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Characterization of a mitochondrial protein binding to single-stranded DNA

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ABSTRACT

A DNA-binding protein from *Xenopus laevis* oocyte mitochondria which has been found associated with the D-loop also shows a strong preference for single-stranded DNA. The binding to polynucleotides is dependent on the base composition, but no sequence specificity was found. This protein, called mtSSB, binds tightly and cooperatively to single-stranded DNA. By its amino-acid composition and its binding properties it appears to be similar to the single-stranded DNA-binding proteins found in prokaryotes.

INTRODUCTION

In the mtDNA of most the vertebrate cells an unusual replicative form has been observed : the displacement-loop (D-loop). It is a structure formed by the synthesis of a short H-strand segment of a relatively discrete length which displaces the parental H-strand (1). In *Xenopus laevis* oocytes 30 to 70 % of the DNA molecules are found to contain D-loops and this frequency is positively correlated with the rate of mitochondriogenesis (2). Promotor elements of both strands (3,4) and the origin of replication for the H-strand (O_H) are located in this region suggesting important functions in the regulation of the replication and/or the expression of the mitochondrial genome. Mitochondrial proteins binding preferentially to this region of mtDNA have already been identified in *Xenopus laevis* oocytes (5,6).

In a previous report (7), we described the isolation from mitochondria of *Xenopus laevis* oocytes of another DNA-binding protein and characterized some of its properties. This protein which binds tightly to the single-stranded part of the D-loop in reconstitution experiments could be also recovered, among others, from mitochondrial nucleoids (7). It could be similar to the 16 Kd protein identified by Van Tuyle and Pavco from rat mtDNA-protein complexes (8) ; in that case it was suggested that it may protect mtDNA against D-loop branch migration.

This work was carried out to characterize further the properties of this

DBP. We studied its differential affinity for DNAs of various forms and base compositions and examined its binding cooperativity with single-stranded DNA (ssDNA). Its amino acid composition was determined. The similarities between this protein and prokaryotic single-stranded DNA binding proteins (SSBs) are discussed.

MATERIALS AND METHODS

Oocytes

Whole ovaries were taken from adult female *Xenopus laevis* and cut into pieces in Barth's medium as modified by Gurdon (9). The animals were purchased from SEREA (Argenton l'Eglise, France).

Materials

DNA cellulose was prepared using native calf thymus DNA according to the procedure of Litman (10). Phosphocellulose (P11) and DEAE cellulose (DE52) were obtained from Whatman. Labelled thymidine was obtained from the CEA (Saclay, France). Hybrid plasmids containing the D-loop region (pX1mB14) or the light strand origin of replication (pX1mB3) were isolated as described in (5). ^{3}H-M13 ssDNA was prepared essentially according to Sanger *et al.* (11) except that the DNA was further purified by CsCl centrifugation ; 10µCi/ml of (methyl-^{3}H) thymidine was added in the culture medium. *Xenopus laevis* γ polymerase was prepared essentially according to the method of Bertazzoni (12).

Isolation of the protein

All operations were carried out in the cold. Mitochondria were prepared as previously described (7) except that solutions contained 0.1 mM phenylmethylsulphonylfluoride (PMSF) instead of 0.1 % BSA. The outer membrane was removed by digitonin according to the procedure of G. Brun *et al.* (13). Purified mitoplasts were lysed with 0.5 % Triton X-100 in 20 mM Tris-HCl pH 7.5, 2 M NaCl, 2 mM DTE, 0,1 mM PMSF. The lysate was centrifuged at 200 000 g for 165 min, the supernatant was dialysed against 20 mM KPO_4 pH 7.5, 2 mM 2-mercaptoethanol, 20 % glycerol and applied to a DEAE-cellulose column equilibrated with the same buffer. The fractions of the flow-through were pooled, dialysed against 25 mM Tris HCl pH 7.5, 90 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 0.09 to 1.5 M KCl. The DBP activity was eluted at about 0.2 M KCl under these conditions (5). The corresponding fractions were pooled and dialysed against 20 mM Tris HCl pH 7.5, 1 mM EDTA, 2 mM 2-mercaptoethanol (Buffer A), 50 mM NaCl, 20 % glycerol and adsorbed onto a DNA-cellulose column. The column was

