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Histones and Nucleosomes in *Cancer* Sperm (Decapod: *Crustacea*) Previously Described as Lacking Basic DNA-Associated Proteins: A New Model of Sperm Chromatin

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ABSTRACT

To date several studies have been carried out which indicate that DNA of crustacean sperm is neither bound nor organized by basic proteins and, contrary to the rest of spermatozoa, do not contain highly packaged chromatin. Since this is the only known case of this type among metazoan cells, we have re-examined the composition, and partially the structure, of the mature sperm chromatin of *Cancer pagurus*, which has previously been described as lacking basic DNA-associated proteins. The results we present here show that: (a) sperm DNA of *C. pagurus* is bound by histones forming nucleosomes of 170 base pairs, (b) the ratio [histones/DNA] in sperm of two *Cancer* species is 0.5 and 0.6 (w/w). This ratio is quite lower than the proportion [proteins/DNA] that we found in other sperm nuclei with histones or protamines, whose value is from 1.0 to 1.2 (w/w), (c) histone H4 is highly acetylated in mature sperm chromatin of *C. pagurus*. Other histones (H3 and H2B) are also acetylated, though the level is much lower than that of histone H4. The low ratio of histones to DNA, along with the high level of acetylation of these proteins, explains the non-compact, decondensed state of the peculiar chromatin in the sperm studied here. In the final section we offer an explanation for the necessity of such decondensed chromatin during gamete fertilization of this species. J. Cell. Biochem. 105: 574–584, 2008.

KEY WORDS: CHROMATIN; ACETYLATION; CRUSTACEA; SPERM

The study of proteins associated to DNA in sperm nuclei is a subject which began over 130 years ago [Miescher, 1874]. The first classification of sperm nuclei according to the type of molecule associated to its DNA was performed by Bloch [1969]. In this work, Bloch established five main categories of sperm nuclei based on the counter ions which are associated to their DNA. The first four categories of nuclei (those containing histones, monoprotamines, diprotamines, and keratinous protamines) have been followed up with additional studies, largely increasing our understanding of the chemical structure of molecules organizing mature sperm DNA [Ausió, 1999]. Furthermore, this molecular characterization helps to explain the vast evolutionary variability they demonstrate [Subirana, 1983; Subirana and Colom, 1987; Daban et al., 1991a,b; Rooney et al., 2000; Lewis et al., 2004; Eirin-Lopez et al., 2006a,b]. Still, the fifth category established in the original work of Bloch remains poorly understood. This category includes sperm nuclei of diverse crustacean species, however representing a great portion of all animal species, in which no basic proteins were found associated to DNA. Nevertheless, the alternative counter ion to basic DNA-interacting proteins is not mentioned in the original work by Bloch.

Around the time period of Bloch's classification of sperm nuclei, diverse species of crustacean sperm were being studied, in an attempt to describe possible proteins associated to the DNA [see for example Chevaillier, 1966, 1967, 1968; Vaughn and Locy, 1968; Vaughn and Hinsch, 1972; Vaughn and Thomson, 1972]. These studies were consistent in describing the peculiarities which are particular to sperm of this taxonomic group, and agree that, for the most part, their cellular components are not homologous to other types of sperm cells [see Langreth, 1969].

Due to the great diversity of crustacean species, in the rest of the introduction we will focus exclusively on sperm of brachyuran decapod crustaceans. Sperm nuclei of these species are positioned in

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the periphery of the cell, surrounding an acrosomal granule approximately spherical in shape [Langreth, 1969; Vaughn and Hinsch, 1972; Hinsch, 1988; Chiba et al., 1992; Medina and Rodríguez, 1992; see also Fig. 1]. In addition, these nuclei exhibit two particular characteristics which differentiate them from other mature sperm nuclei. First, they are not entirely separated from the cytoplasm by a membrane or envelope [Langreth, 1969; Hinsch, 1986], and therefore the cytoplasmic structures which are reminiscent of sperm cells are found immersed within the chromatin itself. (The sperm nuclei of the crustaceans studied here have been designated by several authors under the term *nucleocytoplasm*.) A second characteristic is the apparent decondensed, non-compact chromatin (see Fig. 1). This situation is not at all shared by sperm nuclei of other animal species which contain highly condensed chromatin.

The peripheral position of the nucleus, as well as its decondensed nature, has made difficult the purification of these organelles, and consequently, the analysis of specific counter ions of the DNA. The experiments designed to study the nuclear contents were mainly carried out by specific histochemical staining methods [Alftert and Geschwind, 1953; Bloch and Hew, 1960], even though in some cases an attempt was made to solubilize the chromatin by drastic mechanical methods to analyze the proteins released in the supernatants [Vaughn and Hinsch, 1972]. Specifically in species of the genus *Cancer*, Langreth [1969] observed that the histones apparently leave the nucleus in the most advanced phases of spermiogenesis. Also, Chevaillier [1966, 1967] described that in *Eupagurus lambardus* and *Carcinus maenas* the histones migrate from the nucleus to the acrosomal granule in the latest steps of spermiogenesis. On the other hand, Vaughn and Hinsch [1972] describe only acidic proteins associated to sperm DNA of *Libinia emarginata*.

Due to great interest in understanding which type of counter ions are responsible for organizing the DNA in mature sperm of these animals, we have re-examined the composition and (partially) the structure of mature sperm chromatin of *Cancer pagurus*, since sperm of this species has been described as void of basic DNA-interacting proteins.

MATERIALS AND METHODS

ANIMALS

Male individuals of *C. pagurus* (a decapod brachyuran crab) were purchased live from markets in Barcelona, Spain following proper identification according to their morphological characteristics. Male *Cancer magister* crabs were purchased live from markets in Victoria, British Columbia, Canada, also identified by morphological characteristics. Male individuals of the echinoderm *Holothuria tubulosa* were obtained off the Mediterranean coast of Catalunya, Spain, and the sperm isolated. Both *Patella aspera* and *Murex brandaris* (molluscs) were caught and treated as described elsewhere [Daban et al., 1991a,b; Cáceres et al., 1999, respectively]. The deferential duct was removed from *C. pagurus* and *C. magister*. Fresh sperm and spermatophore suspensions were used for further experimentation. Sperm from *H. tubulosa* were dounce homogenized with ice cold buffer A (0.25 M sucrose, 10 mM Tris pH 7.4,

