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In vivo mitochondrial DNA-protein interactions in sea urchin eggs and embryos

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Abstract Footprinting studies with the purine-modifying agent dimethyl sulphate were performed in *Paracentrotus lividus* eggs and embryos to analyze in vivo the interactions between protein and mitochondrial DNA. Footprinting in the small non-coding region and at the boundary between the ND5 and ND6 genes revealed two strong contact sites corresponding with the in vitro binding sequences of mitochondrial D-loop-Binding Protein (mtDBP). The analysis of the pause region of mtDNA replication showed a strong footprint corresponding with the binding site of the mitochondrial Pause region-Binding Protein-2 (mtPBP-2), but only a very weak signal at the binding site of the mitochondrial Pause region-Binding Protein-1 (mtPBP-1), which in vitro binds DNA with high efficiency. In vitro and in vivo analysis of the 3′ end-region of the two rRNA genes showed no significant protein-DNA interactions, suggesting that, in contrast to mammals, the 3′ ends of sea urchin mitochondrial rRNAs are not generated by a protein-dependent transcription termination event. These and other data support a model in which expression of mitochondrial genes in sea urchins is regulated post-transcriptionally. Footprinting at the five AT-rich consensus regions allowed the detection of a binding site in the non-coding region for an as-yet unidentified protein, mtAT-1BP. The occupancy of this site appears to be developmentally regulated, being detectable in the pluteus larval stage, but not in unfertilized eggs.

Key words Sea urchin · mtDNA · DMS footprinting · DNA-binding proteins

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Introduction

Due to the large number of eggs produced by females and to their external fertilization, the sea urchin represents a useful system for studying mitochondrial DNA (mtDNA) metabolism during embryogenesis. The mtDNA of echinoids has a coding capacity similar to that of vertebrates, as it codes for two rRNAs, 13 polypeptides and 22 tRNAs. However, the distribution of the genes on the two strands and their order are strikingly different (Cantatore and Saccone 1987; Attardi and Schatz 1988; Jacobs et al. 1988; Cantatore et al. 1989; De Giorgi et al. 1996). The two ribosomal RNA genes, 12S and 16S, are not adjacent but are separated by a 3.3-kbp interval containing the genes for ND1, ND2 and for 15 clustered tRNAs; the main non-coding region (NCR), situated in the tRNA gene cluster, is only some 130-bp long. Other shorter, non-coding tracts are scattered in the genome. Three of them are AT-rich; together with two other short AT-rich sequences, located within the 3′ end of ND2 and in the NCR, respectively, they conform to the consensus sequence ATATATAA.

Most of the studies on mtDNA replication and transcription have been performed on *Paracentrotus lividus* and *Strongylocentrotus purpuratus*, two sea urchin species belonging to the order Camarodonta and displaying a high degree of mtDNA sequence similarity (Jacobs et al. 1988; Cantatore et al. 1989). An H-strand replication origin was mapped in the NCR of *S. purpuratus* mtDNA, associated with a D-loop triplex of approximately 80 nt, composed mostly of RNA (Jacobs et al. 1989). The transition point from the RNA primer to DNA was mapped at 15–20 nt downstream from the 3′ end of the $tRNA^{Thr}$ gene. Multi-

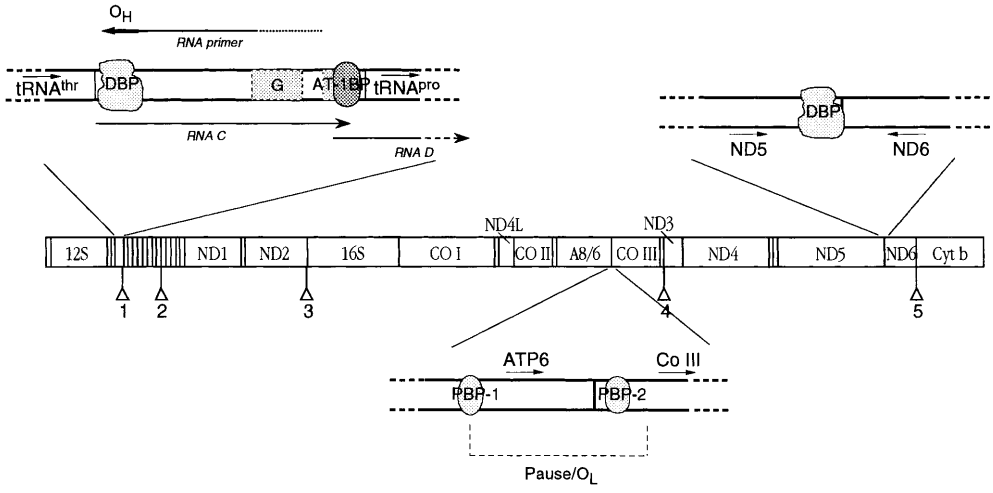


Fig. 1 Schematic linear map of sea urchin mtDNA. Protein and rRNA coding genes are shown. *Triangles* indicate the position of the five AT-rich sequences. The *inserts* show in detail the regions containing the binding sites of mtDBP, mtPBP-1 and mtPBP-2. The binding site for the newly identified mitochondrial AT-1 binding protein (mtAT-1BP) in the NCR, in relation to the 5′ and 3′ ends of the RNAs mapping in this region, is also shown. *Arrows* indicate the transcriptional orientation of the genes.In the NCR the guanine box and the AT-1 sequence are *shadowed*. The 3′ end of the RNA C and the 5′ end of the RNA D are located near or inside the AT-1 region (Cantatore et al. 1990). The replication origins (O_H and O_L) are denoted using the mammalian nomenclature. The direction of H-strand DNA synthesis is indicated by an *arrow*. The RNA primer for H-DNA replication was identified by Jacobs et al. (1989). The *thin line* represents the RNA component, with the *dotted portion* indicating the uncertainty in the 5′-end position; the *thick line* represents the DNA portion of the newly synthesized third strand

ple pause sites for H-strand DNA synthesis were also mapped: the most prominent was located at the junction between the genes for ATPase 6 and COIII. The L-strand replication origin has not formally been mapped; the lagging strand probably initiates from multiple points, one of which appears to be located near the main H-strand replication pause site, at the junction between the genes for ATPase 6 and COIII (Mayhook et al. 1992). Mapping of mitochondrial RNAs (mtRNAs) showed that the 3′ ends of the two rRNAs, 12S and 16S, were each located a few bases inside the adjacent downstream gene (Elliot and Jacobs 1989; Cantatore et al. 1990). Moreover the 5′ ends of several mtRNA precursors mapped within the AT-rich sequences and a large number of high-molecular-weight mtRNAs species was also detected. Finally, steady state levels of mitochondrial mRNAs showed remarkable differences even among transcripts mapping in adjacent positions, such as COII and ATPase 8/6 (Elliot and Jacobs 1989; Cantatore et al. 1990).