eluted stepwise with 600 mM and 1 M NaCl. The DNA-binding activity was recovered in the 1 M NaCl eluate. The fractions were dialysed against buffer A, 50 mM NaCl, 60 % glycerol and kept at -20°C. The purified fraction analysed by SDS PAGE showed a homogeneous band at 15.5 Kd. The molecular weight markers were : phosphorylase B, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lyzozyme (our previous weight estimation at 12.5 Kd was done using basic proteins as markers).

In preliminary experiments the lysis was done with 1 % Triton X-100 in buffer containing 1 M NaCl and the phosphocellulose purification step was omitted. It was found later that some of the protein was still present in the pellet of the lysed mitoplasts (5), hence the purification procedure described above was adopted.

DNA binding assays

Filter binding assays were performed as described (5) in buffer A, 200 mM NaCl, 5 % glycerol, at room temperature.

Sedimentation analysis : increasing amounts of mtSSB were added to ^{3}H-M13 ssDNA, the complexes were allowed to form in buffer A, containing 200 mM NaCl at 25°C ; the mixture was then loaded onto a linear 10-30 % glycerol gradient and centrifuged at 45 000 rpm in a SW60 Beckman rotor for 2 h at 3°C. Fractions were collected and the distribution of radioactivity was determined using a liquid scintillation counter. S values were calculated using the computer program of Young (14).

Amino acid analysis

The protein sample was dialysed against deionized water ; concentrated HCl was added to a final concentration of 6M and the protein sample was hydrolysed for 24 h at 110°C in a sealed tube (no change in methionine content was observed when the hydrolysis was carried out *in vacuo*). Amino acids were analysed using a Biotropik LC 2 000 analyser (15). The results were quantified with a Spectra Physics SP 4 100 integrator. Each analysis was performed with about 10 nmoles of amino acids.

The statistical method used for the comparison of the amino acid composition of several proteins was the factorial analysis of correspondences (16,17). The principle of the method is as follows : each protein sample could be represented by one point in a 15 dimensionnal space where each axis corresponds to an amino acid ; the coordinate of a protein for a given axis - *i.e*, for a given amino acid - is the percentage of this amino acid that this protein sample contains. The comparison of a set of different proteins is changed into the evaluation of the dispersion of as many points in the 15d space. The sta-

tistical method to do that is developed in details for a comparison of ribosomal proteins in (17). The principle is to project the cloud of points on planes defined by 2 axes calculated so that the deformation of the cloud is minimized. In such a system proteins with similar amino acid composition are represented by close points whatever the choice of axis is. To obtain a significant comparison of proteins one has to compare their respective position in planes defined by the axes which represent the maximal spreading of the cloud (*i.e*, with minimal deformation). In practice the two planes carrying the highest inertia are chosen and when two points are close in the two planes the two proteins are considered to have a similar amino acid composition.

RESULTS

Differential affinity of the protein for various forms of DNA

The binding of the protein to various forms of DNA was measured using a filter binding assay. Figure 1 shows that the single-stranded form of pBR328 DNA was retained on the filter with a higher efficiency than supercoiled or linearized DNA (at least 20 fold). This preference for ssDNA was confirmed by competition experiments. The degree of retention of native ^{3}H *E. coli* DNA on filters by a non saturating concentration of protein, in the presence of increasing amounts of unlabelled native or heat-denatured homologous DNA, clearly indicated that this protein binds preferentially to ssDNA (fig. 2). Treatment of *E. coli* DNA with S1 nuclease to remove any single-stranded re-

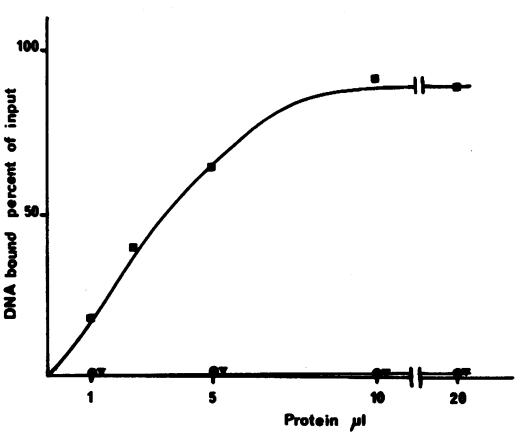

Figure 1 : Affinity of the protein for various forms of DNA. Filter binding assays were performed with ^{3}H-pBR328, either supercoiled (▼), linearized (●) or heat-denatured (■).

gions dramatically reduced the binding affinity of the protein.

