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HISTONES AND NUCLEOSOMES IN CRUSTACEAN SPERM NUCLEI PREVIOUSLY DESCRIBED AS LACKING DNA-ASSOCIATED PROTEINS

Kathryn Kurtz,[1] Fina Martínez,[1] Elsa Fonfría-Subirós,[1,2] Nuria Saperas,[2] Manel Chiva[1]

[1] Department of Physiological Sciences II, Faculty of Medicine, University of Barcelona

Feixa Llarga, s/n. L'Hospitalet de Llobregat 08907. Tel. 934 035 824. Fax: 934 024 268.
klkurt00@smumn.edu, *finamartinez@ub.edu*, *elsafonfria@gmail.com*, *nuria.saperas@upc.edu*, *mchiva@ub.edu.*

[2] Department of Chemical Engineering, Polytechnic University of Catalunya, Barcelona

Abstract

The spermatozoa of several decapod crustaceans have unusual morphologies and atypical chromatin when observed in transmission electron microscopy. These sperm consist of a large electro-dense acrosome, completely surrounded by nearly transparent nuclear material, extending into several lateral arms. In the past, much discussion has been focused on the protein components of the labile, nearly transparent spermatic nucleus of several crab species, but most of the cytochemical studies were performed several decades ago and may present unreliable results, due to the techniques used. Here we revisit the study of how DNA is organized in the spermatic nucleus of crustaceans, using as our model two crab species, *Maja bachidactilae* (family Majidae) and *Cancer pagurus* (family Cancridae), using updated techniques to reconcile the fundamental question of how the chromatin is organized in sperm of these species.

Key words Sperm, nucleosome, chromatin, crustacean, micrococcal nuclease.

Resum

Els espermatozoides de diversos crustacis decàpodes tenen morfologies anormals i la cromatina atípica quan s'observen al microscopi electrònic de transmissió. Aquests espermatozoides consisteixen en un acrosoma molt gran i electrodens que rodeja el nucli per complet. El nucli té braços que s'estenen pels laterals i presenta un aspecte bastant transparent. Al llarg de la història, sempre hi ha hagut controvèrsia pel que fa a la composició proteica de la cromatina del nucli espermàtic, que és molt laxa i gairebé transparent; la majoria d'aquests estudis es van realitzar ja fa temps, i els resultats obtinguts haurien de ser revisats. En aquest treball, estudiem com s'organitza el DNA dins el nucli espermàtic dels crustacis *Maja bachidactilae* (fam. Majidae) i *Cancer pagurus* (fam. Cancridae), utilitzant tècniques més actuals per a donar resposta a una pregunta fonamental que és com s'organitza la cromatina en l'espermatozoide d'aquestes espècies.

INTRODUCTION

Histones are a family of basic proteins found in association with nuclear DNA among nearly all eukaryotes (Vaughn, 1968); a typical eukaryotic nucleus contains about $5x10^9$ bp of DNA associated non-covalently with five types of histones, resulting in a compact packaging of chromatin (van Holde, 1988). The basic repeating subunit of chromatin formed by the DNA-histone complex is the nucleosome, comprised of about 147 base pairs of DNA wrapped around an octamer of core histones (Zheng and Hayes, 2002). Nucleosomal repeats may vary depending on the species, from 160 bp in yeast to over 200 bp in higher organisms, forming nucleosomal arrays (Hayes and Hansen, 2001). The DNA-protein composition affecting chromatin architecture in ripe sperm is also variable among species. There are three major categories of sperm: those containing histones, those containing protamine-like proteins, and those containing protamines (Ausió, 1999). One role of sperm proteins is to pack the genetic material into a small volume, resulting in a more hydrodynamic shape for fecundation and DNA protection (Subirana, 1975). In packaging the chromatin, these nuclear proteins decrease the probability of DNA damage by water, enzymes, and bacteria (Subirana and Puigjaner, 1973). Bloch (1969) has categorized five types of histone changes during spermiogenesis, one being "crustacean type", which is the least under-

stood. Published accounts of these sperm support the conclusion that they lack in nuclear basic proteins associated with DNA (Chevaillier, 1966, 1968; Vaughn, 1968*a* 1968*b*, 1969, 1972; Langreth, 1969; Vaughn and Hinsch, 1970, 1971). In order rectify the question concerning how the lax and seemingly unprotected DNA could be organized in the abnormally incompact nucleus of crustacean sperm, we studied the spermatic chromatin composition and packaging of two crab species, using updated techniques including antibodies and micrococcal nuclease digestions. *Maja brachydactyla*, a species which until now has been unstudied, and *Cancer pagurus*, a crab species which has been shown to have a spermatic nucleus deplete of basic proteins (Langreth, 1969) both exhibit nucleosomal chromatin and histones associated to spermatic DNA.

