 bp labelled *Ava*II-*Dra*I fragment from clone AM65 (see Fig. 1). Various eluates from the heparin-sepharose column were used as follows: no protein (lane 1), 4 μg 0.2−1.0 M KCl eluate (lane 2), 4 μg 0.2- 0.4 M KCl eluate (lane 3) and 4 μg 0.4−1.0 M eluate (lane 4). **(b)** DNase I footprinting of the sense and antisense strands of the *Eco*RI- *Hin*dIII fragment from AM65. Reactions contained: no protein (lane 1), 4 μg (lane 2) and 8 μg (lane 3) of proteins from the 0.2−0.4 M KCl eluate from heparin-sepharose. Footprinted regions are shown in brackets.

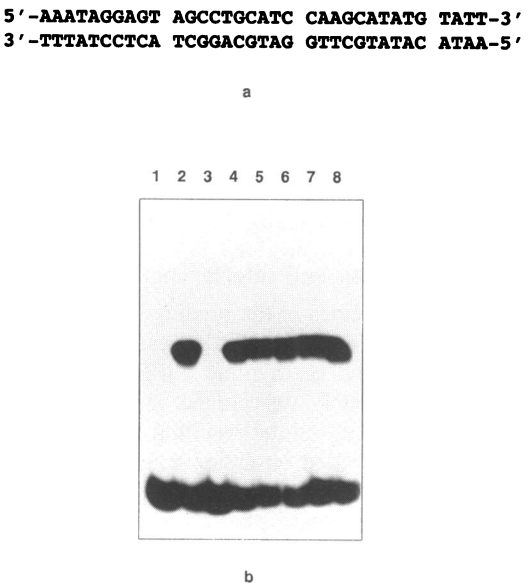

Figure 3. EMSAs with mtPBP1 binding-site oligonucleotide. **(a)** Sequence of oligonucleotide oligo-PBP1, derived from the DNase I footprint. **(b)** EMSAs using oligo-PBP1 and various protein fractions. Lane 1: no protein; lane 2: 4 μg 0.2−0.4 M KCl eluate; lane 3: 4 μg 0.4−1.0 M KCl eluate; lanes 4−8: 4 μg heat-treated protein fractions. Proteins from the 0.2−0.4 M KCl eluate were heated at the indicated temperatures for the indicated lengths of time, and immediately placed on ice for at least 10 minutes prior to incubation with oligo-PBP1. Lane 4: unheated; lane 5: 20°C for 30 min; lane 6: 37°C for 30 min; lane 7: 50°C for 15 min; lane 8: 100°C for 5 min.