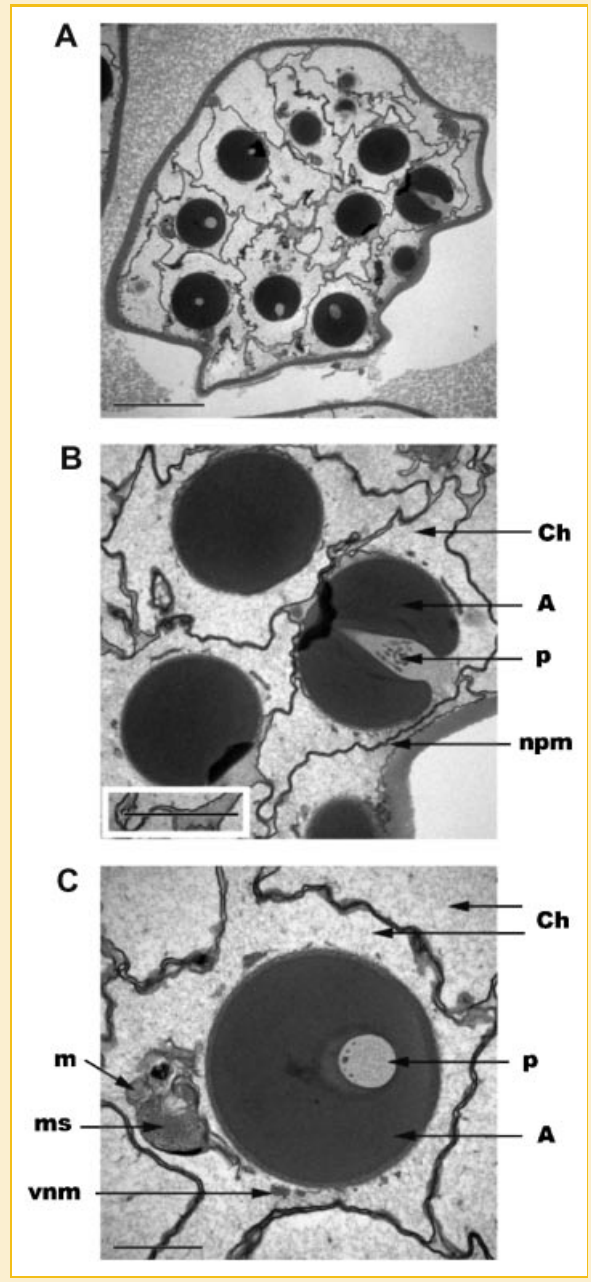

Fig. 1. Sperm structure of *C. pagurus* observed by transmission electron microscopy. A: Spermatophore obtained from the deferential duct of a specimen of *C. pagurus*, consisting of a capsule surrounding several sperm cells contouring around one another to achieve a tightly packed sperm bundle. B: Section cut along the meridian of a mature sperm cell. C: Section cut along the central equator of a mature sperm cell, in which organelles of cytoplasmic origin can be observed, immersed within the chromatin. A, acrosome; Ch, chromatin; m, mitochondrion; ms, membrane system; npm, nucleocytoplasmic membrane; p, perforatorial column; vnm, vessiculate nucleocytoplasmic membrane (nomenclature of organelles is based on that which is used by Tudge et al. [1994] and by Jamieson [1994]). Bars—A: 5 μm; B: 2 μm; C: 1 μm.

3 mM $MgCl_2$, 5 mM $CaCl_2$, 0.1 mM spermine, 0.25 mM spermidine, with 25 mM benzamidine chloride as an inhibitor of proteolysis) and made to 50% glycerol, followed by storage at −20°C until later use.

ELECTRON MICROSCOPY

Small sections of deferential duct have been fixed and embedded in Spur resin for conventional electron microscopy [Giménez-Bonafé et al., 2002].

OBTAINING FREE SPERM CELL POPULATION OF *C. pagurus* AND *C. magister*

The deferential ducts of *C. pagurus* and *C. magister* were gently homogenized with a dounce homogenizer in buffer A, and filtered through four layers of gauze. The filtrate (containing spermatophores and free sperm cells) was stirred during 15 min at 4°C to free sperm cells from spermatophores. Free sperm cells were separated from spermatophore capsules and full spermatophores by sedimentation at unit gravity through a discontinual sucrose gradient of 0.25 M sucrose, 1 M sucrose, and 2.2 M sucrose. The denser material, being full 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 M/1 M sucrose were collected and washed in buffer A. All procedures were performed at 4°C. From this point, the sperm cells freed from spermatophores were used for micrococcal nuclease digestions or extraction of basic proteins. An aliquot of these sperm cells can be seen in Figure 7A,B.

FLUORESCENCE MICROSCOPY

Sperm cell nuclei of *C. pagurus* were stained for 10 min on ice with either Draq 5 (1:1,000 dilution in buffer A) or Topro-3 (1:3,000 dilution in buffer A), and viewed on a Leica optical microscope. The fluorescence resulting from DNA intercalating agents was observed by excitation with a 633 nm red helium neon laser. Alternatively, nuclei of these sperm cells were stained with 0.1 mg/ml Hoesch 33258 in distilled water and observed using an Olympus DP-11 fluorescent microscope, exciting the flourochrome with 295 nm spectra.

EXTRACTION OF NUCLEAR PROTEINS

Free sperm cells were pelleted and extracted with 5 volumes of 0.4 N HCl, or alternatively, with 5% perchloric acid (PCA), and cleared by centrifugation, precipitated and washed with acetone [Saperas et al., 2006].

ANALYSIS OF PROTEINS

Two-dimensional electrophoresis was performed according to the procedure described by Dimitrov and Wolffe [1997] with minor variations, described in Kurtz et al. [2007].

Electrophoresis of native oligonucleosomal fragments was first performed on a 6% acrylamide native gel in TAE (40 mM Tris pH 7.2, 20 mM sodium acetate, 1.5 mM EDTA) buffer. For native two-dimensional analysis, a lane was excised from the first dimension and equilibrated for 5 minutes in buffer containing 2% SDS, 10 mM β-mercaptoethanol, 10 mM Tris pH 7.5, and then placed atop the stacking layer of a 15% SDS gel. The proteins associated to the oligonucleosomal fragments were allowed to resolve at 80 V until loading dye reached the bottom of the gel.

One-dimensional SDS–PAGE and AU-PAGE gels were performed as in Martinez-Soler et al. [2007], and AUT-page gels were performed as in Frehlick et al. [2006].

Amino Acid analyses were carried out after hydrolysis in 6 N HCl at 110°C for 24 h.

Reverse-Phase HPLC was performed following the method described in Ausió [1988].

PROTEIN AND ACETYLATION ANALYSIS BY WESTERN BLOTS

Following electrophoresis, proteins were transferred to a PVDF membrane and probed for the presence of histones H4 or H2A using antisera raised in rabbits as described in Martinez-Soler et al. [2007]. The presence of acetylated histones was detected using the commercial antibodies anti-acetyl lysine (Abcam), anti H4 specifically acetylated in lysine 12 (anti-H4-acK12, Upstate), and anti H4 specifically acetylated in lysine 16 (anti-H4-acK16, Upstate).

The anti-H2A and anti-H4 antisera were prepared 1:1,000 in PBS containing 5% powdered milk and the membrane probed overnight at 4°C with shaking. Commercial antibodies specific for acetylated lysines were prepared in PBS containing 2.5% fetal calf serum and 2.5% powdered skim, at a dilution of 1:250, and incubated at 4°C overnight with shaking. Antibody recognition of all antibodies mentioned was detected using an HRP conjugated goat anti-rabbit secondary antibody diluted 1:3,000 in PBS containing 5% powdered skim milk, and incubated for 1.5 h at room temperature with agitation. HRP detection was performed using an ECL reagent (Amersham).