MATERIALS

Live male *Maja bachidactyla* and *Cancer pagurus* were obtained from fresh sea markets in Barcelona, Spain. Testes and vasa deferentia were immediately dissected and the tissue washed with ice cold buffer A containing 0.25 M sucrose, 10 mM Tris pH 7.4, 5 mM $MgCl^2$, 3 mM $CaCl^2$, 0.25 mM spemidine, 0.1 mM spermine, and 10 mM benzamidine hydrochloride. The homogenate was either processed further to obtain a population of free sperm for further experimentation, or stored at –20º C with 40% glycerol for later use.

METHODS

Electron microscopy

Small sections of testes or vasa deferentia from *Maja bachidactyla* and *Cancer pagurus* have been fixed in 2% paraformaldehyde, with 2.5% glutarlaldehyde in 0.1 M cacoldylate buffer and embedded in Spurr resin for conventional electron microscopy.

Sperm cell purification and protein extraction

Nuclei were obtained from testis and vasa deferentia of *Maja bachidactyla* and *Cancer pagurus* as in Saperas *et al.* (2006). Briefly, tissues of *Maja bachidactyla* and *Cancer pagurus* were homogenized in buffer A (0.25 M sucrose, 3 mM $CaCl^2$, 5 mM $MgCl^2$ 10 mM Tris/HCl pH 7.4, 10 mM benzamidine hydrochloride), filtered and centrifuged. Pellets were washed twice more with buffer A containing 0.25% Triton X-100. Purified nuclei were washed with 1 mM Tris/1 mM EDTA pH 8.0, and basic proteins extracted with 5 volumes of 0.4 N HCl, cleared by centrifugation, precipitated and washed with acetone.

DNA normalization:

DNA of purified sperm cells from *Holothria tubulosa* (positive control of sperm containing histones), *M. brachydactyla*, and *C. pagurus* was quantified using $A^{260\ nm}$ and proteins corresponding to 1mg DNA were extracted with 0.4 N HCl or SDS. Proteins in association with equal amounts of spermatic DNA from these three models were compared among each other in AU gels (not shown) and SDS gels.

Electroforesis

— *SDS-PAGE:* Total protein extraction was performed as in Chiva and Subirana (1987) and analyzed on 15% SDS-PAGE gels.
— *Acetic acid/urea:* Acetic acid/Urea PAGE gel was performed according to Hurley (1977).

Western Blot Analysis

Western blotting was performed using antiserum for histone H2A, or a commercial antibody for histone H4 from calf thymus (Upstate). The antibodies used, as well as their ECL detection of the HRP-conjugated secondary antibody, were handled under the same conditions as those used in Martinez *et al.* (2007).

Obtaining free sperm cell population

A pure sperm cell population was obtained by filtering the sperm homogenate suspended in ice cold buffer A through 4 layers of gauze, and agitating the filtrate for 15 minutes at 4º C to free sperm cells from spermatophores. The sperm cell suspension was separated from spermatophore capsules and full spermatophores by passing the filtrate through a discontinual sucrose gradient of 0.25 M sucrose, 1 M sucrose, and 2.2 M sucrose. The denser material, being spermatophores and spermatophore capsules, sunk to the interphase of 1 M sucrose and 2.2 M sucrose. After allowing full separation of free sperm from spermatophores in the sperm cell suspension, the top layers containing free sperm suspended in 0.25/1 M sucrose were collected and washed in buffer A. From this point, the sperm cells were used for either swelling experiments, micrococcal nuclease digestions, or protein extractions.