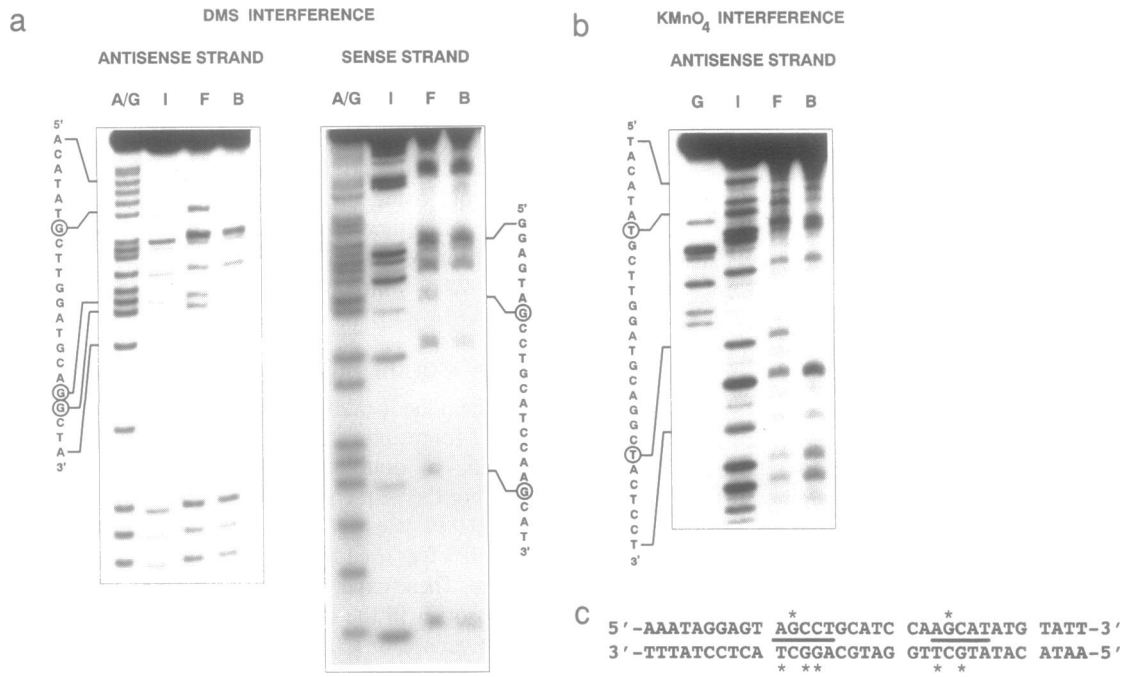

Figure 4. Interference with mtPBP1 binding by DMS and $KMnO_4$ modification. **(a)** Methylation interference analysis of the sense and antisense strands of the cloned *Eco*RI-*Bam*HI fragment containing oligo-PBP1, and **(b)** oxidation interference analysis of the antisense strand of the same fragment. After modification with the respective reagents DNAs were used in a preparative EMSA. Free (F) and bound (B) DNAs were recovered and, together with the input (I) DNA, were cleaved with piperidine and analyzed on a 20% sequencing gel. Modified residues which interfere with mtPBP1 binding are circled. **(c)** Summary of the modification interference data: modified residues from oligo-PBP1 which interfere with mtPBP1 binding are asterisked. The imperfect repeats in which the contacted residues lie are shown by bold lines.

no decrease in its activity was detected after heat treatment (Fig. 3b; lanes 4−8). In subsequent experiments this property of mtPBP1 was exploited, and the 0.2−0.4 M fraction which had been boiled and centrifuged to remove precipitated proteins was used at a concentration of 0.8 mg/ml, providing a further 5-fold purification of mtPBP1. Further purification by Mono-S chromatography, and Southwestern blotting, revealed mtPBP1 to comprise a single polypeptide species of apparent molecular weight 25 kD (data not shown).

Chemical modification and interference analysis

To localize the nucleotides in the binding site acting as contact points for the protein we decided to carry out modification interference experiments on thymine and guanine residues. For this purpose oligo-PBP1 was cloned into the *Sma*I site of pSP6/T7, and after excision the resulting 57 bp *Eco*RI-*Bam*HI fragment was radiolabelled on either strand and used in these experiments. Methylation interference experiments were carried out to probe the protein contact points with the various guanine residues in the binding site. Methylation of the guanine residues at position 12 and 24 on the sense strand, and at positions 13, 14, and 25 on the antisense strand reproducibly interfered with binding (Fig. 4a).

Similarly, C-5/C-6 oxidation interference experiments were carried out to assay for protein contacts with the thymine residues in the binding site. For this, thymine residues on denatured DNA were modified with $KMnO_4$ in the presence of 30 mM Tris−HCl (pH 8.0), which according to Truss *et al.* (24) modifies all thymines regardless of nearest neighbor sequence effects. Under these conditions we found the T at position 15 on the sense strand to be hyporeactive to the oxidizing agent, and, as a consequence, uninformative (data not shown). Other thymine residues on the sense strand were modified normally, but had no effect on protein binding. On the antisense strand modification of the thymine residues at positions 11 and 26 strongly interfered with binding (Fig. 4b). The results of modification interference analysis are summarized in Figure 4c. It is immediately apparent

