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Sperm Nuclei of *Discoglossus pictus* (Anuran Amphibian) Contain a Unique, Histidine-rich Basic Protein

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ABSTRACT—Fractionation with reverse-phase HPLC and electrophoretic analyses revealed that the nuclear basic proteins in sperm of an anuran amphibian, *Discoglossus pictus*, consisted of weakly basic proteins without histones. Amino acid analyses indicated that none of these proteins were histones or protamines because of the low amounts of Lys and Arg. The predominant protein among those fractionated possessed an unusually high content of His and extremely low amounts of Arg and Lys, indicating that this is a unique nuclear basic protein not reported previously. The induction of a highly decondensed state of sperm nuclei upon their incubation in egg extract was accompanied by the removal of most, if not all, of this His-rich protein from the nuclei.

INTRODUCTION

In most animals, sperm chromatin is highly condensed by the interaction of DNA with strongly basic proteins that are different from somatic histones. Despite their broad similarity in function to stabilize the nuclear state of sperm, these basic proteins display a surprising diversity both in molecular weights and amino acid sequences, ranging from the highly specialized protamines, as typically found in mammals and fishes, to the sperm-specific histones found in many invertebrates (Poccia, 1986). This diversity is particularly well-illustrated among different genera of amphibians. In *Bufo japonicus*, for instance, sperm nuclear basic protein (SNBP) is comprised exclusively of protamines (Takamune *et al*., 1991), while in *Xenopus laevis* 6 apparent SNBPs (SP1–6) that exhibit the properties intermediate between protamines and histones coexist with a set of core histones (Yokota *et al*., 1991). The chromatin of *Rana catesbeiana* sperm, on the other hand, is unique in consisting of an irregularly spaced nucleosomal structure by possessing the special histone H1-variants in addition to a full set of core histones (Itoh *et al*., 1997).

In our studies on sperm and egg interactions in *Discoglossus pictus*, we found that the SNBPs of this anuran amphibian are comprised of unique proteins that have not been recorded previously in any form of animal. We describe here their amino acid compositions and electrophoretic mobilities, as well as their possible function in maintaining the condensed state of sperm chromatin.

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MATERIALS AND METHODS

Materials

Sexually mature painted frogs, *Discoglossus pictus* (hereafter referred to as *Discoglossus*), were captured in the neighborhood of Palermo, Italy. South African clawed frogs, *Xenopus laevis* (referred to as *Xenopus*), were obtained from a colony in our laboratory. Sexually mature toads, *Bufo japonicus* (referred to as *Bufo*), were purchased from commercial dealers in the Tokyo area.

Isolation of nuclei from sperm and somatic cells

Mature sperm were obtained from *Discoglossus* as described by Campanella and Gabbiani (1979) with a minor modification, as follows. Adult male frogs were injected in a dorsal lymphatic sac with 200 IU of human chorionic gonadotropin (HCG; Teikokuzoki, Co. Ltd., Japan) in De Boer's solution (DB; 110 mM of NaCl, 1.3 mM each of KCl and $CaCl_2$, pH 7.3 with $NaHCO_3$). Between 24–48 hr later, mature sperm were obtained, either by squeezing the abdomen or by pricking the seminal vesicles after opening the male abdomen, and were suspended in SMT buffer (250 mM sucrose, 2 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4). Sperm were obtained from *Bufo* and *Xenopus* by macerating testes in ice-cold DB. Mature sperm cells were isolated by ultracentrifugation in a 70% Percoll (Pharmacia LKB Biotechnology AB, Sweden) solution containing DB (36,000g, 60 min., 4°C) (*cf*., Yoshizaki, 1987), and were then suspended in SMT buffer.

Mature sperm were demembranated with 0.05% lysolecithin (LC) for 10 min at room temperature as described by Lohka and Masui (1983), and the nuclei were stored at –70°C in SMT buffer at a concentration of 1×10^7 nuclei/ml (*Discoglossus*) or 1×10^8 nuclei/ml (*Xenopus* and *Bufo*).

Erythrocytes of *Discoglossus* were LC-permeabilized in the same way, and the isolated nuclei were suspended in SMT and stored at –70°C at a concentration of 1×10^8 nuclei/ml.

Extraction of nuclear proteins and amino acid analysis

Nuclear proteins were extracted according to Mann *et al*. (1982). Proteins extracted with 0.4 N H_2SO_4 were precipitated with 20% trichloroacetic acid (TCA), washed in cold (–20°C) acetone, and then dried *in vacuo*.