In conclusion, this DBP appeared to bind preferentially to ssDNA - the apparent affinity for purified *E. coli* DNA (7) being probably due to short single-stranded streches ; this mitochondrial single-stranded DNA binding protein will be referred as mtSSB.

Binding specificity

The interaction of the mtSSB with synthetic polynucleotides was studied to look for a possible effect of base composition on the binding affinity. Competition experiments using a fixed concentration of ^{3}H *E. coli* ssDNA and increasing amounts of unlabelled single-stranded polynucleotides were done at a constant non saturating protein concentration. Competition experiments with homopolynucleotides indicated (fig. 3a) that polydT is about 8 times more effective as a competitor than is polydC while polydA and polydG are very poor competitors. Competition with the four random copolymers showed (fig. 3b) that poly(C,T) is about 6 times more effective than polyd(A,C) which is itself twice as effective as polyd(A,T) while polyd(C,G) is a poor competitor. *E. coli* ssDNA included in the experiments as a control was intermediate between polydT and polydC in the first experiment and between polyd(C,T) and polyd(A,C) in the second one. Hence the affinity of the mtSSB for these polynucleotides seems to decrease in the order polydT, poly(C,T) > *E. coli* DNA, polyd(A,C), polydC, polyd(A,T) > polyd(G,C) > polydA, polydG. These observations on the effect of base composition on the binding affinity of the mtSSB for single-stranded

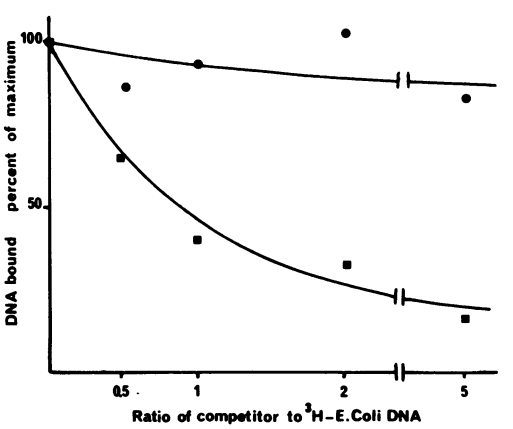

Figure 2 : Affinity of the protein for native or heat-denatured *E. coli* DNA. Filter binding assays were carried out with a mixture of native ^{3}H-*E. coli* DNA and various concentrations of unlabelled competitor DNA : native (●) or heat-denatured (■) *E. coli* DNA.

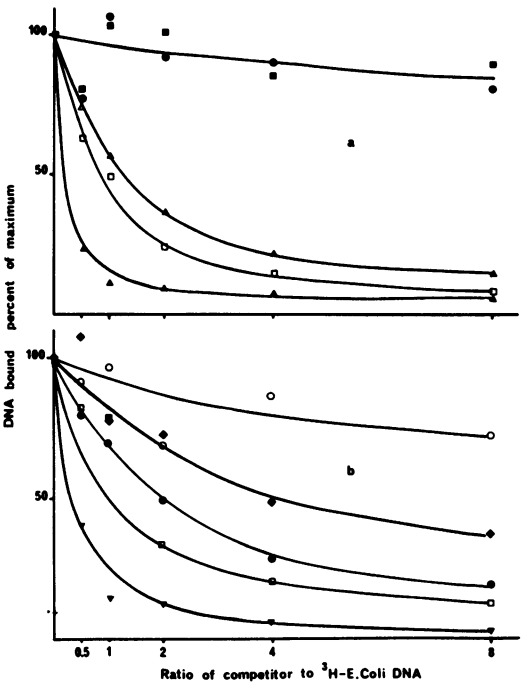

Figure 3 : Affinity of mtSSB for various single-stranded polynucleotides. Filter binding assays were performed with a mixture of heat-denatured *E. coli* ^{3}H-DNA and various concentrations of single-stranded nucleic acids.