Nuclear swelling tests

A free sperm cell population was used to perform swelling tests on both species *Maja brachydactyla* and *Cancer pagurus*. Sperm cells of these species were treated with 0.25% Triton X-100/0.1% non ionic detergent to permeabilize the nuclear membrane. Another cell sample was treated with 1mM Tris pH 7.4 to give the cells a hypotonic shock. A third sperm sample was treated with a solution of 1mM Tris pH 7.4 containing 5 mM EDTA, and a fourth sample was incubated with 5.8×10^{-5} M nucleoplasmine from *Xenopus laevis* as in Salvany *et al.* (2004). Observation of nuclear reaction was done on an Olympus DP-11 fluorescent microscope, having stained the nuclear material with 0.1 mg/ml Hoesch 33258 in distilled water.

Micrococcal nulease digestion

Digestions were performed as in Saperas et al. (2006). Reactions were performed at 37º C for various times in buffer containing 0.25 M Sucrose, 10 mM Tris pH 8.0, 0.5 mM $CaCl_2$, and 5 mM benzamidine hydrochloride, and then halted by the addition EDTA to a final concentration of 10 mM. After centrifugation, the supernatants were collected, and pellets resuspended in 10mM Tris pH 7.5 with 0.1 mM EDTA, centrifuged again, and the second pellet resuspended in the same buffer. DNA fragments were analyzed on 1.1% agarose slab gels, and basic proteins were analyzed with AU-PAGE.

RESULTS AND DISCUSSION

Ultrastructural study of sperm from M. brachydactyla and C. pagurus

Transmission electron microscopy confirms a similar morphology of sperm from these two crab species, demonstrating the typical multistellate sperm morphology described by Moses (1961), Brown (1966), and Hinsch (1969, 1973, 1988). An elecrodense acrosome is surrounded by nuclear material, which is composed of lax chromatin found in characteristic radial arm extensions (figure 1 A,B). DNA appears to lack compaction and organization; it is surrounded by a thick membrane, probably formed by the merging of the nuclear membrane with the double cellular membrane.

Sperm nuclear basic proteins in M. brachydactyla and C. pagurus

Electrophoresis of basic proteins extracted with 0.4 N HCl (not shown) and total proteins extracted with SDS from sperm (figure 3) showed the presence of histones in sperm of both crab species. No significant difference was found in the quantity of histones associated to 1mg DNA in *M. brachydactyla* when compared to sperm nuclei from *H. tubulosa* which are known to contain histones (Cornudella and Rocha, 1979) (not shown). In *C. pagurus*, there seem to be fewer histones globally associated to 1mg of DNA when compared to both *M. brachydactyla* (figure 3, lanes 1 and 3) and *H. tubulosa* (not shown), a noteworthy result deserving further investigation, as this may be important concerning the nucleosomal distribution throughout the nucleus of this crab species. Western Blotting further corroborates and confirms results shown by AU and SDS gels, with antibody recognition of the presence of both histones H2A and H4 in sperm of *M. brachydactyla* and *C. pagurus*, however showing less intensity of antibody signal in *C. pagurus* in comparison to that observed for *M. brachydactyla* (figure 4A, B, lanes 2 and 4).

Swelling of spermatic nucleus of M. brachydactyla and C. pagurus

Free sperm of *M. brachydactyla* and *C. pagurus* were exposed to several solutions and their swelling behavior observed and compared that of sperm under the same conditions, which contain a DNA-histone or DNA-protamine nuclear composition. In solution containing a mix of Triton X-100 and nonionic detergent, which makes the cell membrane more porous, there was no effect on the spermatic nucleus, behaving the same as would a spermatic nucleus containing either histones or protamine (figure 4A, C). The sperm were subject to a hypotonic shock, both with and without the detergent mix, and there was no behavioral change, just as there is no change in sperm containing either histones or protamine. However, when sperm were treated with a hypotonic shock including EDTA, a chelant, the nuclei swelled (figure 4B, D), just as would a nucleus containing histones; nuclei containing protamine associated to DNA are not affected. On the other hand, when nuclei were treated with nucleoplasmin, a nuclear chaperone which reorganizes spermatic DNA by replacing protamine with histones, there was no effect (figure 4A, C); spermatic nuclei containing protamine swell under the effect of nucleoplasmin (Salvany *et al.*, 2004).