The acid-extracted proteins dissolved in 25 mM Tris-HCl (pH 8.5)

were mixed with an equal volume of Q-Sepharose Fast Flow gels (Pharmacia). The unabsorbed proteins were collected as acetone powder, and were fractionated by reverse-phase high performance liquid chromatography (RP-HPLC) with a 150×4.6 mm Finepak SIL 300C_{18}T-7 column (Japan Spectroscopic Co. Ltd., Japan) equilibrated with 0.05% trifluoroacetic acid (TFA). The proteins were eluted with a gradient of acetonitrile (0–50%) in 0.05% TFA at a flow rate of 1 ml/min. The purified proteins were dried *in vacuo*, hydrolyzed with 6 N HCl for 24 hr at 110°C, and subjected to amino acid analysis using a Hitachi-835 amino acid analyzer (Hitachi Co. Ltd., Japan).

Incubation of sperm nuclei in egg-extracts

The egg extract for the incubation of sperm nuclei was prepared as described by Ohsumi *et al.* (1988). Briefly, 10 ml of dejellied mature eggs from *Xenopus* were suspended in ice-cold extraction medium (EM; 100 mM KCl, 2 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4), decanted to remove as much of the EM as possible, and then centrifuged at 10,000g for 10 min (2°C). The semitransparent layer between the top (lipid) and the bottom (yolk and pigment granules) was collected, and served as the 'egg extract' (EE). Twenty μl of LC-permeabilized sperm nuclear suspension (2×10^5 nuclei) was mixed with 200 μl of EE, and incubated at room temperature with frequent vortexing. After the incubation, the nuclei were collected by centrifugation and the proteins were extracted for electrophoretic analyses.

Electrophoresis

Acid/urea/Triton X-100 polyacrylamide gel electrophoresis (AUT-PAGE) was carried out according to Zweidler (1978), using 12% polyacrylamide gels containing 2.5 M urea, 6 mM Triton X-100, and 5% acetic acid. The acetone-dried samples were dissolved in 10 M urea solution. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (1970), using 15% resolving gels and 4.5% stacking gels. The samples were dissolved in SDS-sample buffer (10 mM Tris-HCl, pH 6.8, 1% SDS, 1% 2-mercaptoethanol, 20% glycerol). For two-dimensional analysis, AUT-PAGE was carried out in rectangular tubes (1×2×70 mm), followed by equilibration gels with SDS-sample buffer, and the materials were separated by SDS-PAGE. Gels were stained with 0.05% Coomassie brilliant blue R250.

RESULTS AND DISCUSSION

Fig. 1 shows the AUT-PAGE profiles of nuclear basic proteins from sperm and erythrocytes of *Discoglossus*, in comparison with those from sperm of *Xenopus* and *Bufo*. *Discoglossus* sperm did not possess the core histones that are commonly found in nuclei of somatic cells and sperm of *Xenopus*, but exhibited bands whose mobilities were different from either the sperm-specific proteins (SP1-6) of *Xenopus* or the protamines of *Bufo*. Two dimensional PAGE analyses using AUT- and SDS systems (Fig. 2) also clearly revealed that *Discoglossus* sperm nuclei do not possess either core histones or the H1-type histones found in *Rana catesbeiana* sperm (*cf.*, Itoh *et al.*, 1997). Thus, the nuclear basic proteins of *Discoglossus* sperm appeared unique in that they exhibited electrophoretic mobilities different from those of any sperm nuclear basic proteins recorded previously.

The basic proteins from sperm nuclei and from erythrocyte nuclei of *Discoglossus* were fractionated by reverse-phase HPLC for comparison. All seven fractions obtained from sperm nuclei (designated A–G in Fig. 3) exhibited elution and SDS-PAGE profiles which were distinct from those of the fractions

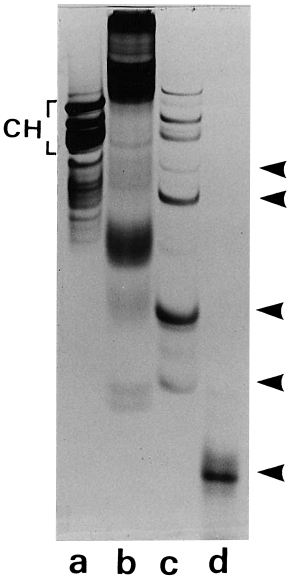

Fig. 1. AUT-PAGE analysis of basic proteins from the nuclei of erythrocytes (**a**) and sperm (**b**) of *Discoglossus* in comparison to those from the nuclei of sperm of *Xenopus* (***c***) and *Bufo* (**d**). Proteins extracted from 1×10^6 (**a**, **c** and **d**) or 1×10^5 (**b**) nuclei were applied to each lane. Direction of electrophoresis was from top (+) to bottom (–). CH, core histones; Arrow heads, sperm-specific proteins of *Xenopus* and *Bufo*.