Table 1. Sense-strand DNA sequences of the binding-site and spacing variants

Variant	DNA Sequence
-	AAATAGGAGT**AGCCT**GCATCCA**AGCAT**ATGTATT
A	AAATAGGAGT**AGCCT**GCATCCc**AGCAT**ATGTATT
B	AAATAGGAGT**AGCtT**GCATCCA**AGCAT**ATGTATT
C	AAATAGGAGT**AGCCT**GgATCCA**AGCcT**ATGTATT
D	AAATAGGAGT**AGCCT**GgATCCA**AGCAT**ATGTATT
E	AAATAGGAGT**AGCCT**GCATCCA**A-CAT**ATGTATT
F	AAATAGGAGT**AGCaT**GCATCCA**AGCAT**ATGTATT
G	AAA-AGGAGT**AGCaT**GgATCCA**AGCAT**ATGTATT
N1	AAATAGGAGT**AGCCT**G**AGCAT**ATGTATT
N2	AAATAGGAGT**AGCCT**CA**AGCAT**ATGTATT
N3	AAATAGGAGT**AGCCT**GCA**AGCAT**ATGTATT
N4	AAATAGGAGT**AGCCT**GGCA**AGCAT**ATGTATT
N5	AAATAGGAGT**AGCCT**GGCCA**AGCAT**ATGTATT
N6	AAATAGGAGT**AGCCT**GGACCA**AGCAT**ATGTATT
N9	AAATAGGAGT**AGCCT**GGATATCCA**AGCAT**ATGTATT
N11	AAATAGGAGT**AGCCT**GGATCGATCCA**AGCAT**ATGTATT