Sequence-specific mtDNA-binding proteins, possibly involved in the control of replication and/or transcription, were also identified. A polypeptide of 40 kDa which binds two homologous sequences of mtDNA was detected in *P. lividus* eggs and embryos (Roberti et al. 1991; Loguercio Polosa et al. 1994). One binding site is located in the NCR at the 3′ end of the D-loop structure, the other encompasses the adjacent 3′ ends of the oppositely transcribed ND5 and ND6 genes. Based on the binding of this protein to the sea urchin mtDNA D-loop region, we designated it mitochondrial D-loop binding protein (mtDBP). MtPBP-1 and mtPBP-2 are two polypeptides, of 25 kDa and 18 kDa respectively, first identified in *S. purpuratus* blastulae (Qureshi and Jacobs 1993a, b). They interact with two sequences located in the region of the major H-strand replication pause site. Figure 1 shows the position of the binding sites of these factors on sea urchin mtDNA. The binding sites for these proteins are conserved between *P. lividus* and *S. purpuratus* (Jacobs et al. 1988; Cantatore et al. 1989) and, moreover, mitochondrial protein extracts from both species contain all three binding activities with similar properties (M. Roberti and S. A. Qureshi, unpublished data).

In spite of this amount of information, many important questions about the mechanism of mitochondrial gene expression in sea urchins are still unanswered. The separation of the two rRNA genes and the overlap between the 3′ end of each rRNA and the 5′ portion of the downstream transcript raises a question about the mechanism generating the 3′ ends of the two ribosomal RNAs. Another point concerns the role of the AT-rich regions; the observation that the 5′ ends of several mtRNAs, or their precursors, map in these sequences suggests that they may act as promoters, but the unavailability of an in vitro transcription system from sea urchin mitochondria means that it has not been possible to test this hypothesis. In this paper we report the results of experiments aimed at answering some of these questions. By using the technique of dimethyl sulphate (DMS) in vivo footprinting we analyzed protein-DNA interactions previously found in vitro. Furthermore, by both in vivo and in vitro approaches we have excluded the presence of protein binding at the 3′ ends of the two rRNA genes, supporting a novel, *post*-transcriptional mechanism for the expression of sea urchin mitochondrial genes. Finally, we detected a developmentally regulated protein-DNA interaction near the AT-rich consensus se-

quence of the mtDNA non-coding region, which may be involved in the control of mtDNA replication.

Materials and methods

In vivo and in vitro DMS treatment of sea urchin mtDNA. About 20 g of *P. lividus* unfertilized eggs and 40-h pluteus larvae were incubated in 50 ml of millipore-filtered sea water containing 0.5–1.0% DMS for 5 min at 22 °C with gentle shaking. They were then placed on ice and washed twice with 4 vol of cold, millipore-filtered sea water. Mitochondria were prepared according to the procedure described by Cantatore et al. (1987). For in vivo methylated mtDNA extraction, the mitochondrial pellet (about 5 mg of protein) was suspended in 400 µl of 50 mM Tris-HCl pH 8.0, 25 mM EDTA, 200 mM NaCl, 0.5% SDS and 0.2 mg/ml of proteinase K and incubated for 30 min at 37 °C. Nucleic acids were extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1), ethanol-precipitated and dried. Control samples of naked (protein-free) DNA were obtained from the same amount of mitochondria, omitting the in vivo DMS treatment. The DNA was suspended in 100 µl of TE buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA), mixed with an equal volume of 100 mM sodium cacodylate pH 7.0 and incubated with 0.1–0.5% DMS for 5 min at 22 °C. The reaction was stopped by adding 50 µl of 1.5 M sodium acetate pH 7.4, 1.0 M β-mercaptoethanol, and the DNA was then ethanol-precipitated and dried. The pellets of in vivo and in vitro methylated DNA were suspended in 100 µl of 1.0 M piperidine and incubated for 30 min at 90 °C. Piperidine treatment was followed by purification through a Sephadex G-50 spin column and by lyophilization. The DMS/piperidine-treated DNA samples were re-suspended in 50 µl of double-distilled water and used for primer extension analysis. In most experiments the piperidine treatment of samples and controls was omitted since preliminary tests showed that the primer extension terminated effectively opposite modified bases, without a requirement for prior cleavage; in this case the DNA pellets were suspended in 100 µl of TE, purified by Sephadex G-50 spin-columns and recovered in 50 µl of double-distilled water.

Primer extension of DMS-treated DNA. Five microliters of DMS-treated DNA (about 1 µg of mtDNA) were used for each extension reaction. The reaction mixture (100 µl) contained also 4 µl of 5 mM dNTPs, 1–5 pmoles of [^{32}P] 5′ end-labeled primer (see figure legends for primer identification), 10 µl of 10×*Taq* buffer (100 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, 500 mM KCl) and 2.5 units of *Taq* DNA polymerase (Boheringer Mannheim). The reaction was carried out in a DNA Thermal Cycler Model 480 (Perkin Elmer); samples were first heated to 94 °C for 5 min, then 25 cycles followed (2 min at the primer annealing temperature, 3 min at 72 °C and 1 min at 94 °C). The reaction was ended with a 2-min annealing step and with a final extension of 10 min at 72 °C. Amplification products were extracted with chloroform-isoamyl alcohol (24:1) and ethanol-precipitated in the presence of 1.0 M ammonium acetate and 5 mM EDTA. The pellet was washed with 70% ethanol, dried, suspended in 6 µl of 98% formamide and loaded on a 6% polyacrylamide-8 M urea sequencing gel alongside sequencing reactions obtained with the same primer. Primer labelling was carried out with γ[^{32}P]ATP and polynucleotide kinase, as described by Sambrook et al. (1989).

Footprint quantitation. The gels were exposed with an intensifying screen for different lengths of time and the desired lanes were scanned densitometrically by using an LKB-Pharmacia Ultroscan-XL Laser densitometer equipped with Gel-Scan-XL Evaluation Software. In most of the experiments, to account for any loading difference, control and test scans were normalized using bands localized in regions lacking observable footprints, adjacent to the reactive areas. Relative levels of protection or hyper-reactivity were then determined using the normalized values. Mean differences between control and test scans greater than 30% (twice the standard deviation of the mean difference among unaffected nucleotides) were scored as being footprinted. The results reported here were each obtained in at least five independent experiments; each different sample of sea urchin eggs and embryos displayed essentially the same in vivo footprinting pattern.

Other methods. Preparation of sea urchin mitochondrial lysates, chromatography and mobility-shift experiments were conducted as described previously (Roberti et al. 1991; Loguercio Polosa et al. 1994).

