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MITOCHONDRIAL DNA-BINDING PROTEINS THAT BIND PREFERENTIALLY TO SUPERCOILED MOLECULES CONTAINING THE D-LOOP REGION OF XENOPUS LAEVIS mtDNA

Bernard MIGNOTTE, Monique BARAT, Janine MARSAULT and Jean-Claude MOUNOLOU

Laboratoire de Biologie Générale, Bât 400,
Université de Paris-Sud, F-91405 ORSAY Cedex, France

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From the bulk of the Xenopus laevis mitochondrial proteins insoluble in 1 % Triton X-100 + 1M NaCl, we have isolated, by DNA-cellulose chromatography, a protein fraction enriched in DNA-binding proteins . This fraction contains proteins showing a specific affinity for supercoiled DNA molecules containing the mitochondrial DNA displacement-loop region, as measured by filter binding and competition assays.

Mitochondrial DNA from vertebrate cells analyzed to date occurs in the form of supercoiled circular molecules of 15-19 Kbp. A peculiar replicative form has been observed in most species : the displacement-loop which is a triplex structure formed by the synthesis of a short, but well defined, H-strand segment displacing the parental H-strand (1,2). The replication proceeds from this D-loop region suggesting two possible levels of replication control : initiation and/or elongation of the D-loop strand.

The rapid turn-over of the D-loop strand in mouse L-cell mtDNA (3), the D-loop frequencies (30-70 % in Xenopus laevis oocyte mtDNA (4)) higher than expected if all D-loop strands were only required for replication, and a clear correlation between the rate of mitochondriogenesis and the D-loop frequency (4) suggest other important functions in the regulation of the expression of the mitochondrial genome. This hypothesis is supported by the fact that the promoters for H and L-strand transcription are probably located in this region (5-7). The nucleotide sequence of this region is known for mammalian and Xenopus laevis mtDNA (7). Potential secondary structures were found near the D-loop strand origin and one repeated sequence, maybe a template stop signal for D-loop strand synthesis, was also described in mamma-

ABBREVIATIONS

D-loop, displacement-loop ; mtSSB, mitochondrial single-strand binding protein ; DBP, DNA binding protein ; $(pBR328)_2$, plasmid pBR328 in its dimeric form ; mitoplast, digitonin treated mitochondria.

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lian (2) and Xenopus laevis (7) mtDNA. At last, the D-loop region shows interspecific but also intraspecific nucleotide sequence variability (8). All these observations suggest the occurence of specific regulation by means of protein mtDNA interactions.

In a previous report (9), we had partially described a Xenopus laevis mitochondrial single strand binding protein which may stabilize the D-loop as the 16 kilodaltons protein reported by Van Tuyle and Pavco does (10). The data reported here concern a search for DBP(s) with specificity for the D-loop region. From the bulk of the mitochondrial proteins insoluble in 1 % Triton + 1 M NaCl, we have isolated a protein fraction enriched in DBP with a specific affinity for supercoiled DNA molecules containing the Xenopus laevis mtDNA D-loop region.

MATERIAL AND METHODS

Oocytes. Whole ovaries were taken from adult female Xenopus laevis and cut into pieces in Barth's medium as modified by Gurdon (11). The animals were purchased from S.E.R.E.A. (Argenton l'Eglise, France).

Material. DNA cellulose was prepared with native calf thymus DNA according to Litman (12). Phosphocellulose (P11) was obtained from Whatman. Labelled thymidine ([methyl-^{3}H] or [methyl-^{14}C] Thymidine) was obtained from the Commissariat à l'Energie Atomique (France).

Plasmids. Two pBR328-mtDNA recombinants were constructed following classical procedure (13) in the E. coli DP50 strain. pXlmB14 corresponds to the plasmid pBR328 with inserted the Bcl I B fragment of Xenopus laevis mitochondrial DNA. This fragment of 4.7 Kbp includes the D-loop region (14). The plasmid pXlmB3 contains the 2.25 Kpb Bcl I C fragment including the L-strand replication origin. These two recombinant plasmids and a pBR328 plasmid in its dimeric form were purified, after amplification with spectinomycin 300 µg/ml, using CsCl/ethidium bromide gradient centrifugation. When necessary plasmids were labelled in vivo by addition of ^{3}H-or ^{14}C-Thymidine (5 or 0.5 µCi/ml) and deoxyadenosine (250 µg/ml) during the amplification step. Biohazard associated with the experiments have been examined by the French National Comitee and the experiments were carried out according to the rules established by this Comitee.