Fig. 1

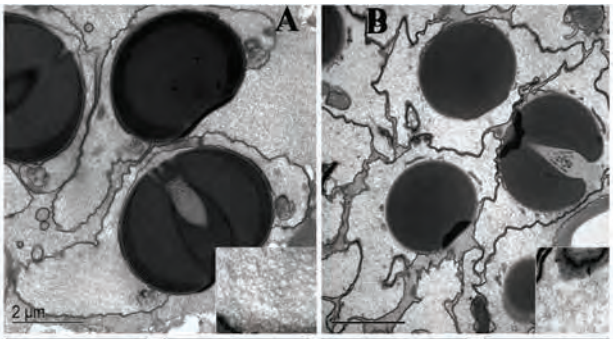


Fig. 2

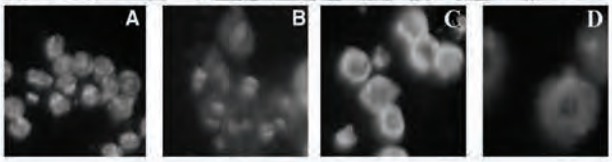
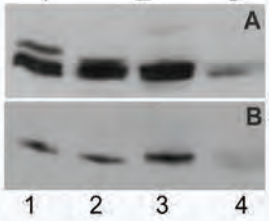