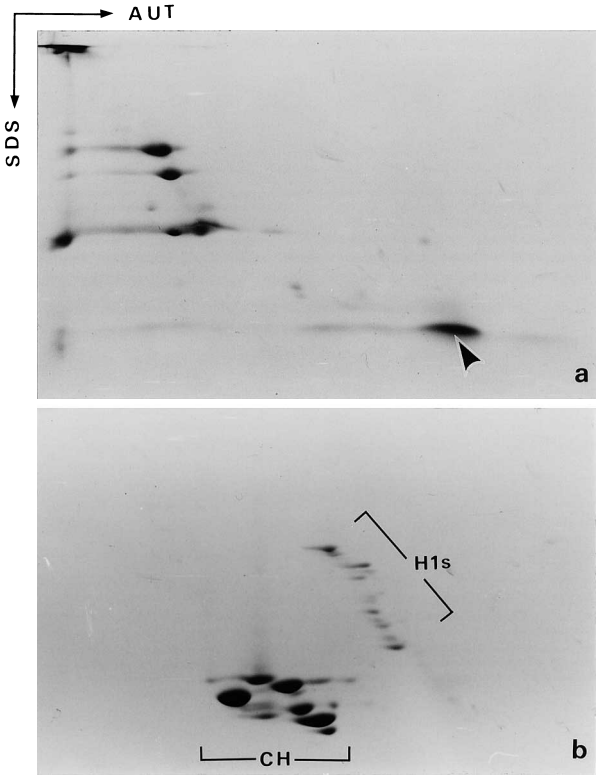

Fig. 2. Two dimensional electrophoresis of acid extracts of the nuclei from sperm (**a**) in comparison to those of erythrocytes (**b**) of *Discoglossus*. Proteins were separated by AUT-PAGE in the horizontal direction from left to right, followed by SDS-PAGE in the vertical direction from top to bottom. Arrowhead in **a**, histidine-rich protein. CH and H1s in **b** refer to core- and H1 histones, respectively.

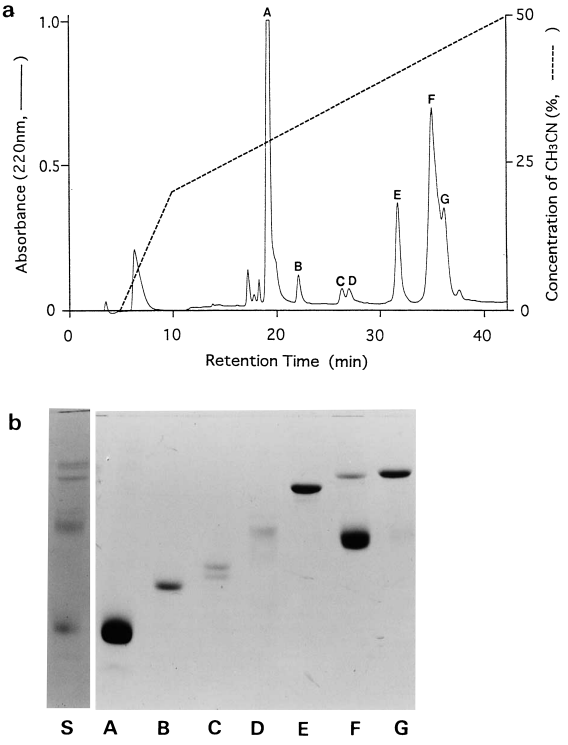

Fig. 3. **a**: Reverse-phase HPLC profiles of nuclear basic proteins from the nuclei of sperm of *Discoglossus*. Acid extracts of proteins unabsorbed to Q- Sepharose under the conditions given in MATERIALS AND METHODS were loaded on a Finepak SIL 300C_{18} T-7 column, and elution was carried out by 10–50% acetonitrile linear gradient in 0.05% trifluoroacetic acid for 50 min, at a flow rate of 1.0 ml/min. **b**: SDS-PAGE analysis of SNBPs (S) and protein fractions A–G shown in **a**.

obtained from somatic cell nuclei (data not shown). The fractions eluted faster than the fraction A (Fig. 3a) possibly represented fast-moving minor bands in AUT-PAGE profile shown in Fig. 1 (lane b), but these fractions were not processed for further analysis.