Results

In vivo footprinting at known sites of protein-DNA interactions

Previous in vitro studies showed that mtDBP binds selectively to two sequences of *P. lividus* mtDNA (Roberti et al. 1991; Loguercio Polosa et al. 1994), one in the NCR (nucleotides 1098–1126) and the other at the boundary of the oppositely transcribed ND5 and ND6 genes (nucleotides 14 028–14 053). Figure 2A shows the result of DMS in vivo footprinting in sea urchin eggs at the two in vitro binding sites of DBP. In the NCR the bases showing altered DMS reactivity were located between positions 1099 and 1115 (nucleotides 1099, 1100, 1105–1110, 1112, 1113, 1115 of the H-strand). At the ND5/ND6 boundary, the affected bases were from 14041 to 14052 (nucleotides 14041–14043, 14045, 14046, 14051, 14052 of the H-strand). In addition, bases other than purines (T-1111, T-1114, T-1117, T-14044, T-14047) showed altered DMS reactivity. This phenomenon, which generally concerns thymines which are 5′ to DMS-reactive bases, has been reported previously by several authors (Sasse Dwight and Gralla 1988; Gutowski and Schreier 1992; Cantatore et al. 1995; Micol et al. 1997); it might be due to an unexplained thymine methylation or to a premature termination of the *Taq* DNA polymerase at a residue preceeding the modified base. To avoid possible misinterpretation of the data we decided not to consider these bases as footprinted. To estimate the protein occupancy at the two regions, the relative level of methylation protection was measured; it can be considered equivalent to the percentage of the sites bound continuously by the protein or to the percentage of the time in which all the available sites are bound. The average level of DMS protection was 80% and 60% at the NCR binding site and at the ND5/ND6 binding site, respectively; these values suggest that in vivo mtDBP binds the DNA specifically and with high efficiency. To test whether the developmental regulation of mtDNA synthesis could be associated with a different binding status of this protein, DMS footprinting experiments on sea urchin embryos at the pluteus stage were carried out. Figure 2B shows that affected purine residues in the NCR were located between positions 1099 and 1118 (nucleotides 1099, 1100, 1106, 1109, 1110, 1113, 1115, 1118), whereas at the ND5/ND6 boundary footprinted purines were detected at positions 14042, 14043, 14045, 14051, 14052. The protein occupancy at both sites was similar to that found in the eggs. From all these data, summarized in Fig. 2C, it ap-

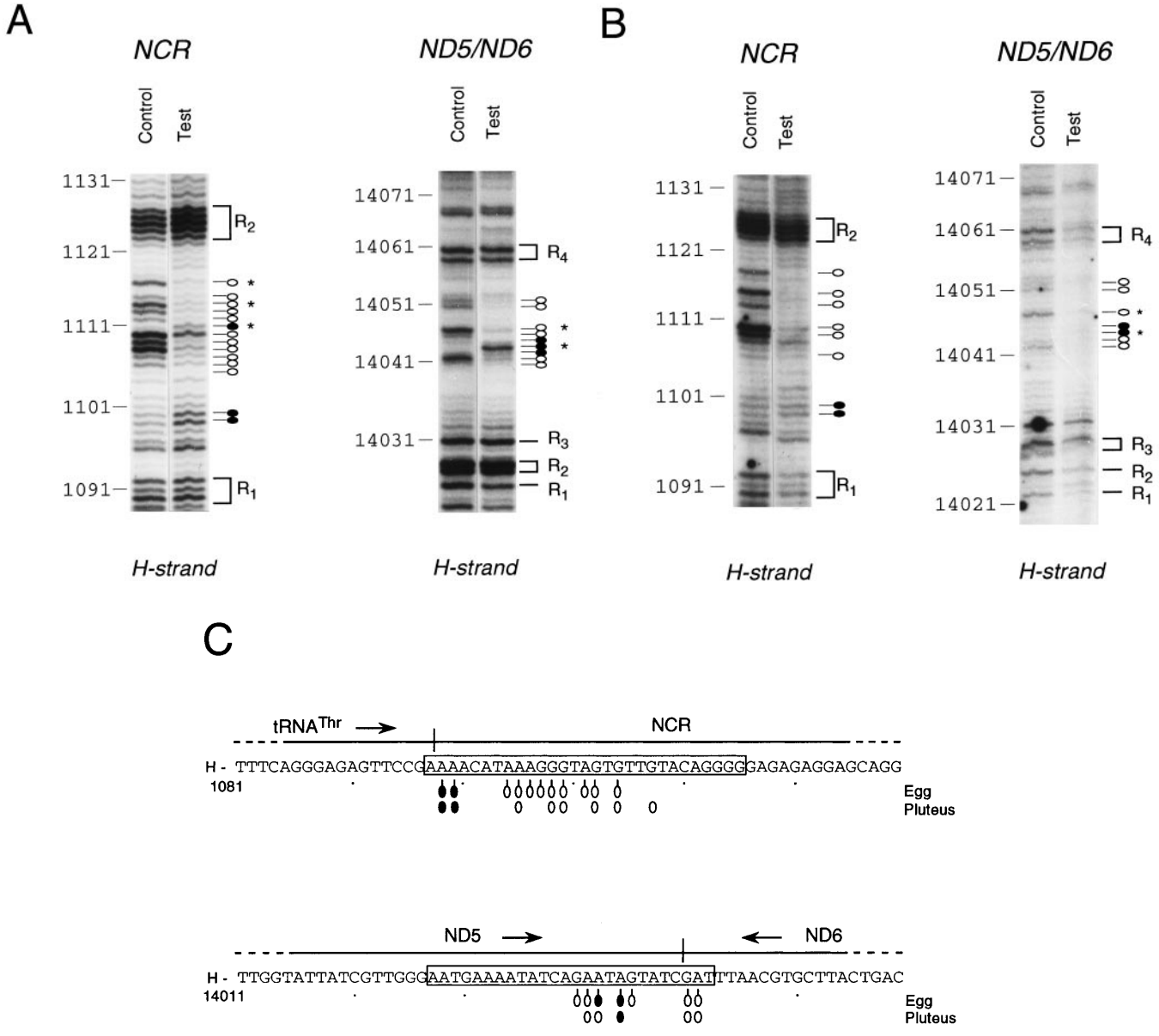
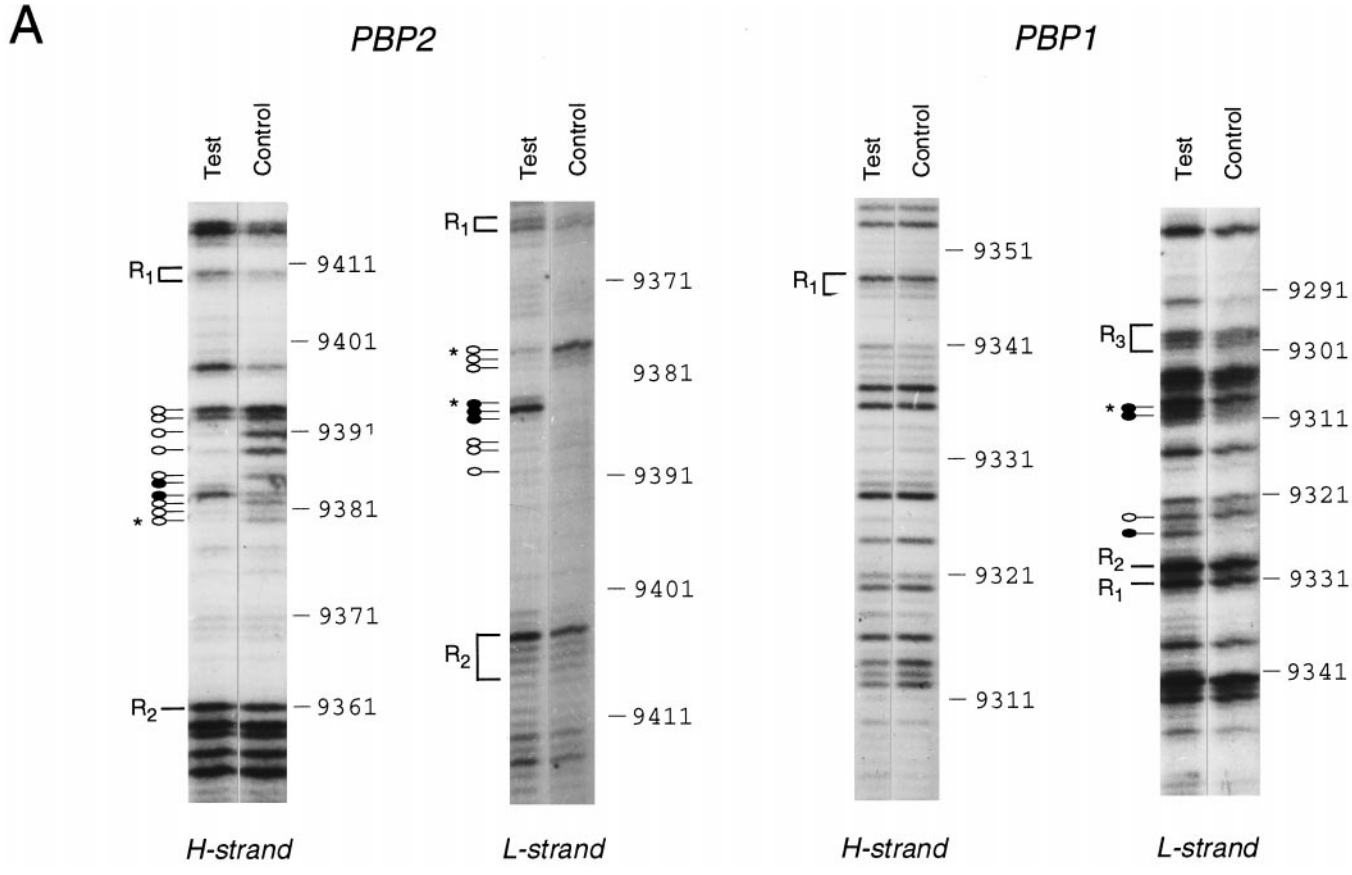