MICROCOCCAL NUCLEASE DIGESTION AND QUANTITATION OF RELEASED PRODUCTS

Digestions were performed as in Saperas et al. [2006]. Sperm chromatin digestions of *H. tubulosa* were performed in parallel with those of *C. pagurus*, since the nucleosome of this species has been previously described in detail [Cornudella and Rocha, 1979]. Briefly, samples of sperm cells or sperm nuclei corresponding to 1 mg/ml of DNA in buffer containing 0.25 M sucrose, 10 mM Tris pH 8.0, 0.5 mM $CaCl_2$, and 5 mM benzamidine hydrochloride were digested at 37°C for various times with micrococcal nuclease (Sigma). The enzyme was added to a concentration of 0.33 U/mg DNA for *H. tubulosa*, which was diminished to 0.11 U/mg DNA for *C. pagurus* to avoid over digestion of the DNA. Reactions were halted by the addition EDTA to a final concentration of 10 mM. After centrifugation (10,000*g* for 10 min), the supernatants (SI) were collected, and pellets washed and centrifuged again under the same conditions. The resulting pellet (P) was resuspended in 10 mM Tris pH 7.5 with 0.1 mM EDTA. Chromatin fragments from the supernatants (SI) and pellets (P) were both DNA and basic protein extracted, as described in Saperas et al. [2006]. DNA fragments were analyzed on 1.1% agarose slab gels, while the corresponding basic proteins were analyzed with AU-PAGE. Nucleosome lengths were

calculated for *H. tubulosa* and *C. pagurus* using the methodology described in Johnson et al. [1976] which was also applied in the study done by Cornudella and Rocha [1979] and used previously in our laboratories [Ribes et al., 2001; Saperas et al., 2006]. Briefly, a standard curve of molecular weight versus migration distance was produced using a molecular weight marker which was included in the DNA electrophoresis along with digested DNA fragments, and used to extrapolate the molecular weight of these fragments in each reaction. The total nucleosome length (linker region + core region) was determined by extrapolating the theoretical mononucleosomal fragment size at time = 0 minutes of the SI fraction. The core size was determined by the limiting fragment size, resistant to further digestion, and the linker length was determined by subtracting the core length from the total nucleosomal length.

For four different species (*C. pagurus*, *H. tubulosa*, *M. brandaris*, and *P. aspera*) aliquots of prepared sperm nuclei, each containing 0.5 mg DNA, were digested in parallel as described above. Following digestion times, EDTA was added to 2 mM and NaCl and PCA were added to final concentrations of 1 M each. The digested chromatin was sedimented and the soluble DNA was quantified by the absorbance at 260 nm using the extinction coefficient of $A_{260} = 20\ cm^2\,mg^{-1}$ DNA. The amount of soluble DNA in each sample was calculated and presented as the percentage of total starting DNA.

PROTEIN/DNA NORMALIZATION

Fresh sperm obtained from spermatophores released from deferential ducts of *C. pagurus* and *C. magister*, or purified nuclei of control species *H. tubulosa*, *P. aspera*, and *M. brandaris* were gently homogenized in ice cold buffer A. Suspensions of sperm cells (*Cp*, *Cm*) or sperm nuclei (*Ht*, *Pa*, *Mb*) were separated into equal aliquots for either DNA or basic protein quantitation, and several replicates were performed for each. Aliquots for DNA quantitation were incubated at 37°C in 10 mM Tris pH 8.0, 0.1 M EDTA, 40 μg/ml RNase, and 0.5% SDS with later addition of proteinase K to 0.1 mg/ml at 50°C until cells or nuclei were fully digested. Following incubation, DNA was isolated with the addition of phenol:cholorform:isoamyl alcohol (25:24:1), and precipitated in ethanol, dried, and solubilized in distilled water. The absorbance of each aliquot was measured at 260 nm, the DNA quantified using the extinction coefficient of $A_{260} = 20\ cm^2\,mg^{-1}$, and the values obtained for each aliquot were averaged. In parallel, basic proteins from sperm aliquots were obtained with 0.4 N HCl solubilization, and precipitated with cold acetone (6 volumes). Sperm basic proteins were analyzed on a 15% SDS gel which included known amounts of chicken erythrocyte histones as a standard which had been previously quantified. *Photoretix 1D* densitometry program was used to analyze the resulting SDS gel, where the band intensities obtained from the sperm samples were compared to those of the quantification standard. The amount of protein present in the sperm samples was extrapolated and averaged. Values were consistent among aliquots for both DNA and basic protein quantification. The relation of protein/DNA was calculated as the dividend of the average protein weight and average DNA weight extracted from sperm aliquots.

RESULTS

DESCRIPTION OF THE NUCLEOCYTOPLASM OF *C. pagurus*

The mature sperm of *C. pagurus* is found tightly grouped into capsules called spermatophores (Fig. 1A). These spermatophores protect the sperm in the spermatheca of the female and allow them to remain during a prolonged period of time without any loss of their functional ability. Longitudinal and transversal sections of sperm can be observed in Figure 1B,C. The chromatin coexists, without complete physical separation from laminar membrane systems, with mitochondria, microtubules, and other components which originally are formed in the cytoplasm of developing spermatids [Hinsch, 1969, 1986; Langreth, 1969; Tudge et al., 1994; Rorandelli et al., 2008]. (Note that these elements are a source of contamination when the chromatin is studied or isolated.) The nuclear membrane has been fused with the plasma membrane, forming a dense envelope that wraps around the majority of the cell. [For more detailed studies on the complete nucleocytoplasmic structures and the substructure of the acrosomal granule one can consult Langreth, 1969; El-Sherief, 1991; Chiba et al., 1992; Jamieson, 1989, 1994.]

BASIC PROTEINS IN THE WHOLE SPERM CELL: PRESENCE OF HISTONES

Due to the absence of tight chromatin packaging and apparent decondensed nature, the sperm nuclei of *C. pagurus* rupture when conventional nuclear purification methods are applied. Additionally, the presence of organelles of cytoplasmic origin which are immersed in the chromatin increase the possibility of proteolysis during experimental handling (see Discussion Section). Since the various *Cancer* species which have been described prior to the present study were concluded to have no histones bound to mature sperm DNA, we carried out an exhaustive examination using diverse electrophretic methods of the basic proteins that were extracted from freshly obtained sperm from the deferential ducts.

Figure 2 (top) shows the total proteins solubilized with 0.4 N HCl from sperm as resolved in SDS–PAGE, acetic acid/urea AU-PAGE, and acetic acid/urea/triton AUT-PAGE. In these different electophoretic systems some extracted basic proteins demonstrate a migration similar to that of histones used as a control. Analysis in polyacrylamide/SDS indicates that two of these proteins (asterisks in Fig. 2) have identical mobilities as histones H3 and H4 from chicken erythrocyte, while another two proteins present slight variations in mobility with respect to histones H2A and H2B. This result is congruent with the fact that histones H3 and H4 are the most evolutionarily conserved [Baxevanis and Landsman, 1996; Piontkivska et al., 2002]. The putative H1 exhibits mobility in SDS–PAGE similar to histones H5 and H1 of chicken erythrocyte. When the proteins are analyzed in high resolution gels of AU-PAGE (Fig. 2: AU), or in AUT gels (Fig. 2: AUT), the electrophoretic development appears more complex since these systems resolve diverse types of histone posttranscriptional modifications [Zweidler, 1978; Dimitrov and Wolffe, 1997]. It is especially interesting to observe that the possible core histones of *C. pagurus* are transformed into more than nine bands in the AU gels, and into an even greater number of bands in AUT gels. Also, we have used AU gels of low resolution to observe the possible presence of a protamine type protein. However, those