Isolation of mitochondrial DNA binding proteins. Mitochondria were prepared as described previously (9). The outer membranes were removed by digitonin according to G. Brun and al. (15). Purified mitoplasts ($\simeq$ 500 mg of proteins) were lysed with 1 % Triton X-100 in 20 mM Tris HCl pH 7.5 1M NaCl, 2 mM Dithioerythritol, 0.1 mM phenylmethylsulphonylfluorid at a protein concentration of about 7 mg/ml. The lysate was centrifuged at 200 000 g for 165 min. The clear supernatant contains the mtSSB previously described (9). The pellet was resuspended in the same buffer which was made 2M NaCl, stirred 15 min. and centrifuged at 200 000g for 165 min. The clear supernatant containing about 3 % of the mitoplast proteins was dialysed against 20 mM Tris HCl pH 7.5, 50 mM NaCl, 1 mM EDTA, 2 mM 2-mercaptoethanol (Buffer A), 20 % glycerol and adsorbed on a calf thymus DNA cellulose column which had been equilibrated with the same buffer. The column was eluted stepwise by 550 mM and 1M NaCl. Fractions containing DNA binding activity as judged by the filter binding assay were dialysed and kept at -20°C in buffer A,60 % glycerol.

DNA binding assay. The nitrocellulose filters (0.45 µm pore sized BA85, Schleicher and Schüll) were washed with 0.3 M NaOH (16) for 10 min. and twice

with deionised water for 5 min. They were then stored in buffer A.5 % glycerol at least one night. Routinely the plasmids ^{14}C-pXlmB14 (32 ng ; 450 cpm) and/or ^{3}H-(pBR328)$_2$ (170 ng ; 2100 cpm) were incubated with the protein solution in buffer A, 5 % glycerol and 50 µg/ml BSA (total volume 0.2 ml) at 30°C for 10 min. The reaction mixture was filtered through a nitrocellulose filter pre-washed with 10 ml of the binding buffer at a flow rate of about 3 ml/min. The buffer was then washed with 10 ml of the same buffer and dried ; ^{14}C and ^{3}H radioactivity were determined in toluene PPO-POPOP scintillation solution using LKB rackbeta II counter. Appropriate control experiments were also carried out in the absence of DBP to determine the background (2-5 % of input supercoiled DNA and less than 1 % for linear double or single stranded ones).

Protein electrophoresis. Protein electrophoresis was performed on 11 % polyacrylamide slab gels containing 0.1 % Na Dod SO_4 using the system of Laemmli (17). Protein bands were revealed by the silver staining method (18).

Phosphocellulose chromatography. An aliquot of the fraction was diluted to 25 mM Tris pH 7.5, 100 mM KCl, 2 mM 2-mercaptoethanol, 0.2 mM EDTA, 20 % glycerol and loaded onto a phosphocellulose column equilibrated with the same buffer. Adsorbed proteins were eluted using a linear KCl gradient from 0.1 to 0.75M KCl followed by a 1.5M KCl step. The fractions collected in tubes containing 50 µg/ml BSA were used in DNA binding assays.

RESULTS

Isolation of DNA binding proteins. Since it has been proposed that mtDNA is associated with the inner mitochondrial membrane (19, 20, 21, Barat et al. submitted) maybe near the replication origin (22), purification was done from the bulk of proteins unsolubilized in 1 % Triton and 1 M NaCl in order to enrich in membrane bound and DNA tightly bound proteins. Material solubilized with 2 M NaCl was chromatographied on DNA cellulose column (see material & Method). Preliminary studies suggesting a possible DBP activity specific for mtDNA in the fraction eluted at 1 M NaCl, this fraction was studied in details.

In vitro DNA binding properties of the fraction. To detect DBPs with a specific affinity for the Xenopus laevis mitochondrial D-loop region, two kinds of competition experiments were done at 50 mM NaCl using dimeric pBR328 and plasmids pXlmB3 and pXLmB14 described in material and methods. In the first one, increasing concentrations of proteins were added to a mixture of ^{14}C labelled pXlmB14 and ^{3}H labelled (pBR328)$_2$ and the percentage of DNA retained on filters was measured. In this case supercoiled ^{14}C-pXlmB14 was retained with an efficiency about 10 times higher than supercoiled ^{3}H-(pBR328)$_2$ (fig. 1a) ; with linear native or heat-denatured plasmids only weak binding was found (fig. 1b and c). In control experiments done with others mtDBPs the percentage of ^{14}C DNA retained was about three time higher than the one of ^{3}H DNA. The presence of about 40 % of oligomeric plasmids in the ^{14}C-pXlmB14 preparation is probably responsible for this result, although the plasmid pBR328 has been used in its dimeric from (9.8 Kbp v.s. 9.6 Kbp for monomeric pXlmB14). Then in order to