a) polydA : (●) ; polydT (▲) ; polydG (■) ; polydC : (△) ; *E. coli* ssDNA : (□).
b) polyd(A,T) : (◆) ; polyd(G,C) : (○) ; polyd(C,T) : (▼) ; polyd(A,C) : (●) ; *E. coli* ssDNA : (□).

DNA led us to test if this protein binds preferentially to single-stranded DNA associated with the D-loop region ; competition experiments were carried out using a fixed amount of ^{3}H-ss-pBR328 and increasing amounts of ss-plasmids with or without the D-loop region, pX1mB14 and pBR328 - pX1mB3 respectively (fig. 4). The relative affinity for these 3 plasmids was very similar. The results indicated that the binding of the mtSSB to ssDNA is affected by the general base composition but does not show a sequence specificity for the D-loop region.

Binding cooperativity

Although of limited sensitivity, the filter binding assay can be used to detect cooperative binding of proteins to DNA. Since the retention of DNA on a filter increases as a function of the amount of added protein, the shape of

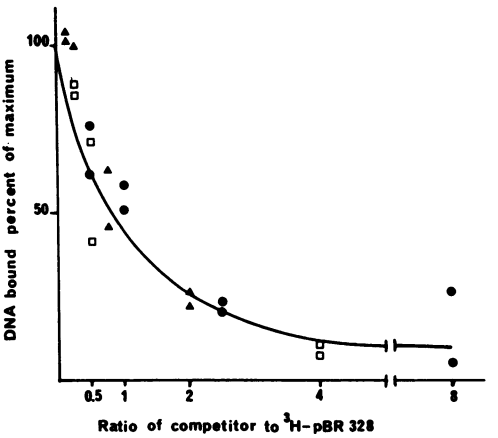

Figure 4 : Affinity of the mtSSB for single-stranded plasmids with or without the D-loop region. Filter binding assays were performed with heat-denatured linear ^{3}H-pBR328 in the presence of various concentrations of unlabelled heat-denatured linear plasmids, pBR328 : (▲), pX1mB3 : (●), pX1mB14 : (□).

the titration curve obtained using defined conditions (18) can show whether the binding is cooperative. A precise titration curve was made using heat-denatured pX1mB14 DNA ; fig. 5 shows that it is sigmoidal. If the efficiency of retention of protein-DNA complexes containing one or several protein molecules was the same, this would indicate that a cooperative binding was occuring. This possibility was investigated further using velocity sedimentation

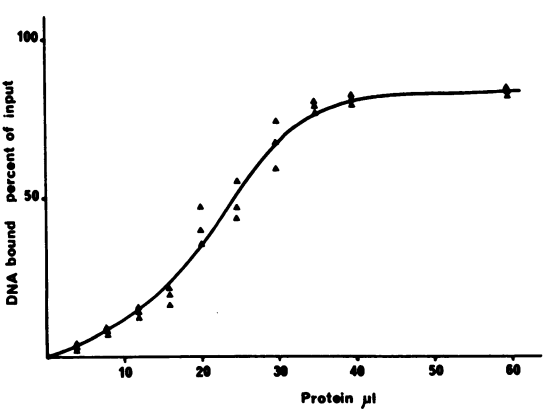

Figure 5 : Titration of mtSSB-ssDNA complexes. The fraction of single-stranded pX1mB14 retained on filter was measured in the presence of various amounts of mtSSB.