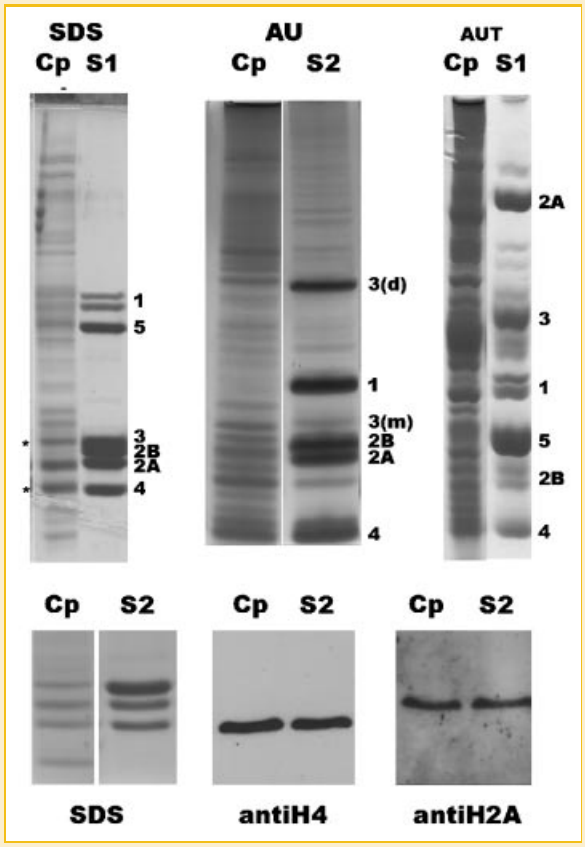

Fig. 2. Basic proteins in the sperm of *C. pagurus*. Electrophoretic analysis of solubilized proteins with 0.4 N HCl from sperm of *C. pagurus*. Top (left to right). Polyacrylamide–SDS gels (SDS), polyacrylamide–acetic acid–urea (AU) and polyacrylamide–acetic acid–urea–triton (AUT). From 1 to 5: Histones H1, H2A, H2B, H3, H4, and H5; 3(d) Dimer of histone H3; 3(m) monomer of histone H3. Asterisks mark histones H3 and H4 of *C. pagurus* sperm. Bottom: Immunodetection of basic proteins from *Cancer* sperm with anti-H4 and anti-H2A antisera. Cp, *C. pagurus*, S1, Standard of histones from chicken erythrocyte; S2, Standard of histones from lamprey sperm [Saperas et al., 1994].

results (not shown) demonstrate that sperm of *C. pagurus* do not contain protamine.

In order to unmistakably verify the identity of the histones in this sperm, we carried out Western blot analyses of the proteins resolved in SDS gels, using an antiserum specific for histone H4 (indicator of the tetramer $[H3\text{-}H4]_2$) and one specific for histone H2A (indicator of the dimer [H2A-H2B]). The results of these Western blots, which are shown in Figure 2 (bottom) assures that the extracted proteins of the sperm are indeed nucleosomal core histones. Histone H1 will be described later in this work.

THE HISTONES OF *C. pagurus* SPERM ARE BOUND TO DNA

Currently, it is known that in practically all cells of eukaryotic organisms, except for some protozoa such as dinoflagelates [Bodansky et al., 1979; Herzog and Soyer, 1981], the DNA is organized via its interaction with basic proteins, mainly histones (and/or SNBPs in the sperm nucleus). However, evidence contradicting this biological principle has been shown in sperm nuclei of crustaceans, and consequently it is imperative to this study whether the histones we describe here are interacting with DNA in the nucleocytoplasm, or if they have left their position in the nucleus.

Due to the impossibility of obtaining pure nuclei, we used sperm released from spermatophores and permeabilized with 0.25% Triton X-100/0.1% Nonidet NP-40. The permeabilized cells were incubated with micrococcal nuclease (MNase) at different times. Since the nucleocytoplasm occupies a peripheral position in the sperm cell (see Fig. 1), the chromatin is accessible to nuclease digestion, and the enzyme acts to release chromatin fragments to the supernatant during the reaction. The digestion product of the MNase digestion has been examined in a two-dimensional gel system, in which the first dimension separates chromatin fragments (protein–DNA complexes), and the second dimension resolves the protein content of these chromatin fragments. In Figure 3A it is shown that the chromatin fragments are organized in sizes corresponding to the multiple nucleosome subunits (M, D, T in Fig. 3A), which clearly contain the complete endowment of core histones.

To study in more detail the nucleosome size of sperm chromatin of *C. pagurus*, we have analyzed the DNA fragments released at different incubation times when treated with MNase. As a comparative control, we used sperm nuclei from *H. tubulosa*, which contain histones forming nucleosomes of a known size [Cornudella and Rocha, 1979; Azorin et al., 1985]. The result (which can be appreciated in Fig. 3B and in Table I) indicates that the nucleosome size in mature sperm of *C. pagurus* is composed of 170 base pairs of DNA (core: 145 bp; linker: 25 bp, see Table I). This small nucleosomal size can easily be observed in Figure 3B (top) where oligonucleosomal fragments of *C. pagurus* have a faster migration than those of *H. tubulosa* (compare fragment mobilities in S portion of *C. pagurus* to those in lane H). Figure 3B also shows that in *C. pagurus* sperm, all DNA is readily MNase digestible, since after just a few minutes of incubation with the enzyme no large DNA fragments were left undigested in the supernatants or pellets. This result indicates that there are no existing regions of DNA protected by a protamine or any other type of special protein. Although the kinetics of the MNase digestion do not allow the proportion of DNA free from histones to be known with exact precision, the analysis of the soluble DNA fraction in cold 1M PCA offers an estimation of the proportion of DNA readily accessible to digestion, and therefore with a high probability of being unbound to any histones. The fraction of cold-acid soluble DNA (1M PCA) obtained at various times for sperm of *C. pagurus* is compared in Figure 4 with the equivalent digestion kinetics of sperm nuclei containing histones, protamines, or protamine-like proteins. In this figure, it can be observed that the sperm chromatin of *C. pagurus* is digested at a faster rate and in greater proportion to the other shown chromatin types, with nearly 50% of the genome accessible to enzyme digestion.