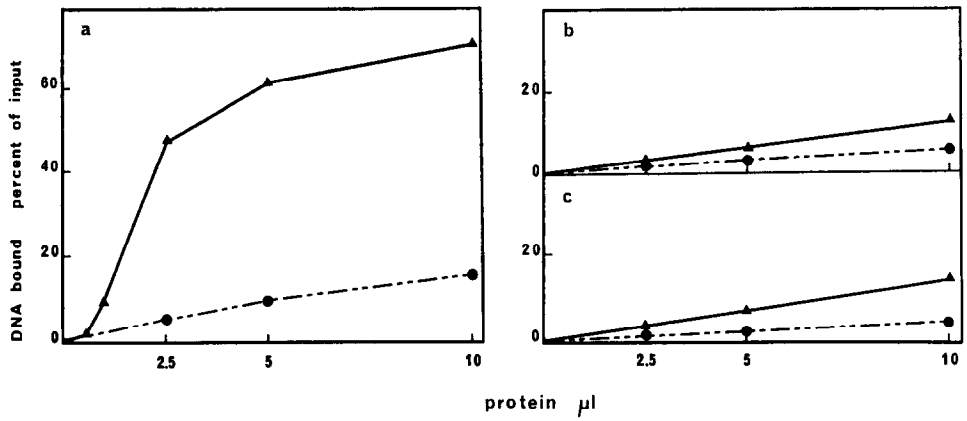

Figure 1 : Binding of proteins to a mixture of ^{14}C-pXlmB14 (▲—▲) and ^{3}H-(pBR328)$_2$ (●--●) a) supercoiled DNA. b) DNA linearized with the restriction endonuclease pVUII. c) linearized and heat-denatured DNA.

confirm the specificity for pXlmB14 a second kind of competition experiments, using fixed supercoiled ^{14}C-pXlmB14 concentration and increasing amounts of supercoiled cold competitor DNA of unique size, was done at a constant not saturating protein concentration (fig. 2) : (pBR328)$_2$ and pXlmB3 plasmids were found poor competitors compared to pXlmB14. In control experiments with other mtDBPs the competition was similar for the 3 plasmids. This result

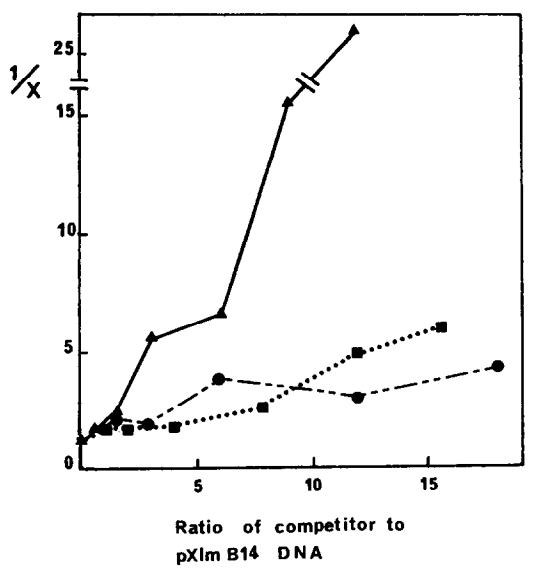

Figure 2 : Competition experiments. Binding experiments were carried out with a mixture of ^{14}C-pXlmB14 and various concentrations of cold competitor DNA ; pXlmB14 : (▲—▲) ; pXlmB3 : (■····■) ; (pBR328)$_2$: (●--●). X is the ratio of the amount of pXlmB14 retained in the presence competitor to the amount retained in the absence of competitor.

shows that proteins contained in the fraction exhibit specificity for supercoiled pXlmB14. This specific binding is sensitive to ionic strength with two maxima at 50 and 500 mM (fig. 3). This result reflects probably the heterogeneity of the fraction but it may be also the consequence of some conformational change of the protein(s) and/or of DNA when varying the salt concentration.