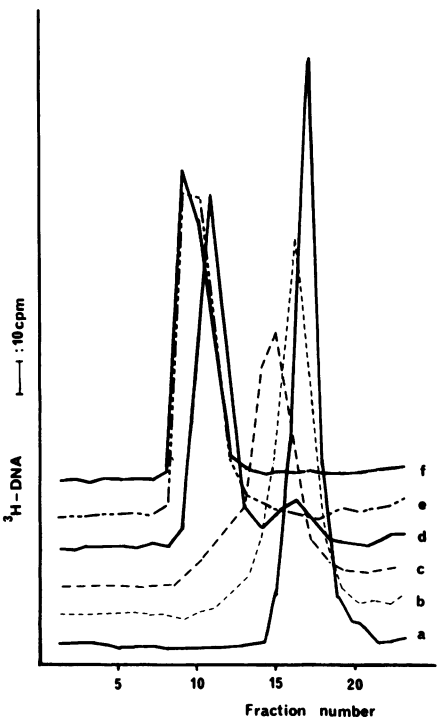

Figure 6 : Binding cooperativity. Sedimentation analysis on glycerol gradient of DNA-protein complexes formed by M13 ssDNA and increasing amounts of mtSSB.a:free DNA, b-f : DNA was incubated with respectively 0.5, 1, 2, 4 and 8 µl of protein solution.

experiments. Complexes formed from a fixed amount of labelled M13 DNA (in its single-stranded form) and increasing amounts of mtSSB were sedimented through glycerol gradients. At high ratio of protein to DNA a sedimentation coefficient of 56 S has been calculated for DNA protein complexes (fig. 6 e-f) compared to 24 S for free DNA (a). At lower ratios an asymetric peak (b-c) or two distinct peaks (d) were obtained ; in this latter case one peak corresponds to free DNA, the other one to a complex of protein and DNA. These data are in agreement with the filter assay results and show that the binding of mtSSB to ssDNA is indeed cooperative.

Stability of the DNA protein complex

The fate of preformed ^{3}H-ssDNA-protein complexes after the addition of a large excess of unlabelled homologous DNA was studied. After 90 min in standard conditions no detectable dissociation of the initial complexes could be observed (table 1) indicating that the binding was fairly stable. On the other hand the binding of mtSSB to DNA is temperature sensitive. Routinely 25°C was used as the standard incubation temperature because previous results had shown that the percentage of DNA retained on a filter was not significantly diffe-

Table 1 : Stability of the complexes of mtSSB and ssDNA :

Time after adding 10 fold excess of unlabelled ssDNA (min.)		0	10.3	1	2	5	10	20	40	90
^{3}H ssDNA bound (percent of input)	Experiment 1	14	13	13	14					
	Experiment 2				15	13	14	16	16	17

A non saturating amount of mtSSB was incubated with *E. coli* ^{3}H-ssDNA for 10 min at 25°C, then a 10 fold excess of unlabelled homologous ssDNA was added. The mixture was filtered after various times as indicated.

rent over the range 0°C to at least 30°C. However, the preformed complexes are not stable to high temperatures (table 2) : after 10 min at 55°C they were completely dissociated as judged by the filter binding assay. This result could have been due to the denaturation of the protein since its binding activity is completely lost after 1 min at 60°C.

Biological properties of the mtSSB

No ATPase, nuclease, topoisomerase or polymerase activity could be detected associated with this protein. Preliminary observations, using activated calf thymus DNA as a template, suggest that there may be an effect on the polymerizing activity of the homologous γ DNA polymerase since in its presence the rate of the reaction was inhibited by 40 %.

Amino acid analysis

The amino acid content of the mtSSB was determined (table 3). This composition was compared with that of other non-sequence specific DBPs described in the literature (19-37) by a statistical method (see Materials and Methods). A graphical representation of the data is shown in fig. 7. Several groups of homology can clearly be made. In terms of amino acid composition the mtSSB is related to the other SSBs (group A) and quite different from the *Xenopus laevis* DBP 1, the histone H1 and the HMGs 1, 2, 14 and 17 (group C), while the nucleosome core histones, the bacterial histone-like proteins and the nuclear

Table 2 : Effect of the heat on the stability of the complex :

Temperature (°C)	30	37	45	55	65
^{3}H ssDNA bound (percent of input)	50	36	30	<1	<1

After incubation under standard conditions the preformed complexes were incubated at the indicated temperature for 10 min and then filtered.