CHARACTERISTICS OF HISTONES FROM THE CHROMATIN OF *C. pagurus*

Amino acid composition. The histones of *C. pagurus* have been purified by reverse phase HPLC (see Materials and Methods Section) and the results shown in Figure 5. The behavior in HPLC of the core

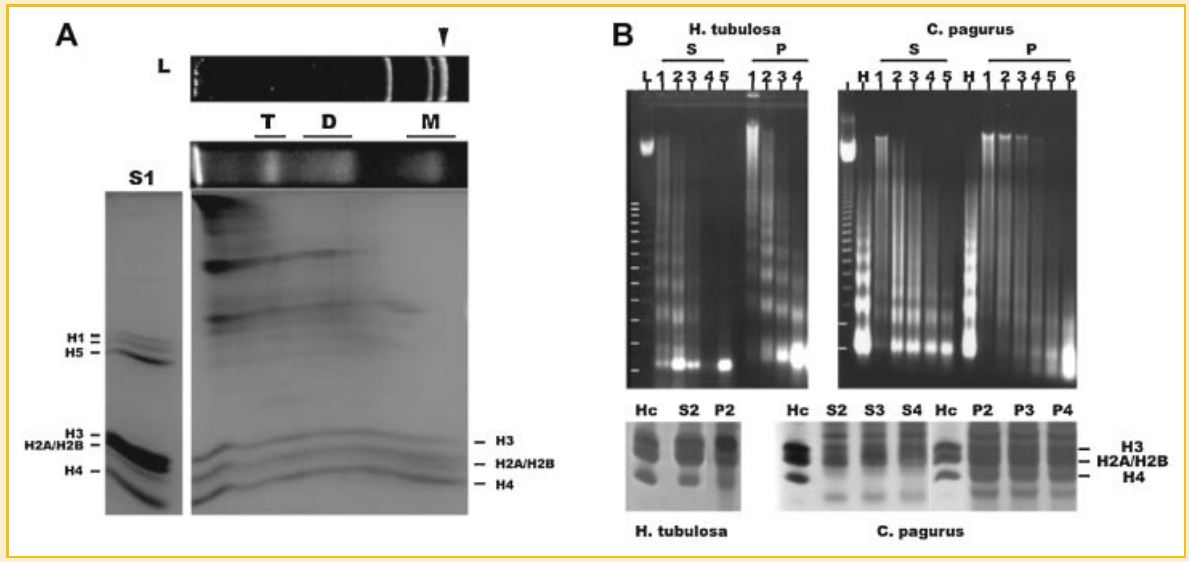

Fig. 3. Sperm chromatin of *Cancer*. A: Two-dimensional gel resolving the protein fraction of chromatin fragments. The first dimension (from left to right) resolves the trimer (T), dimer (D), and monomer (M), of the nucleosome subunit. The arrow on the ladder (L) indicates the core particle size. The second dimension corresponds to electrophoresis in SDS which allows identification of core histones bound to the chromatin fragments (H1 isoforms are not identified in this gel and will be analyzed in Fig. 5). S1, standard of chicken erythrocyte histones. B: (top) Kinetics of *Holothuria* and *Cancer* MNase digestion of sperm nuclei. L, ladder indicating multiples of 123 base pairs. S, supernatants; P, pellets; from 1 to 6: incubation times corresponding, respectively, to 2, 5, 10, 20, 40, and 120 min. H, pellet obtained from *H. tubulosa* sperm chromatin digestion at 20 minutes serving as a nucleosomal repeat marker to compare with nucleosomes observed in *C. pagurus* sperm digests. Bottom: Proteins in supernatants (S) and in pellets (P) at different times. Hc: Histone core standard.

histones studied in *Cancer* (Fig. 5A,C) is practically identical to other histones of sperm or somatic origin studied in our laboratories [Saperas et al., 1994; Roccini et al., 1996]. However, HPLC chromatography of the fraction of histones soluble in 5% PCA (histone H1 and other closely related proteins, Fig. 5B) only partially purified some histone H1 forms and other additional or contaminating proteins (Fig. 5D).

The core histones and the main H1 fraction which were purified have amino acid compositions which are very similar to somatic histones of calf thymus (Table II). Sperm nuclei of *C. pagurus* contain at least three isoforms of histone H1 (a, d, e in Fig. 5B,D and in Table II). These H1 isoforms are identified as such based on their amino acid compositions. Two of these (d, e) exhibit mobilities in SDS which are nearly the same as H1/H5 of chicken erythrocyte. One isoform (a, Fig. 5B) has a much slower electrophoretic mobility than H1/H5 despite its amino acid composition identifying this fraction as an H1 isoform. Some proteins obtained in the 5% PCA soluble fraction also have mobilities in the H1/H5 region, but are too acidic in nature to be considered H1 proteins, and therefore are designated as H1-like (b, c in Fig. 5B,D and in Table II).

TABLE I. Nucleosome Size of Crustacean Sperm Chromatin From Genus *Cancer*, Compared to That of *H. tubulosa*, Used as a Control

	Core (bp)	Linker (bp)	Nucleosome (bp)
Holothuria (1)	145	82	227
Holothuria (2)	145	80	225
Cancer (2)	145	20–25	165–170

(1) From Cornudella and Rocha [1979]; the rest (2) are from the present work.

Histone acetylation. In Figure 2 the examined histones can be observed in AU and AUT gels, which have been resolved into several bands. This behavior corresponds to the different posttranslational modifications of these proteins. In this section we analyze the acetylation state of *C. pagurus* sperm histones. On one hand, the proteins extracted with 0.4 N HCl have been separated by two-dimensional electrophoresis according to a variation of the method described by Dimitrov and Wolffe [1997] and used previously in our

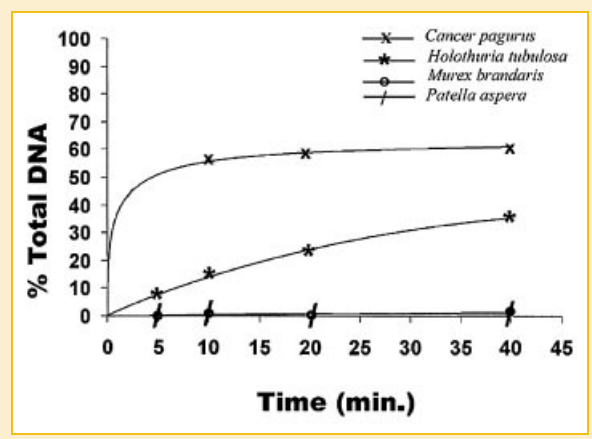

Fig. 4. Kinetics of MNase digestion in sperm of various chromatin compositions. Percent of sperm DNA which is soluble in the presence of cold 1 M PCA from various species presenting different sperm chromatin types. *C. pagurus* (X) (decondensed sperm chromatin); *H. tubulosa* (*) (sperm chromatin containing histones); *P. aspera* (/) (sperm chromatin containing protamine-like proteins); *M. brandaris* (o) (sperm chromatin containing protamine).