Fig. 2 A–C In vivo DMS footprinting at the binding sites of mtDBP, at different developmental stages. In vivo (*test*) and in vitro (*control*) DMS-treated DNA from *P. lividus* eggs (panel **A**) and plutei (panel **B**) was prepared as described in the text. Primers were *DBP-1-For* (L-1030–1054) and *DBP-2-For* (L-13935–13954), visualizing H-strand bases in the NCR and in the ND5/ND6 binding sites, respectively. MtDNA positions (Cantatore et al. 1989) were deduced from the control G-ladder and from sequencing reactions run alongside (data not shown). Sites of in vivo methylation hypersensitivity are indicated by *filled circles*; *open circles* indicate the sites of methylation protection. The *asterisks* indicate nucleotides other than purines reacting with DMS; they were not considered as footprinted in the summary (panel **C**). The bands designated by R_n in each panel serve as a reference for normalization. **C** Positions of the in vivo footprinted bases at the mtDBP binding sites, at different developmental stages. *Filled* and *open circles* indicate the sites of DMS altered reactivity as deduced from panels **A** and **B**. The regions footprinted by DNase I are *boxed*; the polarity of the $tRNA^{Thr}$, ND5 and ND6 genes is shown; part of the NCR is also shown

Fig. 3 A–C In vivo DMS footprinting at the replication pause region of *P. lividus* mtDNA, at different developmental stages. In vivo (*test*) and in vitro (*control*) DMS-treated DNA from *P. lividus* eggs (panel **A**) and plutei (panel **B**) was prepared as described in the text. Primers were *PBP-For* (L-9259–9278) and *PBP-Rev* (H-9464–9445), visualizing H-strand and L-strand footprinted bases, respectively. MtDNA positions (Cantatore et al. 1989) were deduced from the control G-ladder and from sequencing reactions run alongside (data not shown). Symbols are the same as in Fig. 2. **C** H and L-strand positions of in vivo footprinted bases in the replication pause region at the developmental stages analyzed. The sites of DMS altered reactivity are deduced from panels **A** and **B**. The two *boxed* regions are those protected from DNase I cleavage in in vitro footprinting. The polarity of the ATPase 6 and COIII genes is shown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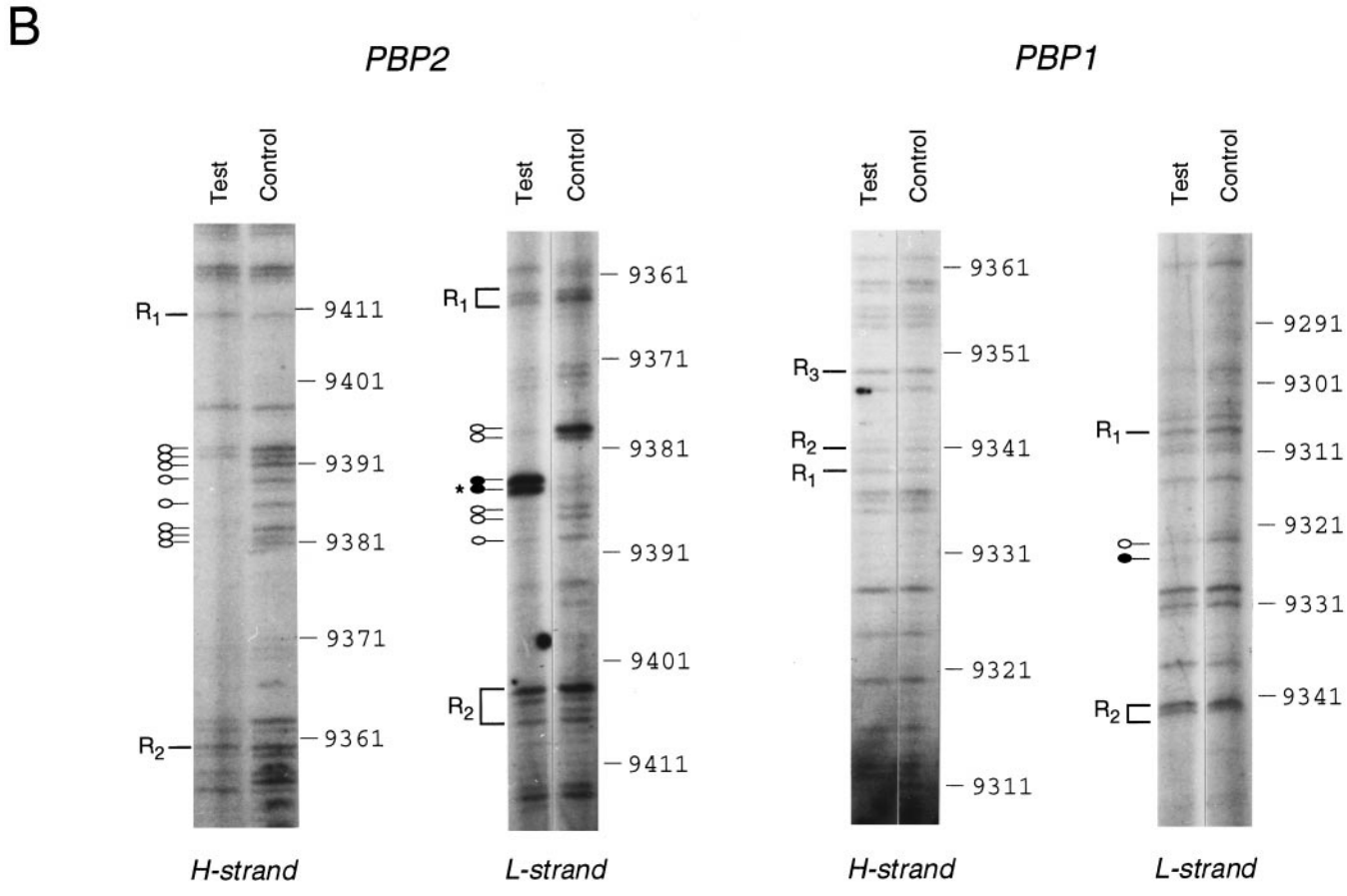
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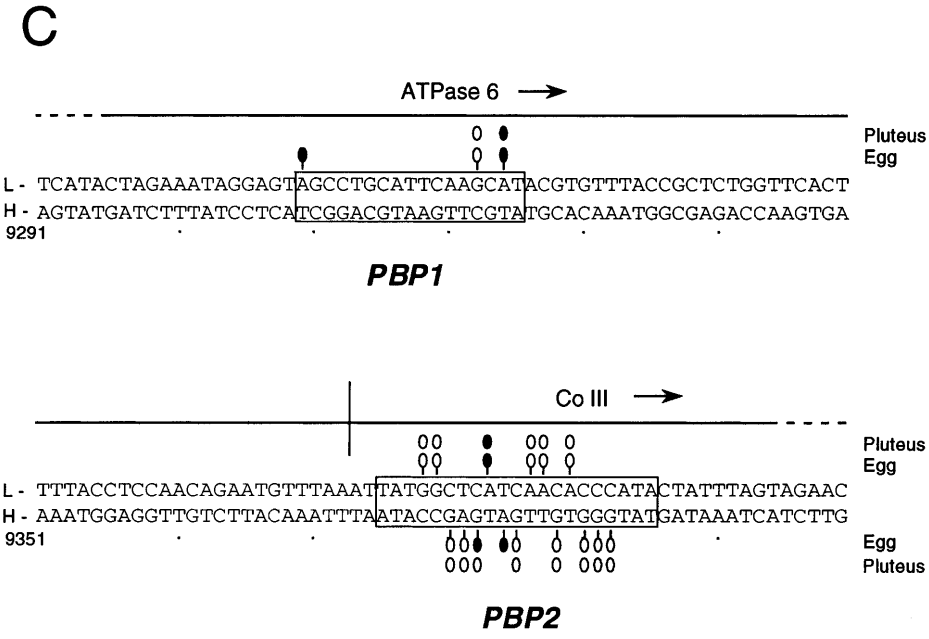