Table 3 : Amino acid composition of the mtSSB :

Asx	11.9	Ala	6.0	Tyr	1.4	Values are expressed as moles per 100 moles amino acids recovered. Asx means Asn + Asp, Glx means Gln + Glu, Trp and Cys were destroyed by the acid hydrolysis and Pro was not detected by the method used. For details see Materials and Methods.
Thr	10.9	Val	8.5	Phe	3.4	
Ser	8.9	Met	0.0	His	2.1	
Glx	8.9	Ile	4.8	Lys	7.9	
Gly	9.2	Leu	9.5	Arg	7.5	

AK protein are at an intermediate position (group B). It should also be noted that the yeast mitochondrial protein HM is not similar to the *Xenopus laevis* mtSSB. The content of lysine and, to a lesser extent, of alanine has a major importance in the distribution of the proteins : the lysine represents 7.4 % ± 1.8 % of the 17 amino acids analysed for the group A (the SSBs), 12.0 ± 3.0 % for the group B and 27.2 ± 5.2 % for the group C, while mtSSB contains 7.9 % of lysine.

DISCUSSION

In a previous report (7) we partially characterized a *Xenopus laevis* mitochondrial protein which binds to the single-stranded part of the D-loop. The data reported here show clearly that this protein has a much higher affinity for single-stranded DNA than for double-stranded supercoiled or linear DNA. The apparent affinity for native DNA previously observed (7) was probably due to short single-stranded stretches.

The interaction of this mitochondrial single-stranded DNA binding protein (mtSSB) with different polynucleotides was studied to look for possible effects of the base composition on the binding affinity. Competition experiments (fig. 3a) showed that polydT is much more efficient than the other homopolymers as a competitor. Such a preferential binding to polydT seems to be a general feature of prokaryotic SSBs (38,39) ; this observation may be due, at least in part, to enhanced stacking interaction between the aromatic amino acids and the thymine residues. PolydC is about 8 times less effective than polydT as a competitor but is itself several orders of magnitude more effective than polydA or polydG. These data are in good agreement with the results of experiments done with random copolymers : polyd(C,T) is the most effective competitor of the four tested polynucleotides (fig. 3b). Hence the net binding constant of the mtSSB to polynucleotides appears to be positively correlated with the pyrimidine content of the polynucleotides. There is an apparent absence of preferential binding to the plasmids pXlmB14 containing the D-loop region (fig. 4). However it has been shown that there is a significant cluster of pyrimidines