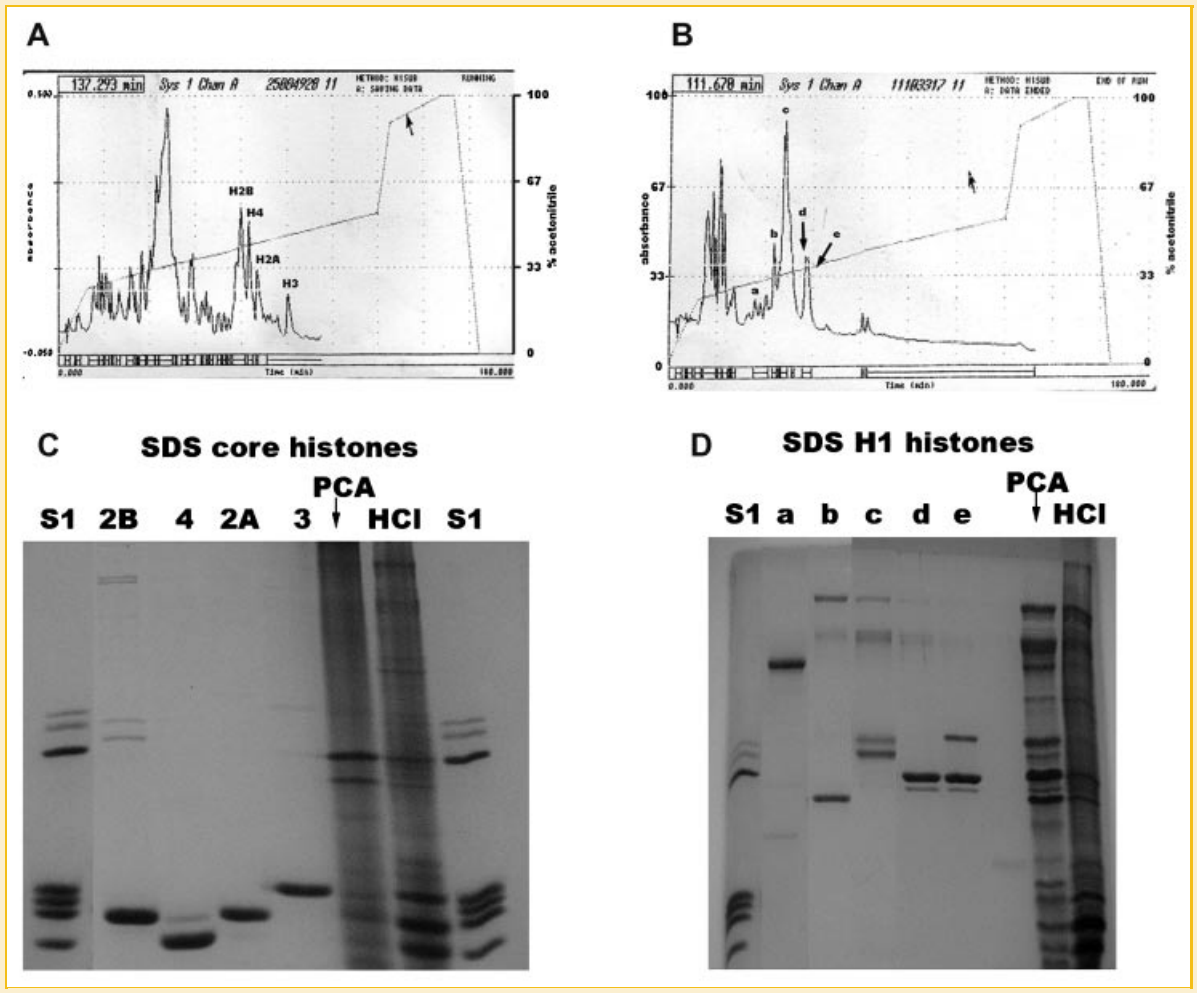

Fig. 5. Purification of histones from *Cancer* sperm. A: HPLC chromatogram and (C) Electrophoresis in SDS, of core histones purified by reverse-phase HPLC. B: HPLC chromatogram and (D) Electrophoresis in SDS, of proteins soluble in 5% PCA, separated by reverse-phase HPLC. S1, histone standard from chicken erythrocyte; HCl, total basic sperm proteins solubilized with 0.4 N HCl; PCA, sperm proteins solubilized with 5% perchloric acid (H1 and family).

TABLE II. Amino Acid Composition (mol%) of the Histones of *C. pagurus* (Cp) Compared to the Amino Acid Composition of Histones From Calf Thymus (Ct)

	H2A		H2B		H3		H4		H1				H1 Like	
	Ct	Cp	Ct	Cp	Ct	Cp	Ct	Cp	Ct	Cp (a)	Cp (d)	Cp (e)	Cp (b)	Cp (c)
Lys	10.2	10.1	14.1	13.8	9.7	9.0	11.4	8.5	26.8	18.3	16.1	20.3	13.9	7.0
His	3.1	1.8	2.3	2.3	1.4	1.5	2.2	2.3	0.0	1.1	0.6	0.5	0.8	1.6
Arg	9.4	8.6	6.9	6.4	13.0	12.8	12.8	12.2	1.8	4.3	2.1	4.7	2.4	2.9
Asp	6.2	7.5	5.0	6.9	4.2	4.3	5.2	6.0	2.5	5.8	6.2	5.9	8.4	11.3
Thr	3.9	3.0	6.4	5.2	6.8	6.3	6.3	6.4	5.6	10.3	6.0	7.5	7.6	8.3
Ser	3.4	5.1	10.4	8.4	3.6	3.7	2.2	3.2	5.6	6.7	4.8	6.1	6.0	7.1
Glu	9.8	9.3	8.7	8.5	11.6	11.8	6.9	7.5	3.7	4.6	8.2	1.6	14.1	13.0
Pro	4.1	4.7	4.9	3.3	4.6	4.8	1.5	2.3	9.2	9.4	10.6	10.6	9.1	8.4
Gly	10.8	11.1	5.4	5.5	5.4	6.2	14.9	14.7	7.2	5.3	5.0	5.1	5.0	4.9
Ala	12.9	9.7	10.8	11.5	13.3	12.0	7.7	7.4	24.3	9.5	21.2	19.3	9.9	4.5
Cys	0.0	0.0	0.0	0.0	1.0	0.3	0.0	0.0	0.0	0	0.0	0	0.0	0.0
Val	6.3	6.8	7.5	6.4	4.4	5.3	8.2	7.7	5.4	9.2	8.2	8.4	11.2	10.3
Met	0.0	1.5	1.5	2.2	1.1	1.8	1.0	1.5	0.0	0.2	1.0	0	0.9	2.4
Ile	3.9	5.1	5.1	6.4	5.3	5.3	5.7	5.7	1.5	4.8	3.1	3.5	4.6	6.1
Leu	12.4	10.8	4.9	6.0	9.1	9.0	8.2	7.9	4.5	6.8	4.5	4.2	4.3	6.8
Tyr	2.2	2.9	4.0	4.5	2.2	2.7	3.8	4.4	0.9	0.9	1.5	1.1	1.0	1.9
Phe	0.9	1.8	1.6	2.5	3.3	3.3	2.1	2.4	0.9	2.7	0.9	1.2	0.8	1.7

H1 and H1 related proteins a, d, e and b, c (respectively) correspond to protiens a–e of Figure 5.