pears that, despite minor differences, mtDBP occupies the same binding sites in eggs and plutei, and with a similar efficiency.

MtPBP-1 and mtPBP-2, two other DNA-binding proteins of 25 kDa and 18 kDa, respectively, interact with sequences located in the major H-strand replication pause site, near the boundary between the genes for ATPase 6 and COIII (Qureshi and Jacobs 1993a, b). Figure 3A shows, in eggs, a region with altered DMS reactivity, located between nucleotides 9379 and 9393. The affected purines are 9381–9383, 9385, 9386, 9389, 9391–9393 on the H-strand and 9379, 9380, 9384, 9387, 9388, 9390 on the L-strand. The footprinted region lies in the binding site of mtPBP-2 (nucleotides 9376–9396) detected by in vitro footprinting in *S. purpuratus* (Qureshi and Jacobs 1993b) and in *P. lividus* (M. Roberti and S. A. Qureshi, unpublished observations). The estimate of the protein occupancy gives a value of about 80%, showing that the protein binds the DNA in vivo with high efficiency. Three more purines, A-9310, G-9323 and A-9325 (L-strand), showed a slightly altered DMS reactivity. These bases are contained within the in vitro binding site of mtPBP-1, located in *P. lividus* between nucleotides 9310 and 9326 (Qureshi and Jacobs 1993a; M. Roberti and S. A. Qureshi, unpublished observations). The much weaker reaction at this site indicates that mtPBP-1 binds to DNA in vivo with low efficiency. In vivo footprinting experiments at the ATPase 6/COIII boundary on embryos at the pluteus stage were also performed. Figure 3B shows that the DMS reactivity pattern in plutei was similar to that found in eggs, with an occupancy at the mtPBP-2 site of about 80%, and with only a weak signal detected once again in the mtPBP-1 binding site. The results of in vivo footprinting in the pause region for leading-strand synthesis are summarized in Fig. 3C.

In vivo footprinting at the 3′ end of the 12S and 16S rRNA genes

In mammalian mitochondria, the termination of the ribosomal transcription unit depends on the binding of a protein factor, mTERF, which contacts a sequence placed a few bases downstream from the 3′ end of the 16S rRNA gene (Kruse et al. 1989). In order to investigate the existence in sea urchins of a DNA-binding protein interacting in the region of the 3′ ends of the two rRNA genes, we prepared a mitochondrial lysate from sea urchin eggs (Roberti et al. 1991) and fractionated it on a Heparin-Sepharose column. The fractions that eluted between 0.2 and 1.0 M KCl were collected and used in band-shift experiments with mtDNA probes. Probes were two fragments (positions 863–1080 and 5763–5929), obtained by PCR, encompassing the 3′ ends of the 12S and 16S ribosomal RNA genes, respectively. These experiments did not reveal any DNA-binding activity (data not shown). As this result could be due to a low concentration of the protein factor or to the chromatographic conditions employed, we carried out DMS in vivo footprinting in the same regions of the mitochondrial genome on sea urchin eggs and plutei. The results of these experiments, shown in Fig. 4, revealed no consistent and reproducible alterations in the pattern of DMS reactivity of these regions of mtDNA extracted from eggs. As a similar result was obtained with plutei (data not shown), we suggest that there are no protein factors bound adjacent to the 3′ ends of the rRNA genes at either stage.