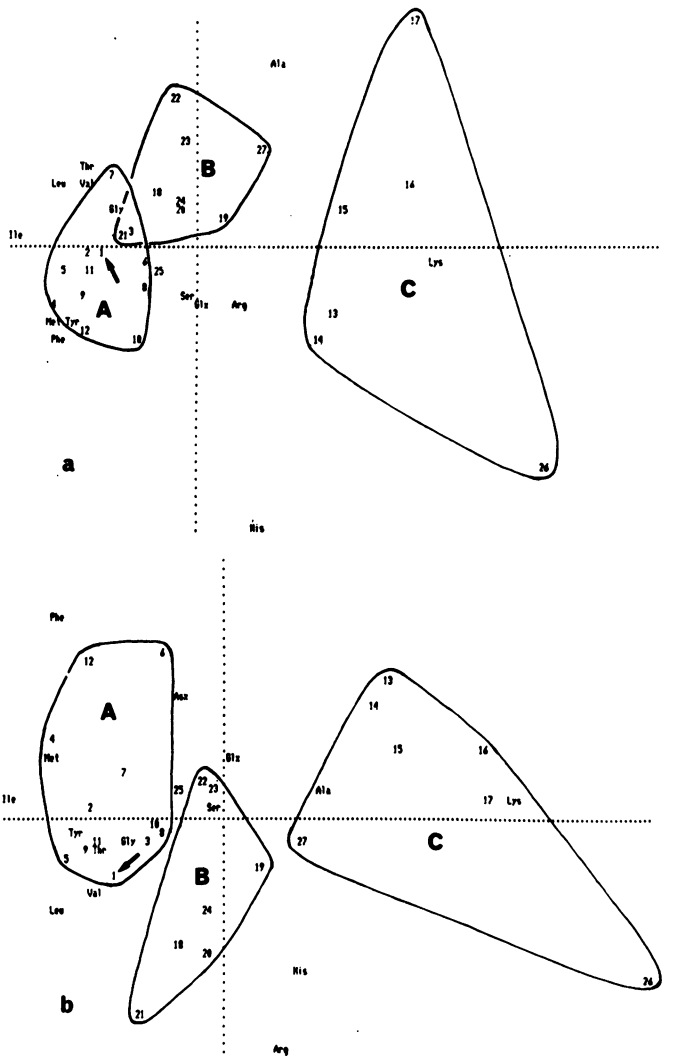

Figure 7 : Amino acid composition comparison : graphical representation of the factorial analysis of correspondences. The first and second axes (a) or the first and third axes (b) are drawn in the plane of the paper (see Materials and Methods). The percentage of the inertia on the different axes are the following : axis 1 : 35.7 %, axis 2 : 17.0 %, axis 3 : 13.4 %.
Numbers indicate the projection of the amino acid composition of one protein on the plane.1 : *Xenopus laevis* mtSSB, 2 : *E. coli* SSB (19), 3 : F factor SSB (20), 4 : λ SSB (21), 5 : fd gene 5 protein (22), 6 : T4 gene 32 protein (23), 7 : pf1 SSB (24), 8 : Ad5 DBP (25), 9 : mouse HD1 (26), 10 and 11 : calf HD1 and LSE (27), 12 : yeast SSB1 (28), 13 to 16 : calf HMG1, HMG2, HMG14 and HMG17 (29), 17 : calf H1 (30), 18 to 21 : Xenopus H2A, H2B, H3 and H4 (31,32), 22 and 23 : *E. coli* HU1 and HU2 (33), 24 : *E. coli* H (34), 25 : yeast HM (mitochondrial) (25), 26 : Xenopus DBP1 (36), 27 : calf AK (37).

near the 5' end of the D-loop (40) and it may be that while this region binds mtSSB with high affinity the presence of the rest of the plasmid masks this preferential binding. The velocity sedimentation analysis shows, in agreement with the filter binding assay results, that the binding to ssDNA proceeds cooperatively. The cooperativity which seems to be a general property of the prokaryotic SSBs could enhance its preferential binding to ssDNA (38). The binding of mtSSB to DNA is stable as evidenced by its elution from the DNA cellulose column at 1M NaCl and the lack of dissociation of the complex after 90 min under the standard conditions.

No enzymatic activity has yet been assigned to this protein. Preliminary results indicate some inhibition of the polymerizing activity of the *Xenopus laevis* γ polymerase in *in vitro* experiments but further experiments are required to elucidate the exact mechanism of this inhibition.

The amino acid composition of the mtSSB is close to that of other types of mtSSBs (fig. 7) with the exception of HMG1 which is known to be a SSB (41) but HMG1 is anyway rather different from other proteins. If we assume that the similarities between the different SSBs is a reflection of some common structural constraints, then the differences observed in the case of HMG1 may be related to its reported unique role during chromatin replication (42). Determination of the amino acid sequence of the mtSSB will be necessary to confirm possible structural homologies with the other SSBs and to study the protein-DNA interaction at a molecular level.

In conclusion, this *Xenopus laevis* mtSSB shows many similarities with other SSBs ; however it is clearly different from the SSBs previously purified from eukaryotic cells (38) which are presumably involved in nuclear DNA replication. In contrast to the eukaryotic SSBs but like prokaryotic SSBs the mtSSB is polymeric (7) and binds cooperatively to ssDNA suggesting that it could participate to the replication of mitochondrial nucleoids in a way similar to that of prokaryotic SSBs.

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ABBREVIATIONS

SSB : single-stranded DNA-binding protein, mtSSB : mitochondrial SSB, H-strand : heavy-strand, ssDNA : single-stranded DNA, DBP : DNA-binding protein.

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