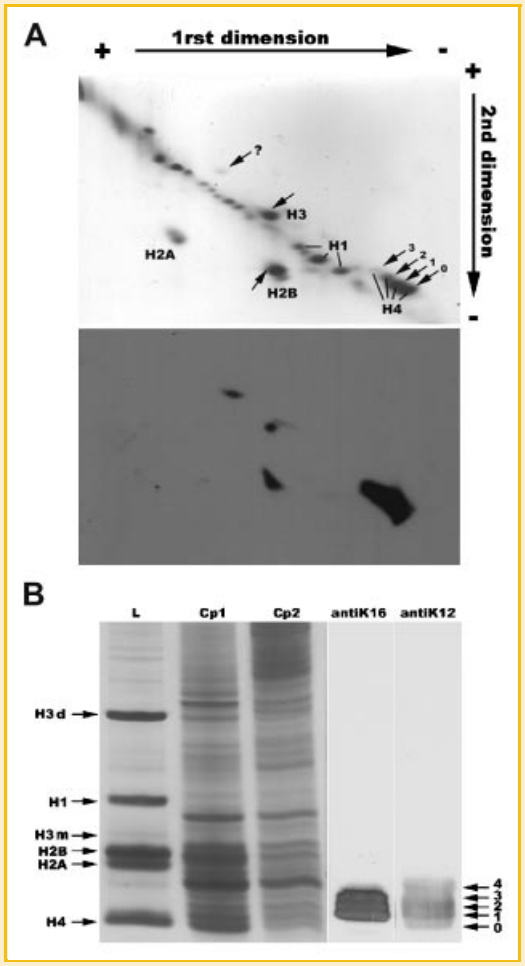

Fig. 6. Acetylation of histones from *Cancer* sperm. A: Two-dimensional electrophoresis [a variation of the method used in Dimitrov and Wolffe, 1997, described in Kurtz et al., 2007] of proteins extracted from sperm of *C. pagurus* with 0.4 N HCl (top) and Western blotted with anti-acetyl lysine antibody (bottom). In the 2D gel, the histones have been identified, along with their acetylated forms. B: Mono-, di-, and triacetylated isoforms of histone H4 are demonstrated by Western blotting using antibodies specific for acetylated residues on the amino-terminal tail of H4 (anti-H4-acK12 and anti-H4-acK16). The proteins were resolved AU-PAGE. L, sperm histones of lamprey; Cp 1 and 2, histones of *C. pagurus*: Cp1 corresponds to the immunoblot for anti-H4-acK16 and Cp2 corresponds to the immunoblot for anti-H4-acK12; 0, 1, 2, 3, 4: non, mono, di, tri, and tetra acetylated forms of histone H4.

lab to identify acetylated forms of spermatic histones [Kurtz et al., 2007]. Then, the histones were analyzed by Western blot with an anti-acetyl lysine antibody, which detects any protein possessing acetylated lysine residues. The results of these experiments (Fig. 6A) show that histone H4 has a high level of acetylation in mature sperm, while histones H2B and H3, along with an unidentified protein, appear to have low levels of acetylation. Histone H4 acetylation (mono-, di-, and triacetylated forms) is unquestionably proven using antibodies recognizing specific acetylated lysine residues on the amino terminal tail of histone H4 (anti-H4-acK12 and anti-H4-acK16, Fig. 6B).

Proportion of histones/DNA. To complete the analysis of *Cancer* sperm chromatin, we estimated the histones/DNA (w/w) ratio as well as the ratios of basic protein/DNA from other sperm types; we describe the method used for this estimation in Materials and Methods Section. In these experiments, we quantified the relation of histones/DNA (w/w) in sperm from two species of the genus *Cancer* (*C. pagurus* and *C. magister*), and the basic protein/DNA ratio in sperm nuclei of the following three control species: (a) *H. tubulosa* whose sperm contain histones; (b) *P. aspera*, whose sperm contain specific proteins of protamine-like type (PL); and (c) *M. brandaris*, whose sperm contain protamine. The obtained ratios are compared in Table III. It is interesting to observe that while all three control species (*Ht*, *Pa*, *Mb*) have a basic protein/DNA ratio of approximately 1/1 (w/w) (despite all having different types of basic proteins), the amount of histone in sperm of two *Cancer* species is only 0.5 and 0.6 per unit weight of DNA.

DISCUSSION

A DECONDENSED CHROMATIN STRUCTURE IN THE SPERM OF *Cancer* IN THE PRESENCE OF A LOW HISTONE/DNA RATIO

In contrast to what is observed in sperm nuclei of most organisms studied to date, the nucleocytoplasm of the decapod crab *Cancer* exhibits a decondensed organization [Langreth, 1969; Tudge et al., 1994]. This is despite the association of DNA with histones, forming nucleosome structures with a sort linker DNA (see Table I), as has been described in the previous section.

In spite the fact that sperm histones of *Cancer* possess a composition practically indistinguishable from somatic histones of calf thymus, our results provide an explanation for the decondensed chromatin state. First is the low proportion of histones per DNA content. While other sperm nuclei have a proportion of DNA-interacting proteins/DNA of approximately 1/1 (w/w) sperm chromatin of *Cancer* has a much lower value of approximately 0.5–0.6 histone/DNA (w/w). The histone–DNA association results in a nucleosome organization with a short linker DNA size (25 bp) which is much shorter than the average linker found in somatic cells [van Holde, 1988] and in other sperm cells (Table I). This suggests that in *Cancer* sperm, chromatin is organized into short oligonucleosomal arrays, consisting of closely packed nucleosomes interspersed in the midst of non-nucleosomally organized DNA, accounting for

TABLE III. Comparison of the Ratio of DNA Interacting Proteins/DNA (w/w) in Sperm With Different Sperm Nuclear Basic Proteins Types

	Cancer pagurus	*Cancer magister*	*Holothuria tubulosa* (H)	*Patella aspera* (P-L)	*Murex brandaris* (P)
Nuclear proteins/DNA (w/w)	0.5	0.6	1.0	1.0	1.2

Sperm of both *Cancer* species contains chromatin with histones, sperm of *H. tubulosa* contains histones, sperm of *P. aspera* contains protamine-like proteins, and sperm of *M. brandaris* contains protamines.