In vivo footprinting at the AT consensus regions

Sea urchin mtDNA contains five AT-rich sequences located in different positions of the genome. The possibility that

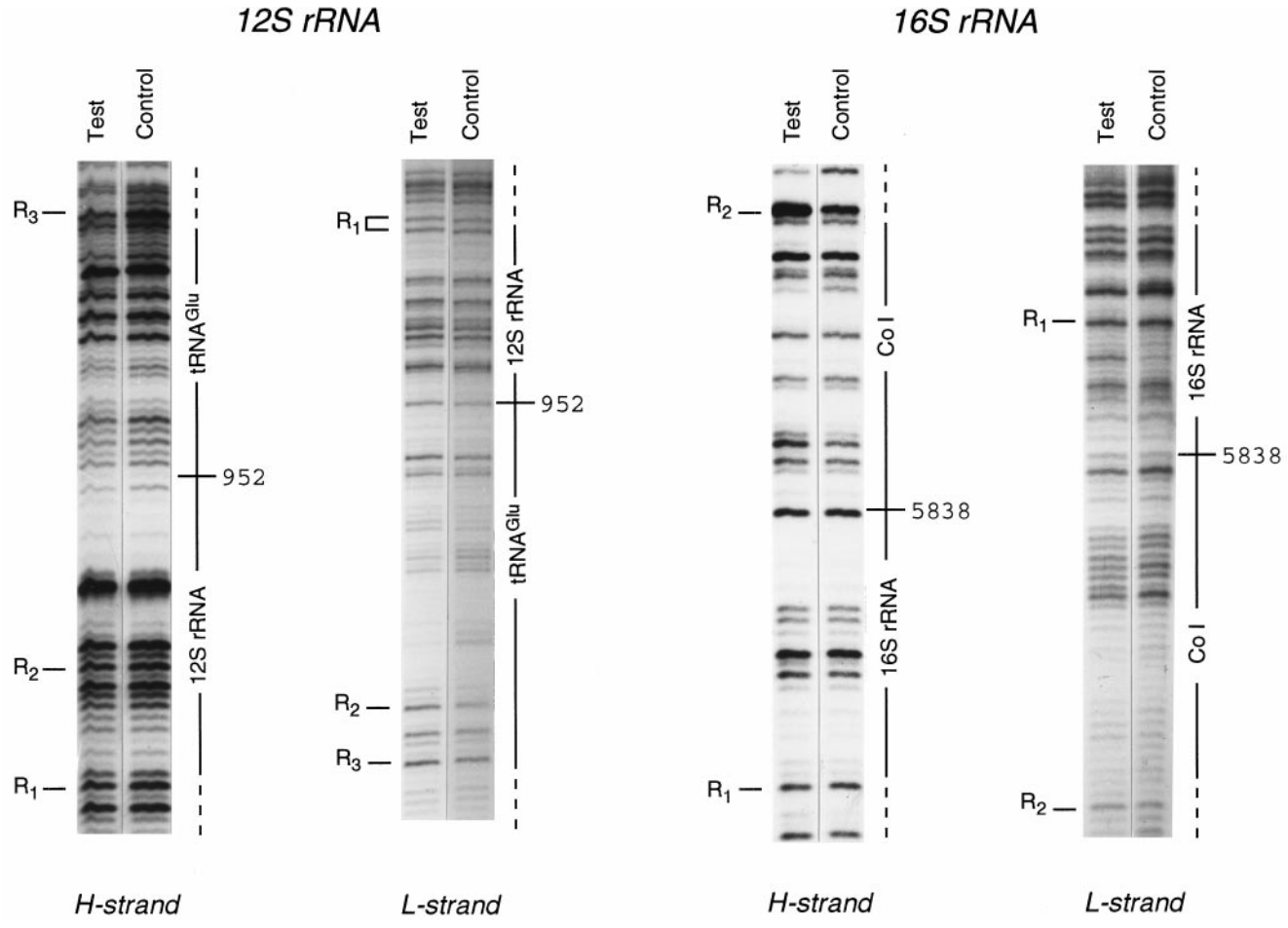

Fig. 4 In vivo DMS footprinting at the 3′ end of 12S and 16S rRNA genes. In vivo (*test*) and in vitro (*control*) DMS-treated DNA from *P. lividus* eggs was annealed with primers *12S-For* (L-863–882) and *12S-Rev* (H-1080–1061), to visualize H-strand and L-strand residues encompassing the 3′ end of the 12S rRNA gene. Primers *16S-For* (L-5763–5782) and *16S-Rev* (H-5929–5910) were used to visualize H-strand and L-strand residues encompassing the 3′ end of the 16S rRNA gene. MtDNA positions (Cantatore et al. 1989) were deduced from the control G-ladder and from sequencing reactions run alongside (data not shown). The positions of the 12S rRNA/$tRNA^{Glu}$ and 16S rRNA/COI boundaries are indicated

the AT consensus regions could function as *cis*-regulatory elements by interacting with DNA-binding proteins was tested by DMS in vivo footprinting. Five oligonucleotides lying upstream of or downstream from the five AT-rich sequences were used to extend the in vivo modified DNA from eggs and plutei. In pluteus mtDNA a block of hypomethylated purines (1217, 1220, 1222, 1224) was detected (Fig. 5B). The footprinted nucleotides (Fig. 5C) are located in the NCR, near to the 5′ end of the $tRNA^{Pro}$ gene, and comprise part of the AT-1 consensus (placed between 1212 and 1220). The DNA-binding activity was present only in the pluteus larval stage, as the DMS footprint was essentially absent in eggs (Fig. 5A). This represents the first case of a possible developmental variation of a DNA-binding activity in sea urchin mitochondria. No footprint was detected at the other AT-rich regions at either developmental stage (data not shown).

Discussion

DMS in vivo footprinting is a technique which provides crucial information on protein-DNA interactions. It has a sensitivity higher than in vitro techniques, and this allows the detection of interactions not visible by in vitro methods. In mammalian mitochondria a careful analysis of the *in organello* methylation pattern allowed the confirmation of all the protein-DNA interactions described in vitro, and permitted the identification of novel interactions in the D-loop of mammals (Ghivizzani et al. 1994; Cantatore et al. 1995; Ammini et al. 1996). Recently, similar studies have made it possible to assess the action of ethidium bromide and ATP on human mitochondrial transcription (Micol et al. 1997) and to identify protein binding sites in the 3′ D-loop region of human and rat mtDNA (Roberti et al. 1998). In the present paper we used DMS footprinting to analyze in vivo the interactions between proteins and sea urchin mtDNA. We first estimated the protein occupancy in the non-coding region (NCR) and at the boundary