In Table 1 are compared the amino acid compositions of fractions A–G shown in Fig. 3 with those of sperm nuclear proteins from other anuran species reported previously (Yokota *et al*., 1991; Takamune *et al*., 1991; Itoh *et al*., 1997). It is clear that none of the proteins from *Discoglossus* sperm were similar in amino acid compositions to those from sperm nuclei from other amphibian species so far reported (cited in Table 1). We could not determine whether the minor fractions (B–G) represented distinct molecular entities or fragments derived from the major fraction A. In any event, all of the minor proteins were of weaker basicity (*i.e*., they contained a lower amount of total basic amino acids than do somatic histones), and were composed of relatively smaller amounts of Lys than are histones (*cf*., Itoh *et al*., 1997). Unlike the sperm from *Bufo* or *Xenopus* which possessed Arg-rich, more typically sperm-specific nuclear basic proteins (Takamune *et al*., 1991; Yokota *et al*., 1991), the sperm proteins from *Discoglossus* contained extremely small amounts of Arg. Above all, the predominating basic protein in *Discoglossus* sperm (fraction A, Table 1) was characterized by an unusually high content of His, which is apparently the major contributor to the observed basicity of this protein.

In an attempt to elucidate the role of these unique basic proteins in the condensed state of the sperm chromatin, sperm nuclei were incubated in egg extract from *Xenopus*. The

Table 1. Amino acid composition of fractions A-G (*cf*., Fig. 3) isolated from the sperm of *Discoglossus pictus*, in comparison with sperm nuclear basic proteins from other anuran amphibians. The numbers indicate the molar percentages of amino acids, calculated from the average of three independent determinations.

Amino acid	Fraction							*Rana*[1]	*Bufo*[2]	*Xenopus*[3]
	A	B	C	D	E	F	G			
Asx	2.4	4.8	8.6	7.8	9.6	12.7	9.7	3.4	2.7	5.2
Thr	11.3	8.7	3.8	3.9	5.4	6.5	3.9	4.5	6.9	11.5
Ser	8.8	10.6	10.1	8.4	6.3	7.2	7.5	8.4	6.0	14.1
Glx	6.1	9.1	14.4	11.7	12.3	10.8	14.0	3.1	3.9	2.6
Gly	1.5	4.4	8.8	10.5	5.6	6.6	10.2	6.7	0.9	6.4
Ala	8.1	7.1	5.9	7.0	8.9	7.3	7.9	19.8	4.7	11.5
Cys	3.5	2.2	0.5	1.4	1.7	3.4	1.6	0	0	0
Val	10.9	7.9	5.8	4.8	6.8	5.3	5.9	6.0	5.1	1.3
Met	1.1	0.1	1.9	2.5	1.7	1.2	0.7	0	0	3.9
Ile	5.9	1.2	2.7	5.3	4.1	4.1	3.9	1.6	0	0
Leu	3.7	7.2	8.8	6.8	5.3	5.9	6.1	4.0	0	1.3
Tyr	2.6	2.2	1.9	1.6	4.1	6.6	3.6	0.8	2.6	2.6
Phe	0.2	0.2	1.0	1.1	3.4	4.8	3.6	0	0	0
Lys	2.5	15.0	7.7	8.8	8.9	7.0	5.7	29.6	5.2	1.3
His	20.8	4.4	6.3	5.6	1.8	2.4	1.3	0.7	9.7	1.3
Arg	0.6	0.5	5.9	6.1	5.8	3.8	4.1	1.5	42.3	35.9
Pro	9.9	14.2	5.7	6.8	28.3	4.7	10.5	10.1	10.0	1.3
Basic amino acids	23.9	19.9	19.9	20.5	16.5	13.2	11.1	31.8	57.2	38.5
Lys/Arg ratio	4.2	30.0	1.3	1.4	1.5	1.8	1.4	19.7	0.1	0.1>

[1] Sperm-specific H1 variant in *Rana catesbeiana*, cited from Itoh *et al*. (1997).
[2] Protamine in *Bufo japonicus*, cited from Takamune *et al*. (1991).
[3] Sperm-specific protein SP4 in *Xenopus laevis*, cited from Yokota *et al*. (1991).