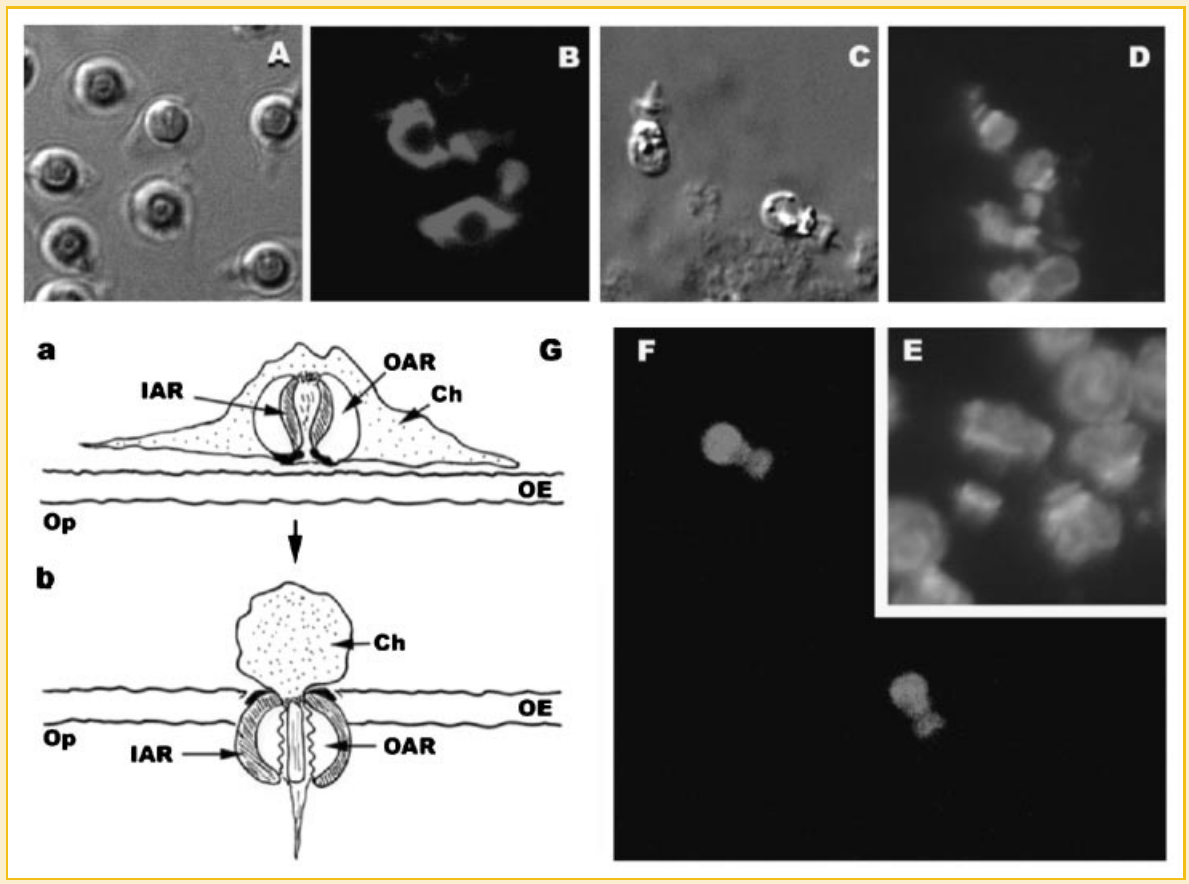

Fig. 7. Change in sperm shape during the activation of *C. pagurus* spermatozoa. Light micrograph of *C. pagurus* sperm cells (A), showing Draq 5 epifluorescence of the cell nuclei in the peripheral position (B). When the sperm cells are activated (spontaneously in buffer A, or by adding calcium) the acrosome everts, and the nucleus changes shape (light micrograph in C, and epifluorescent staining by Topro-3 in F, or by Hoechst reagent in D, E). Image G shows a simplified schematic representation of the activation process of the *C. pagurus* spermatozoon (a) before activation; (b) after activation. IAR.– inner acrosomal region; OAR, outer acrosomal region; Ch, chromatin; OE, oocyte envelope; Op, oocyte cytoplasm.

approximately 50% of the genomic DNA (see Fig. 4). This type of chromatin organization seems evident as well, considering that in a canonical nucleosome organization of 200 bp with one histone H1 per nucleosome the (w/w) histone/DNA ratio is equal to one. The histone-free regions appear to be stabilized mainly by metal ions.

The HPLC analysis of a 5% PCA extract from *Cancer* sperm (Fig. 5B,D) allowed us to identify three fractions (a, d, and e in Fig. 5B,D) that have an amino acid composition rich in alanine, lysine, and proline which is characteristic of members of the histone H1 family (Table II). However, the results presented here suggest that these histones are present in the oligonucleosomal chromatin fraction in sub-stoichiometric amounts (i.e., less or no more than 1 H1 per nucleosome). Also, though we have properly used proteolytic inhibitors, the possibility of degradation during chromatin isolation cannot be discarded, since the bands corresponding to fractions a, d, and e from Figure 5D and Table II are very faint in Figure 3A. The seemingly low proportion of H1 in this sperm chromatin is not surprising, considering the result that the nucleosomal repeat length in *Cancer* (170 bp) is very close to that of the chromatosome (160–168 bp) [Simpson, 1978; van Holde, 1988].

However, even in the presence of histone H1 it is very unlikely that the oligonucleosomal structures just described adopt a higher order folded structure such as that exhibited by the chromatin of organisms that contain histones with a canonical histone/DNA ratio in their mature sperm [Williams and Langmore, 1991]. Furthermore, preliminary results from our lab suggest that the linker histone fraction associated with *Cancer* chromatin is phosphorylated (results not shown). Linker histone phosphorylation has also been shown to lead to unfolding of the chromatin fiber [Horn et al., 2002].

In addition, the histones of *Cancer* sperm chromatin exhibit a high extent of acetylation of H4 (together with a minor acetylation of H2B and H3) (see Fig. 6). Histone acetylation leads to unfolding of nucleosome arrays [Garcia-Ramirez et al., 1995] and its role in spermiogenesis in other species has been linked to chromatin relaxation prior to displacement of histones by protamines (or other SNBP) [Oliva and Dixon, 1991; Kurtz et al., 2007]. However, acetylation of histones in organisms that retain histones in their spermatozoa has never been described before. Thus, the highly unfolded chromatin characteristic of *Cancer* sperm is unique and is not shared by any other species with condensed sperm nuclei.

BIOLOGICAL IMPLICATIONS OF A DECONDENSED CHROMATIN ORGANIZATION FOR FERTILIZATION

All the structural characteristics of *Cancer* chromatin described above suggest a decondensed chromatin organization, which is most likely quite flexible. This ''most likely'' has important biological implications that reflect the very peculiar mode of fertilization of this organism. In contrast to most other spermatozoa, the sperm of crustaceans lack flagella. Therefore, the mechanism of oocyte penetration by the sperm appears to involve an eversion of the acrosomal granule that takes place when it contacts the oocyte coating [Hinsch, 1971; Brown, 1976; Goudeau, 1982; Medina and Rodríguez, 1992]. This process results in a modification of the relative position of cellular components, as the sperm nucleus is pulled forward passing through the relatively narrow internal canal created for its own passage through the acrosome (see Fig. 7). In spite of many aspects of this process which still are not fully understood (or have not been studied), the sperm nucleus must undergo important changes in shape during fertilization. A condensed nucleus in which chromatin is highly compact would lack the flexibility necessary to allow it to pass through the narrow acrosomal canal. This type of sperm chromatin organization would be at the opposite end of the highly rigid organization resulting from the interaction of DNA with cysteine-rich SNBPs [see, e.g., Giménez-Bonafé et al., 2002].

CONSIDERATIONS OF PREVIOUS STUDIES

One final aspect to consider has to do with the discrepancy between the results reported by us and previous work which reported a complete lack of histones or any other SNBPs in the sperm of brachyuran crabs, including *Cancer*. Most of the earlier works were carried out using histochemical staining methods and electrophoretic analysis. The relatively low histone/DNA ratio and the occurrence of histone acetylation possibly hampered the resolution of the staining histochemical analysis. Also, the electrophoretic analysis of the SNBPs of this organism is not trivial. SDS electrophoresis of total *Cancer* sperm protein extracts (not shown) reveals a highly complex electrophoretic pattern in which histones are almost undetectable in the background of a multiplicity of proteins likely arising from the large acrosomal granule. Only when basic proteins from sperm were extracted with 0.4 N HCl (Fig. 2 SDS) or when chromatin was highly purified (Fig. 3) could histones be clearly visualized. Furthermore, we have noticed that release of mature sperm from spermatophores during purification elicits a proteolytic activity that results in important histone degradation unless careful manipulation and adequate use of protease inhibitors are used.

The main conclusion of the present study is that *Cancer* sperm nuclei which had previously been considered deplete of SNBPs, do indeed contain nucleosomes, albeit with an unusually unfolded and unique chromatin organization. This should be considered as a chromatin adaptation to the peculiar fertilization process of the gametes involved.

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