Fig 3

Fig. 4



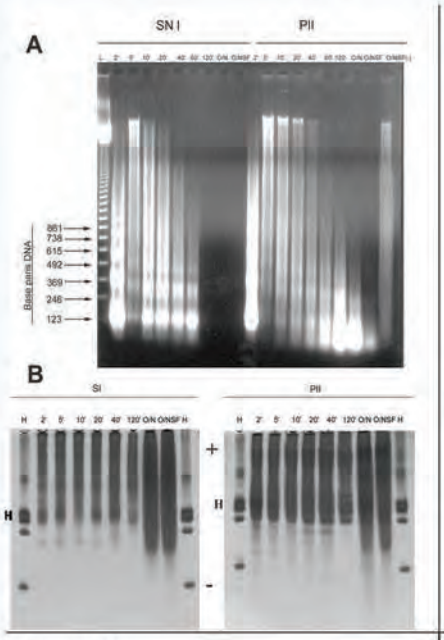


Figure 5

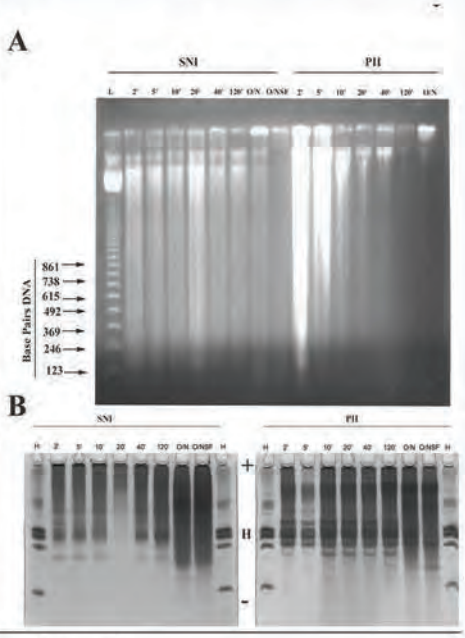


Figure 6

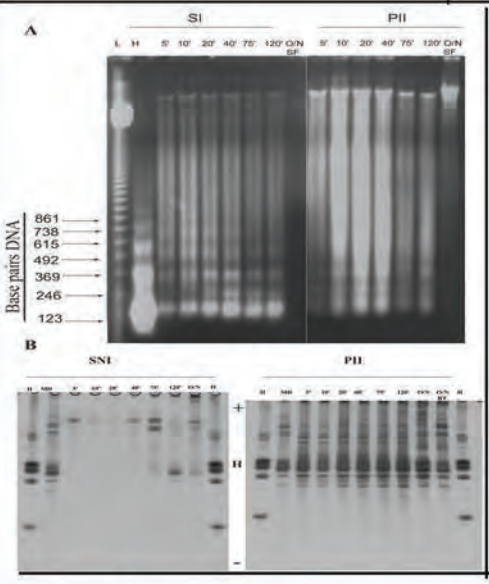


Figure 7

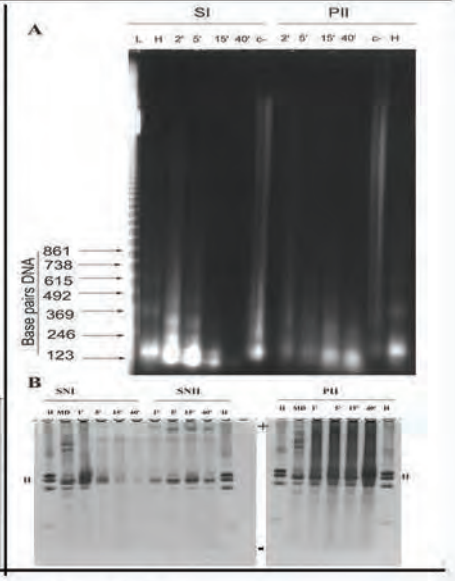


Figure 8

Figure 1 Ultrastructure of sperm, spermatic chromatin, and spermatophore from *M. brachydactyla* (A) and *C. pagurus* (B). Insets: Amplified detail of the chromatin fibers show a slightly more compact chromatin structure in *M. brachidactyla* (A) than in *C. pagurus* (B), which has a more sparse fiber distribution within the nucleus. Amplifications of insets is equal.

Figure 2 Swelling behavior of nuclei from *C. pagurus* (A,B) and. *M. brachydactyla* (C,D). Swelling is observed with the treatment of Tris/EDTA, shown in B, D. Non-swelling is observed with Tris, Tris/non-ionic detergent, Tris/Triton X-100, and nucleoplasmin, as shown in image A and C.

Figure 3 SDS gel showing equal amounts of SDS total protein exract from 1 mg DNA. Lane 1: total proteins from vasa deferentia of *M. brachydactyla*; lane 2: Standard of histones from lampreay testes; lane 3: total proteins extracted from vasa deferentia of *C. pagurus*..

Figure 4 A.- H2A recognition in western blot analysis. B: H4 recognition in western blot analyisis. Both blots contain basic proteins extracts from from 1 mg DNA taken from testes or deferent tissue, and separated on acetic acid/urea gels before tranfered to PVDF membranes and antibodies treatment. Lane 1.- total basic proteins from testes of *M. brachydactyla*. Lane 2: total basic proteins from vasa deferentia of *M. brachydactyla*; lane 3: total basic proteins from testes of *C. pagurus*; lane 4: total basic proteins extracted from vasa deferentia of *C. pagurus*.

Figure 5 Nuclease Digestions of sperm from *C. pagurus*. A: 1.1% agarose gel resolving DNA fragments liberated in digest for different times, and compared to standard 123 DNA base pair ladder. B: Basic proteins associated to DNA fragments liberated in digestion (SI) and basic proteins associated to unfragmented DNA (PII). Time intervals are indicated at the head of both DNA and protein gels. ON SF: Sample contained sperm in spermatophore instead of free sperm, left to digest overnight (O/N). H.- Histones.

Figure 6 Internal nuclease digestions of sperm from *C. pagurus*. A: 1.1% agarose gel resolving DNA fragments liberated in digest for different times, and compared to standard 123 DNA base pair ladder. B: Basic proteins associated to DNA fragments liberated in digestion (SI) and basic proteins associated to unfragmented DNA (PII). Time intervals are indicated at the head of both DNA and protein gels. ON SF: Sample contained sperm in spermatophore instead of free sperm, left to digest overnight (O/N). H: Histones.

Figure 7 Internal nuclease activity of sperm from *M. brachydactyla*. A: 1.1% agarose gel resolving DNA fragments liberated in internal digestion activity allowed for different times, and compared to standard 123 DNA base pair ladder. B: Basic proteins associated to DNA fragments liberated in internal digestion (SI) and basic proteins associated to unfragmented DNA (PII). Time intervals are indicated at the head of both DNA and protein gels. ON SF: Sample contained sperm in spermatophore instead of free sperm, left to digest overnight (O/N). H: Histones.

Figure 8 Nuclease Digestions of sperm from *M. brachydactyla*. A: 1.1% agarose gel resolving DNA fragments liberated in internal digestion activity allowed for different times, and compared to standard 123 DNA base pair ladder. B: Basic proteins associated to DNA fragments liberated in internal digestion (SI) and basic proteins associated to unfragmented DNA (PII). Time intervals are indicated at the head of both DNA and protein gels. ON SF: Sample contained sperm in spermatophore instead of free sperm, left to digest overnight (O/N). H: Histones.