Composition of the fraction. Electrophoresis of the fraction on denaturing polyacrylamide gels (fig. 4) revealed one predominant polypeptide which shows apparently the same electrophoretic mobility as the mtSSB previously described (15,500 daltons) (9), and 7 minor bands with molecular weight ranging between 40,000 and 90,000 daltons. The major polypeptide represents about 0.001 % of the mitoplast proteins. This result led us to test the affinity of proteins contained in the fraction for supercoiled, linear and heat-denatured pBR328 in conditions where the purified mtSSB retains heat-denatured DNA with an efficiency more than 20 times higher than supercoiled or double-stranded linear ones (fig. 5a). With the fraction described here, (Fig. 5b) single stranded DNA is retained with an efficiency about 2.5 times higher than supercoiled one, while double-stranded linear DNA is only weakly retained. This result shows that if the mtSSB is one of the components of the fraction, other proteins with affinity for supercoiled molecules are also present. It is unlikely that RNA polymerase could be responsible for the affinity for supercoiled pXlmB14 since Xenopus laevis and rat mitochondrial RNA polymerases (23, 24) show different chromatographic properties than the proteins described here ; furthermore, in preliminary experiments no measurable RNA polymerase activity could be found in the fraction. In the sensitive assay used for polyoma virus T antigen (25) no ATPase activity could be detected either.

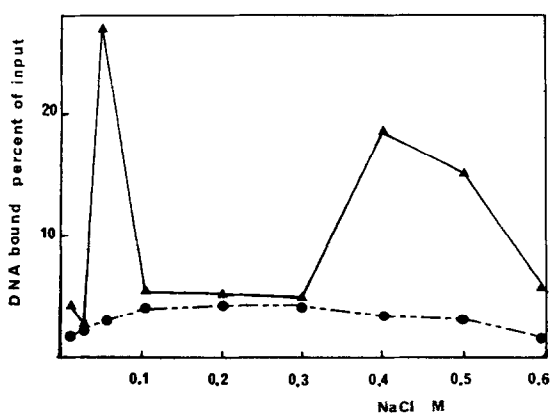

Figure 3 : Effect of ionic strength. Binding experiments with a mixture of ^{14}C-pXlmB14 (▲—▲) and ^{3}H-(pBR328)$_2$ (●- -●) were done at various NaCl concentrations (mean of 5 experiments).

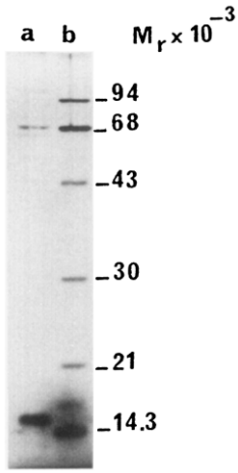

Figure 4 : NaDod SO_4 polyacrylamide gel electrophoresis pattern of the fraction, lane a. Markers (phosphorylase B ; BSA ; Ovalbumin ; carbonic Anhydrase : Soybean Trypsin Inhibitor ; lysozyme) lane b.

Analysis of the fraction by phosphocellulose chromatography. In order to verify the possible presence of mtSSB and to check if the affinity for supercoiled pX1mB14 could be related to this protein the fraction was chromatographied on a phosphocellulose column (fig. 6). Binding experiments were done with denatured (linear) ^{3}H-pBR328 to detect the mtSSB and with a mixture of supercoiled ^{14}C-pX1mB14 and ^{3}H-$(pBR328)_2$ to determine the elution profile of the proteins showing a specific affinity for mtDNA. The single stranded DNA binding activity was eluted as a major peak at about 150 mM KCl but some activity was also found in the flow-through. DNA binding assay performed

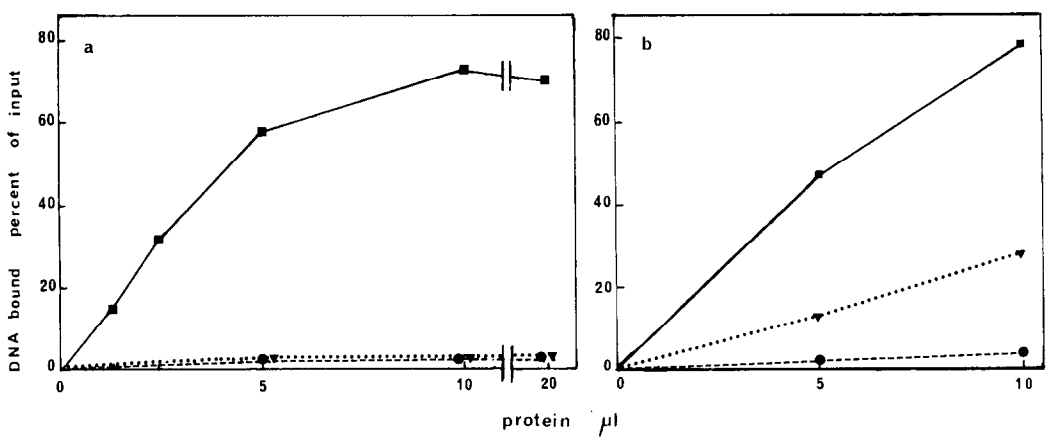

Figure 5 : DNA binding activity with supercoiled (▼····▼), linearized (●--●) and heat-denatured (■——■) pBR328 in buffer A, 200 mM NaCl, 5 % glycerol.
a. increasing amounts of purified mtSSB.
b. increasing amounts of the fraction.