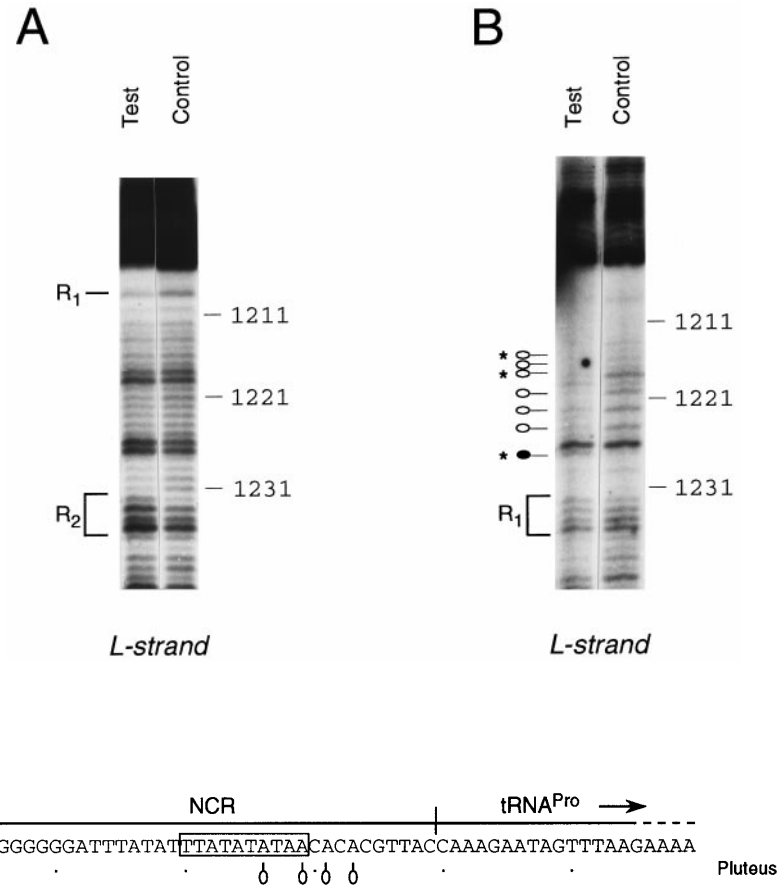

Fig. 5A–C In vivo DMS footprinting near the AT consensus sequence located in the NCR of *P.lividus* mtDNA, at different developmental stages. In vivo (*test*) and in vitro (*control*) DMS-treated DNA from *P. lividus* eggs (panel **A**) and plutei (panel **B**) was annealed with primer *AT-1-Rev* (H-1315–1296) to visualize footprinted bases on the L-strand. MtDNA positions (Cantatore et al. 1989) were deduced from the control G-ladder and from sequencing reactions run alongside (data not shown). Symbols are the same as in Fig. 2. **C** L-strand sequence positions of the sites of in vivo footprinted bases near the AT-1 consensus at the developmental stages analyzed. The sites of DMS altered reactivity are indicated as deduced from panel **B**. The AT consensus is *boxed*; the polarity of the $tRNA^{Pro}$ gene is shown

between the ND5 and ND6 genes and detected two strong contact sites (Fig. 2), which corresponded to the in vitro binding sequences of mtDBP (Roberti et al. 1991; Loguercio Polosa et al. 1994; Fig. 1). This indicates that both interactions have a functional significance, thus excluding that the in vitro binding observed at the two sites was an artefact due to sequence similarity (about 70%). As the NCR-binding site contains the transition point from the RNA primer to the nascent H-strand DNA, plus the termination site of the D-strand, mtDBP could function in the regulation of D-loop formation and/or expansion. A protein-induced block of D-loop expansion might constitute a regulatory mechanism for mtDNA replication. This process is active during oogenesis, quiescent during early development, and resumes at the pluteus stage (Pikò 1969; Pikò and Taylor 1987). The observation that the binding of mtDBP does not change during development suggests that its binding *per se* is not the mechanism regulating D-loop expansion. However, mtDBP might still have a regulatory role, since *post*-translational modification may take place on a domain of the DNA-bound protein. A similar situation has been described for UV and TPA activation of the c-*jun* promoter (Rozek and Pfeifer 1993; Herr et al. 1994) and for growth-factor activation of c-*fos* (Herrera et al. 1989). In this light, the minor, but reproducible, variations in the footprint observed at the different developmental stages could be significant. The second mtDBP binding site was located at the boundary of the 3′ ends of the oppositely transcribed ND5 and ND6 genes. Present data do not support any particular role for this interaction. In principle, a function in mtDNA replication or transcriptional termination should be considered. A substantial difference between in vitro and in vivo footprints concerns the H-strand replication pause region. In vitro studies in both *S. purpuratus* and *P. lividus* (Qureshi and Jacobs 1993a, b; M. Roberti and S. A. Qureshi, unpublished data, summarized in Fig. 1) showed the existence of two proteins, mtPBP-1 and mtPBP-2, having similar abundance and able to bind independently and with high affinity to two sequences lying at the boundary between the ATPase 6 and COIII genes. Our results show that, whereas the mtPBP-2 binding site appears to be almost fully occupied in vivo, the mtPBP-1 binding site has a very low occupancy. This may be due to the presence of some other factors interfering with the binding of mtPBP-1 to DNA. Alternatively, the binding of mtPBP-1 and mtPBP-2 in vivo may not be independent, and may even exhibit mutual interference. A further possibility is that mtPBP-1 has other, higher affinity, sites in the mitochondrial genome, that compete with the binding site in the pause region identified from in vitro studies. The observation that here, too, binding does not change during early embryogenesis suggests that

these proteins may be not involved in developmental regulation of mtDNA synthesis or transcription.

The presumed absence of protein binding at the 3′ ends of the rRNA genes suggests that in sea urchins, contrary to mammals, the 3′ ends of the rRNAs are not generated by a protein-dependent termination event. On the basis of sequence conservation of a heptamer motif contained in the mTERF binding site Valverde et al. (1994) suggested the existence of a conserved, protein-dependent, mitochondrial rRNA transcription termination mechanism in all animals. The data reported here show that this principle does not apply to sea urchins. This may reflect the difference in gene organization between sea urchins and other animal mitochondrial genomes. Relevant features include: (1) the separation of the two rRNA genes; (2) the orientation in the opposite direction of the heptamer motif found at the 3′ end of the 16S rRNA, and (3) the absence of such a motif at the 3′ end of the 12S rRNA gene. The lack of protein-dependent transcription termination at the 3′ ends of the rRNA genes suggests that the 3′ termini of these transcripts are generated by endonucleolytic cleavage of a polycystronic transcript, and supports the idea that mitochondrial gene expression is regulated post-transcriptionally in sea urchins (Elliot and Jacobs 1989; Cantatore et al. 1990; Jacobs 1989a, b). Therefore, mtRNA synthesis in sea urchins might depend on multiple, partially overlapping transcription units. According to this mechanism the mitochondrial genes would be transcribed as larger precursors, from which flanking sequences are excised during RNA processing. Such sequences might play a role in transcript maturation and may also be the coding sequences of another gene. This hypothesis is supported by the detection of a large number of relatively abundant, overlapping high-molecular-weight transcripts (Cantatore et al. 1990), mostly representing the flanking sequences discarded during RNA processing. Furthermore, the considerable difference in the abundance of mRNAs mapping in adjacent positions and the overlaps between the 3′ ends of the two rRNAs and the 5′ ends of the downstream genes (Elliot and Jacobs 1989; Cantatore et al. 1990) are consistent with this view of multiple transcription units that are modulated by post-transcriptional processing events, rather than by transcriptional initiation and termination.

With the aim of identifying new protein-DNA interactions at other putative regulatory regions we analyzed the methylation patterns at the five AT-consensus regions, which had previously been suggested to serve as bidirectional promoter elements, based on similarity with the yeast nonanucleotide promoter (Jacobs et al. 1990). In agreement with this hypothesis, the 5′ ends of several high-molecular-weight transcripts were mapped to some of these sequences (Elliot and Jacobs 1989; Cantatore et al. 1990). The analysis of the footprinting pattern at the five AT-rich regions revealed only one clear case of protein binding. It concerns seven nucleotides located in the NCR, overlapping the AT-1 consensus. This part of the NCR may have an important role in regulating mtDNA metabolism. It contains (Fig. 1) the overlapping 3′ and 5′ ends of two RNA species encoded by the H-strand (RNA C and D according to Cantatore et al. 1990) and presumably the 5′ end of an L-strand-encoded RNA which acts as a primer for mtDNA leading-strand replication (Jacobs et al. 1989). Importantly, this site seems to be occupied efficiently at the pluteus stage, but much less so, or not at all, in the unfertilized egg. As protein binding is inferred to occur only at a stage when mtDNA replication is active, it could be directly related to mtDNA synthesis. The absence of protein binding in vivo at the other four AT consensus sequences indicates that if they do, indeed, function as promoters, then they interact only transiently or weakly with RNA polymerase. By implication, the environment of the AT sequence located in the the NCR must differ, since it alone is bound by a protein at the pluteus stage. The bound protein must therefore recognize other features than just the AT consensus: perhaps structural distortions provoked by the adjacent polyguanosine tract, the specific gene sequences abutting the AT consensus, or else other proteins bound in this region.