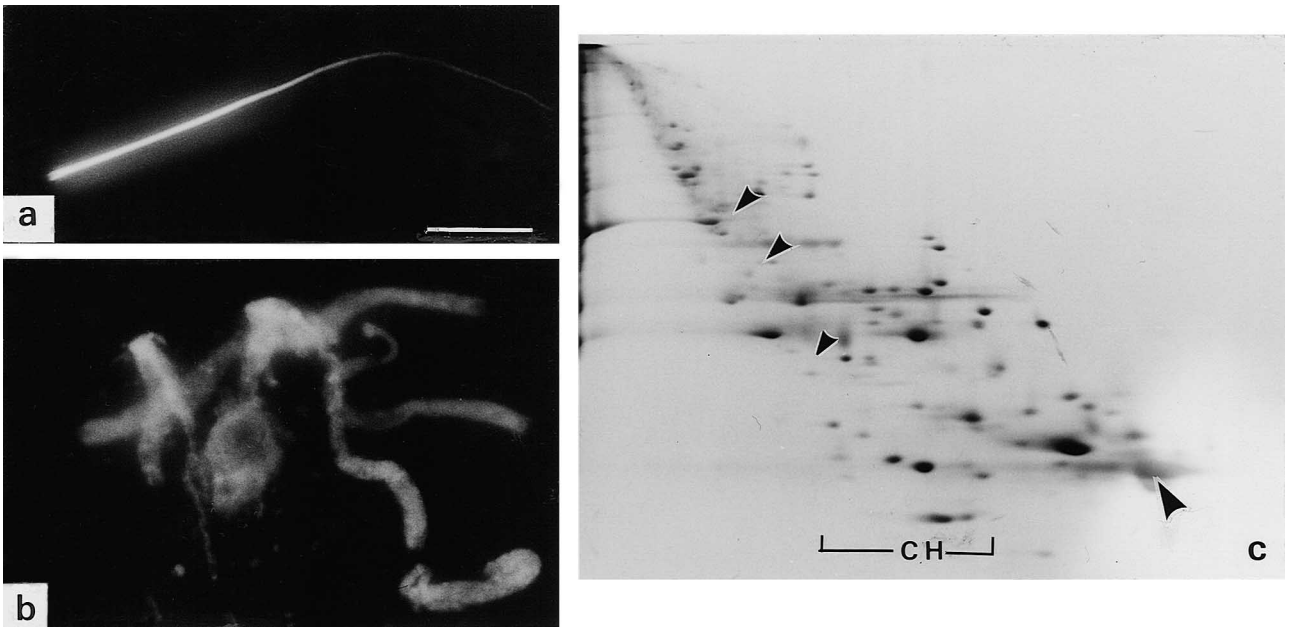

Fig. 4. **a** and **b**, Hoechst-stained sperm nuclei of *Discoglossus* at 0 (**a**) and 60 (**b**) min following incubation with *Xenopus* egg extract, showing prominent decondensation in **b**. bar, 10 μm; **c**, Two dimensional-PAGE of acid extracts from the nuclei of *Discoglossus* sperm which were incubated with *Xenopus* egg extract, showing a total or partial loss of sperm-specific proteins and accumulation of core histones (CH). Large and small arrowheads indicate the positions of Histidine-rich and other minor sperm proteins, respectively. Electrophoresis conditions same with those given in Fig. 2.

nuclei of *Discoglossus* sperm decondensed well during 60 min of incubation (Fig. 4a, b), as did those of homologous *Xenopus* sperm. Two dimensional PAGE analyses of sperm nuclei after incubation in the egg extracts (Fig. 4c) revealed that His-rich protein was reduced but a certain amount remained unremoved, concomitant with a total loss of other minor SNBPs. This loss and/or reduction of the *Discoglossus* perm proteins was accompanied by the partial acquisition of histones from the egg extracts (Fig. 4c). We also induced the decondensation of *Discoglossus* sperm nuclei without complete loss of His-rich protein after incubation in a high concentration of *Xenopus* nucleoplasmin (data not shown), a result that is reminiscent of the partial removal of sperm-specific H1-type proteins from *Rana catesbeiana* (*cf.*, Itoh *et al.*, 1997).

Thus, our results demonstrate the existence of a unique type of sperm nuclear basic protein which has not been reported in any form of animal (*cf.*, Kasinsky, 1989). Further studies are needed to clarify both the primary structure of this His-rich protein, and how it contributes to the condensation of chromatin in this unusually giant sperm (*cf.*, Campanella and Gabbiani (1979).

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