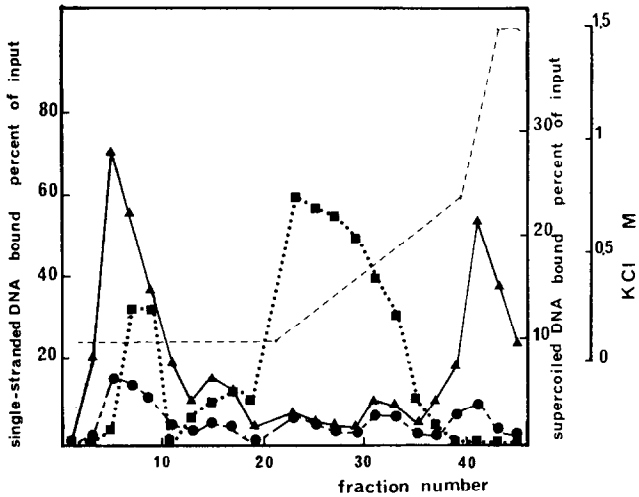

Figure 6 : Phosphocellulose chromatography. DNA binding activity was assayed in two conditions : (1) binding to a mixture of supercoiled ^{14}C-pX1mB14 and ^{3}H $(pBR328)_2$ in buffer A, 5 % glycerol and 400 mM NaCl (in this condition the effect of the salt added with the fraction is negligible) and (2) binding to single-stranded pBR328 (linearized at the pVUII site) in 20 mM $NaPO_4$, 500 mM NaCl, 1 mM EDTA 2 mM, 2-mercaptoethanol, 5 % glycerol (optimal conditions for the mtSSB). ^{14}C-pX1mB14 (▲—▲) ; ^{3}H-$(pBR328)_2$ (●--●) ; heat-denatured pBR328 (■···■).

with a mixture of supercoiled ^{14}C-pX1mB14 and ^{3}H-$(pBR328)_2$ showed two peaks of activity ; in both peaks the higher affinity for ^{14}C-pX1mB14 was still found, one corresponds to proteins not adsorbed on the column, the other to those eluted with the 1.5M KCl step. Since (a) the single-strand binding activity shows the same behaviour on phosphocellulose as the purified mtSSB, (b) the proteins of the fraction are eluted at 1M NaCl from DNA cellulose as the mtSSB and (c) the major band found on denaturing polyacrylamide gels has the same electrophoretic mobility as the mtSSB, we conclude that this protein is present in the fraction. However, supercoiled pX1mB14 binding acticity is not eluted from the phosphocellulose column at the same position as the single-strand one indicating that the fraction contains mtSSB and proteins with specificity for supercoiled pX1mB14. The yield of the chromatography (7 % for pX1mB14 binding activity and 9 % for single strand one) did not allow us to detect any band on polyacrylamide gel. It is not yet clear if supercoiled pX1mB14 binding activity is due to one or two proteins since two peaks of activity have been observed with the same $^{14}C/^{3}H$ ratio. It is possible that these two peaks correspond to the same polypeptide associated (or not) with others in a complex.

DISCUSSION

The peculiarity of the D-loop region of the mtDNA has led us to postulate the existence of DBPs able to recognize specifically this fragment.

Proteins with such a property have been found among the Xenopus laevis oocyte mitochondrial proteins insoluble in 1 % Triton X100, 1M NaCl. However once isolated these proteins are eluted at 1M NaCl from the DNA cellulose column. The necessity of an ionic strength to release proteins from the lysate higher than the one used to elute a DNA cellulose column is a peculiar observation. It may be expected in the case of proteins binding at specific sequences as it has been observed for T antigen of SV40 (26) or in the case of structures stabilized by protein-protein interactions as it has been found for histones (27). Another plausible interpretation is that these DBPs are membrane bound proteins dissociated at 2M NaCl (3 % of the mitoplasts proteins are solubilized at this step). A similar situation was observed in E. coli : a membrane derived DBP binds specifically to two sites within oriC and it has been suggested to play a role in the attachment of the chromosome to the membrane (28). We intend to carry on further purifications of these DBP and to examine their putative role in the replication and the expression of the mtDNA.

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