Micrococcal nuclease digestions

The results obtained on the assessment of micrococcal nuclease digestion of chromatin from mature sperm cells of *M. brachydactyla* and *C. pagurus* show that there is indeed a nucleo-histone complex organizing the spermatic chromatin, thus hindering enzymatic access to the chromatin in these areas. Sperm chromatin is converted into nucleosome oligomers with the addition of enzyme (figures 5 and 8). Histones associated with these nucleosomal fragments are liberated in the digestion (Figures 5 and 8). However, incubation of the nuclei without enzyme showed chromatin degradation; a digestion pattern similar to that of DNase I (figures 6A and 7A). There is a more defined synergic effect of the internal enzyme and added micrococcal nuclease activity in *M. brachydactyla* (figure 8), as the DNA is more rapidly digested than that of *C. pagurus* (figure 6) in the positive control with enzyme added. Further experimentation needs to be done to eliminate the prospect that the DNA digestion observed in samples without added enzyme could be due contamination of enzymes from the hepatopancreas. However, the possibility of internal DNase activity in sperm nuclei of crabs is worth further investigation, especially concerning the function this could have in fertilization biology of these animals.

Determination of DNA repeat lengths was done by comparing the resulting DNA fragment motilities to a standard curve derived from DNA fragments of 123 base pair multiples. The resulting sized fragments were extrapolated to what their sizes would be at time 0. This method of nucleosome calculation was performed first on a positive control model, *Holothuria tubulosa*, as has been described by Cor-

nudella and Rocha (1979), and our method produced the same nucleosome size as that which has been published, which is 225 bp. The nucleosome size for both *M. brachydactyla* and *C. pagurus* is about 170 bp, and the core is 145 bp.

One aspect to consider when analyzing the nucleosome found in sperm of these two species is the size of linker DNA. This is the area of the nucleosome with most variability. Within chromatin, the basic unit consists of the core particle and histone H1, with 166 bp of DNA wrapped around the histone octamer and associated with the globular region of H1; this complex is called a chromatosome, or chromatin particle (Bharah *et al.*, 2003). The rest of the DNA included in the nucleosome is called linker DNA, associated with the tail domains of linker histone H1, which folds nucleosomes into higher order a chromatin structure (Widom, 1998). If the nucleosome size corresponds to the size of the chomatosome, then linker DNA portions in the nucleosomes of the crab species studied here are practically nonexistent.

Here we disprove the previous reports that the sperm nucleus of crabs is void of basic proteins. With the use of micrococcal nuclease digestions, we show that spermatic DNA is organized into nucleosomes, and that histones are associated with the DNA fragments protected from enzymatic digestion. Some possible reasons for the discrepancy in our results compared with the literature may have to do with the fixation methods used in staining of basic proteins, on which many have based their conclusion that there is an absence of basic proteins in crustacean sperm nuclei (Chavaillier, 1966; Langreth, 1969; Vaughn and Hinsch, 1972). We believe the chemical fixation method could cause artifactual histone migration and chromatin aggregation, and hence unreliable results, both in ultrastructural studies using transmission electron microscopy, as well as cytological and histochemical studies performed on fixed cell nuclei. Further, chromatin of crab sperm has been observed to be highly sensitive by Chevaillier (1968), showing that the chromatin fibers clump unless stabilized by heavy metal ions. Considering sensitivity of sperm from these crabs, buffers used for sperm purification, such as sea water, later replaced by EDTA in the washing technique of Vaugh and Hinsch (1972) exposes the sperm to harsh conditions. It is possible that these sperm, exposed to high ionic strengths and chelating agents or pH changes in the techniques used by earlier investigators, weakened electrostatic histone-DNA affinity, hence resulting in histone migration to other areas of the sperm, such as the acrosome, which has been shown to contain basic proteins according to Vaughn and Hinsch (1972). However, a study done by Sellos and le Gal (1981) found the presence of somatic-like histones in sperm of the crustacean *Palaemon serratus*, and their sperm purification method and buffers used are practically identical to those used in our washing method described here.

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