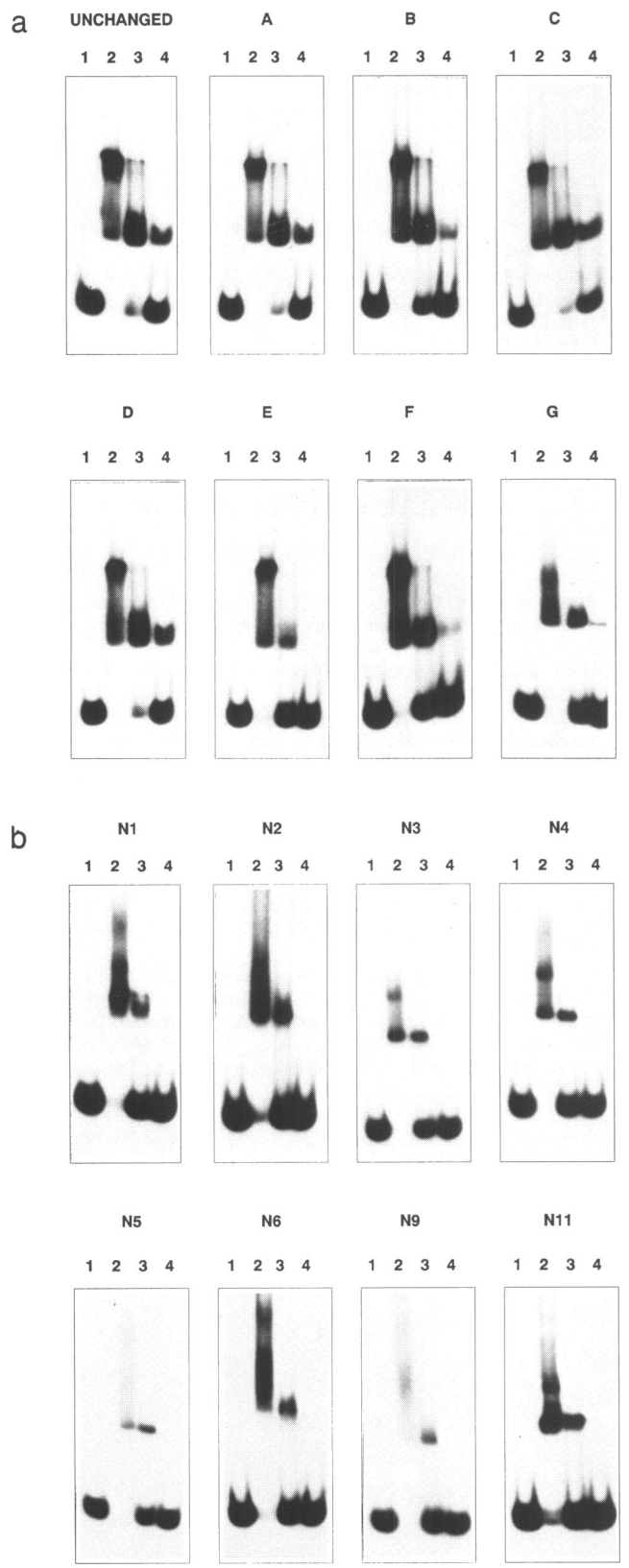

Figure 5. EMSAs with variant binding-site probes. **(a)** Binding-site variants A–G (shown in Table 1) along with the fragment containing the wild-type sequence (unchanged) were used in EMSAs, either with (lanes 1) no protein, or (lanes 2–4) 0.8 μg of boiled 0.2–0.4 M KCl protein fraction, in the presence of 0, 0.1 and 1 μg respectively of poly(dA-dT).poly(dA-dT) as nonspecific competitor DNA. **(b)** EMSAs using the eight spacing variants shown in Table 1. Binding conditions were the same as described above.

that the contacted residues lie within two copies of the imperfectly repeated sequence AGC(C/A)T, separated by 7 bp. These repeats lie approximately one turn of the DNA double helix apart.

EMSAs with binding site variants

In order to understand the consequences for mtPBP1 binding of various point mutations in the binding site, as well as the effect of altered spacing between the two imperfect repeats, we constructed two sets of binding-site variants, using the methodology outlined in Materials and Methods. We obtained programmed as well as unprogrammed mutations in the binding site, as summarized in Table 1, where mutated bases are shown in lower case. The relative strength of mtPBP1 binding to the variant sites was assessed by EMSA (Fig. 5), carried out in the presence of increasing amounts of the non-specific competitor poly(dA-dT).poly(dA-dT). Variants A and D behaved the same way as the original binding site, indicating that the C to G mutation at position 17, which creates a *Bam*HI site between the imperfect repeats, is inert. This *Bam*HI site was used in the creation of the spacing variants. The binding of mtPBP1 to variant C, in which both copies of the repeated sequence are AGCCT, appeared to be slightly stronger than to the original site, in which the second copy is AGCAT. All other site variants bound relatively weakly, as shown by their inability to bind mtPBP1 in the presence of 1 μg of the nonspecific competitor. EMSAs with the spacing variants indicated that none of them could interact with mtPBP1 to form a complex in the presence of 1 μg of the nonspecific competitor (Fig. 5b). The 7 bp spacing between the repeats is therefore critical for tight binding. Oligonucleotides containing either of the two half-sites in isolation were also bound only weakly by mtPBP1, even in the complete absence of a nonspecific competitor (data not shown), indicating that the presence of both half-sites is essential.

DISCUSSION

In this study we have identified a protein which binds specifically and strongly to a DNA sequence close to the A6/COIII gene boundary of the sea urchin mitochondrial genome. Since this region contains a strong pause site for leading-strand DNA replication (18), we propose to refer to the protein as mitochondrial pause-region binding protein or mtPBP1.