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References

Ammini CV, Ghivizzani SC, Madsen CS, Hauswirth WW (1996) Genomic footprinting of mitochondrial DNA. II. In vivo analysis of protein-mitochondrial DNA interactions in *Xenopus laevis* eggs and embryos. Methods Enzymol 264:23–36

Attardi G, Schatz G (1988) Biogenesis of mitochondria. Annu Rev Cell Biol 4:289–333

Cantatore P, Saccone C (1987) Organization, structure, and evolution of mammalian mitochondrial genes. Int Rev Cytol 108:149–208

Cantatore P, Roberti M, Morisco P, Rainaldi G, Gadaleta MN, Saccone C (1987) A novel gene order in the *Paracentrotus lividus* mitochondrial genome. Gene 53:41–54

Cantatore P, Roberti M, Rainaldi G, Gadaleta MN, Saccone C (1989) The complete nucleotide sequence, the gene organization and the genetic code of the mitochondrial genome of *Paracentrotus lividus*. J Biol Chem 264:10965–10975

Cantatore P, Roberti M, Loguercio Polosa P, Mustich A, Gadaleta MN (1990) Mapping and characterization of *Paracentrotus lividus* mitochondrial transcripts: multiple and overlapping transcription units. Curr Genet 17:235–245

Cantatore P, Daddabbo L, Fracasso F, Gadaleta MN (1995) Identification by *in organello* footprinting of protein contact sites and of single-stranded sequences in the regulatory region of rat liver mitochondrial DNA. J Biol Chem 270:25020–25027

De Giorgi C, Martiradonna A, Lanave C, Saccone C (1996) Complete sequence of the mitochondrial DNA in the sea urchin *Arbacia lixula*: conserved features of the echinoid mitochondrial genome. Mol Phylog Evol 5:323–332

Elliott DJ, Jacobs HT (1989) Mutually exclusive synthetic pathways for sea urchin mitochondrial rRNA and mRNA. Mol Cell Biol 9:1069–1082

Ghivizzani SC, Madsen CS, Nelen MR, Chandramohan VA, Hauswirth WW (1994) *In organello* footprint analysis of human mitochondrial DNA: human mitochondrial transcription factor A interactions at the origin of replication. Mol Cell Biol 14:7717–7730

Gutowski JC, Schreier HJ (1992) Interaction of the *Bacillus subtilis glnRA* repressor with operator and promoter sequences in vivo. J Bacteriol 174:671–681

Herr I, van Dam H, Angel P (1994) Binding of promoter associated AP-1 is not altered during induction and subsequent repression of the c-*jun* promoter by TPA and UV irradiation. Carcinogenesis 15:1105–1113

Herrera RE, Shaw PE, Nordheim A (1989) Occupation of the c-*fos* serum response element in vivo by a multi-protein complex is unaltered by growth factor induction. Nature 340:68–70

Jacobs HT (1989a) The developmental regulation of mitochondrial biogenesis. Bull Mol Biol Med 14:89–112

Jacobs HT (1989b) Do ribosomes regulate mitochondrial RNA synthesis? BioEssays 11:27–34

Jacobs HT, Elliott DJ, Math VB, Farquharson A (1988) Nucleotide sequence and gene organization of sea urchin mitochondrial DNA. J Mol Biol 202:185–217

Jacobs HT, Herbert ER, Rankine J (1989) Sea urchin egg mitochondrial DNA contains a short displacement loop (D-loop) in the replication origin region. Nucleic Acids Res 17:8949–8965

Jacobs HT, Elliott DJ, Mayhook AG, Segreto DT, Rinaldi A-M (1990) Homology between the mechanisms of mitochondrial gene regulation in echinoderms and fungi. In: Quagliariello E, Papa S, Palmieri F, Saccone C (eds) Structure, function and biogenesis of energy transfer systems, Elsevier Science Publishers B. V. (Biomedical Division), pp 145–151

Kruse B, Narasimhan N, Attardi G (1989) Termination of transcription in human mitochondria: identification and purification of a DNA-binding protein factor that promotes termination. Cell 58:391–397

Loguercio Polosa P, Roberti M, Mustich A, Gadaleta MN, Cantatore P (1994) Purification and characterization of a mitochondrial DNA-binding protein that binds to double-stranded and single-stranded sequences of *Paracentrotus lividus* mitochondrial DNA. Curr Genet 25:350–356

Mayhook AG, Rinaldi A-M, Jacobs HT (1992) Replication origins and pause sites in sea urchin mitochondrial DNA. Proc R Soc Lond B 248:85–94

Micol V, Fernández-Silva P, Attardi G (1997) Functional analysis of in vivo and *in organello* footprinting of HeLa cell mitochondrial DNA in relationship to ATP and ethidium bromide effects on transcription. J Biol Chem 272:18896–18904

Pikò L (1969) Absence of synthesis of mitochondrial DNA during early development in sea urchin. Am Zool 9:1118

Pikò L, Taylor KD (1987) Amounts of mitochondrial DNA and abundance of some mitochondrial gene transcripts in early mouse embryos. Dev Biol 123:364–374

Qureshi SA, Jacobs HT (1993a) Characterization of a high-affinity binding site for a DNA-binding protein from sea urchin embryo mitochondria. Nucleic Acids Res 21:811–816

Qureshi SA, Jacobs HT (1993b) Two distinct, sequence-specific DNA-binding proteins interact independently with the major replication pause region of sea urchin mtDNA. Nucleic Acids Res 21:2801–2808

Roberti M, Mustich A, Gadaleta MN, Cantatore P (1991) Identification of two homologous mitochondrial DNA sequences, which bind strongly and specifically to a mitochondrial protein of *Paracentrotus lividus*. Nucleic Acids Res 19:6249–6254

Roberti M, Musicco C, Loguercio Polosa P, Milella F, Gadaleta MN, Cantatore P (1998) Multiple protein binding sites in the TAS-region of human and rat mitochondrial DNA. Biochem Biophys Res Commun 243:36–40

Rozek D, Pfeifer G (1993) In vivo protein-DNA interactions at the c-*jun* promoter: preformed complexes mediate the UV response. Mol Cell Biol 13:5490–5499

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Sasse-Dwight S, Gralla JD (1988) Probing co-operative DNA-binding in vivo. The lac O_1:O_3 interaction. J Mol Biol 202:107–119

Valverde JR, Marco R, Garesse R (1994) A conserved heptamer motif for ribosomal RNA transcription termination in animal mitochondria. Proc Natl Acad Sci USA 91:5368–5371