Modification interference experiments have identified the binding site for mtPBP1 in *S. purpuratus* mtDNA as the sequence 5′- AGCCT(N_7)AGCAT-3′. All five guanine residues (on both strands), as well as the two antisense-strand thymines which are represented in both copies of the imperfect repeat, are involved in binding to mtPBP1. No information was obtained from these experiments, concerning the two antisense-strand thymine residues. Although the oxidation of the C-5/C-6 double bond of these residues does not interfere directly with binding, it is possible that the protein may utilize these residues indirectly, for example by making contacts with the complementary adenines. The fact that these two A-T pairs add to the symmetry of the binding site supports the view that they are likely to be involved in recognition. In addition, the 7 bp spacing between the imperfect repeats, approximately equivalent to a helical turn, is crucial for strong binding.

Since the two half-sites are almost symmetrical, and binding is enhanced by converting both copies to AGCCT, it is plausible that mtPBP1 interacts with its site as a dimer. This type of interaction is common among DNA-binding proteins, for example

the catabolite activator protein (CAP) of *E.coli*, which binds as a dimer to two symmetrical half-sites separated by 6 bp, and yields a single retarded complex on EMSA (25). Like many such proteins, the binding of mtPBP1 is dramatically weakened if the spacing is perturbed. A binding site comprising short direct repeats with a constrained spacing resembles that for the phage lambda cII repressor (26), and also recalls the architecture of the binding sites for the nuclear receptor family (27).

The binding site for mtPBP1 is highly conserved in the related sea urchin species *Paracentrotus lividus* (28), diverged from *S. purpuratus* by at least 40 Myr (29), suggesting that it is of functional significance. More strikingly, the sequence AGC(C/A)T$(N)_7$AGC(C/A)T occurs once, and only once, in all deuterostome mitochondrial genomes we have examined, though not always in the same location. In mouse, as in sea urchins, it is found in the 3′ end of A6, where amino acid sequence constraints arguably favour its occurrence. However, in human and *Xenopus* mtDNAs it occurs in two unrelated regions of ND4, and in bovine mtDNA in the D-loop. We are unable to evaluate the possible significance of this observation, other than to note that the given sequence would be expected to occur by chance only about once every 250,000 base-pairs.

The relationship between mtPBP1 and DNA-binding proteins studied in other systems remains to be determined. Its thermostability recalls that of mtTF1, although it does not form the characteristic laddered pattern of complexes which mtTF1 exhibits on EMSA (2), and its binding-site preference appears to be much higher. There is also no reason to associate it with the 40 kD mitochondrial protein from *P.lividus*, studied by Roberti *et al.* (30), as there is no similarity in the binding sites for the two proteins.

The location of the binding-site for mtPBP1 does not suggest any obvious role for this protein in transcription. Although we cannot exclude the possibility that it acts as a termination factor, we have been unable to detect any 3′ ends of transcripts of the A6 gene, other than precisely at the gene boundary (31).

A possible function for mtPBP1 in DNA replication is suggested by the proximity of its binding site to the major pause site for leading strand replication, and a prominent lagging-strand origin (18). We suggest, by analogy with the *tus* protein of *E.coli*, which functions in the termination of chromosomal and plasmid replication (32), that mtPBP1, when bound, might stall an advancing replication complex by antagonizing the action of its associated DNA helicase. This could facilitate the formation on the displaced strand of the priming structure for lagging-strand initiation, for example, by impeding the access of single-strand binding protein, or by preventing interference from upstream sequences. Alternatively, the local presence of DNA polymerase and other components of the replication machinery might allow the efficient initiation of lagging-strand synthesis. To test these ideas we are attempting to purify mtPBP1 to homogeneity, in order to assay its activities *in vitro*.

ACKNOWLEDGEMENTS

We are indebted to Frank Calzone (University of California, Irvine) for the provision of frozen lysates of *S.purpuratus* blastulae, and of lab facilities for preparing mitochondria from these lysates. We also thank Andrew Mayhook, for supplying the clone AM65, which he constructed. We are also grateful for financial support from the Royal Society, Medical Research Council, the European Community and NATO.

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