Two Highly Specialized Histone H1 Proteins Are the Major Chromosomal Proteins of the Sperm of the Sea Anemone *Urticina (Tealia) crassicornis*[†]

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ABSTRACT: The sperm nuclear basic proteins (SNBPs) from a sea anemone (*Urticina crassicornis*) have been isolated and characterized for the first time. They consist of two sperm-specific members of the histone H1 family with M_r 22 700 and 24 600. They amount to about 60−70% of the total chromosomal sperm proteins. Their amino acid composition and the primary structure of their trypsin-resistant core indicate a strong relation to histone H5 from the nucleated erythrocytes of birds and amphibians as well as to other highly sperm-specific H1-like (PL-I) proteins from phylogenetically distant groups. The major presence of histone H1-like protein in the sperm of an organism belonging to such a low phylogenetic group provides experimental support to the hypothesis that SNBPs may all have evolved from a primitive histone precursor.

From the point of view of their nuclear protein composition, spermatozoa can be grouped in three major categories. At one end of this classification would be those sperm cells whose nuclear composition consists mainly of histones either of somatic-like or of sperm-specific origin. At the other end would be the sperm cells that contain highly arginine-rich (≥50% arginine) protamines as a result of the partial or complete replacement/displacement of histones during spermiogenesis (Oliva & Dixon, 1991). In the middle of this classification are the sperm cells which consist of an intermediate kind of protein, that shares structural and compositional similarities to both histones and protamines. These latter kinds of proteins exhibit a significant extent of structural variability with an amino acid composition rich in both lysine and arginine (arginine + lysine ≥ 45%). This protein composition is equivalent to the *Mytilus* type defined by Bloch (1969). We have recently shown (Ausio et al., 1987; Carlos et al., 1993a,b) that despite their structural variability, some of these intermediate proteins are related in one way or another to histones of the histone H1 family. A PL-I (PL = protamine-like)[1] protein with compositional and structural features resembling those of histone H1 as well as protamines has been identified in several bivalve molluscs (Ausio, 1986, 1992). The fact that sperm-specific intermediate proteins (and possibly protamines) are related to protein members of the heterogeneous histone H1 family (Ausio, 1995) is not surprising. Highly specific histone H1 variants with a compositional arginine increase (for instance, histone H5 and H1^{s}) are also found in other terminally differentiating cell systems such as in the nucleated erythrocytes of fish, amphibians, reptiles, and birds (Miki & Neelin, 1977; Rutledge et al., 1984).

In fact, H1 could be considered a nuclear marker for cell differentiation that modulates gene expression in somatic cells either by folding chromatin into its higher order structure or by rearrangement of its attachment sites to the nuclear matrix (Flickinger, 1994). Histone H1 mediated gene silencing in the sperm can be accomplished in several different ways. The equilibrium unfolded → folded chromatin could be displaced toward the folded state either by increasing the stoichiometric ratio of histone H1 versus core histones and/or by highly sperm-specific H1 histones such as those found in the sperm of echinoderms (Zalenskaya & Zalensky, 1980; Zalenskaya et al., 1980; Poccia, 1991). Finally, in the case of PL-I protein, the same goal could be achieved by replacing the core histones and tightly compacting chromatin in a similar way as in the case of protamines. An example of these last two situations can be found in the primitive sperm of the sea anemone *Urticina crassicornis* as it is described next.

MATERIALS AND METHODS

Living Organisms. The specimens of *Urticina crassicornis* (O. F. Müller, 1776) were collected at Ten Mile Point (Victoria, British Columbia) and were kept in seawater tanks at the University of Victoria.

Sperm Collection and Extraction of the Nuclear Proteins. Small incisions were made in the outer body wall with the help of a scalpel, and the sperm that was spontaneously flowing out of the body was collected with a Pasteur pipet and processed for nuclear protein extraction as described elsewhere (Ausio, 1986).

Extraction and Isolation of Somatic Histone H1. Somatic histone H1.S_m was extracted from tissue of the distal part of the secondary septum (in the region of the testicular cysts) of immature individuals. The tissue was quickly homogeneized in 0.15 M NaCl/10 mM Tris-HCl, pH 7.5, containing 0.5% Triton X-100, 20 μg/mL TLCK, 100 μg/mL TPCK, and 0.3 mM PMSF. After homogeneization, the sample was centrifuged for 10 min at 10000*g* at 4 °C. This step was repeated once more, and the pellet was extracted with 0.4 N HCl. Fractionation of somatic histones was carried out by

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[1] Abbreviations: PL, protamine-like protein; SNBP, sperm nuclear basic protein; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; PCA, perchloric acid; TFA, trifluoroacetic acid; MALDi, matrix-assisted laser desorption ionization.

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HPLC (see next) as described elsewhere (Saperas et al., 1994).

Gel Electrophoresis. Urea (2.5 M)−acetic acid (5%) polyacrylamide gel electrophoresis (PAGE) was carried out as described elsewhere (Jutglar et al., 1990). SDS−PAGE were prepared according to Laemmli (1970).

Two-dimension electrophoresis (Acetic acid−urea−triton, first dimension, and SDS, second dimension) was carried out as described in Ausio (1988).

HPLC Fractionation. HPLC was carried out on 5 μm (25 × 0.46 cm) Vydac C_4 or C_{18} columns, with 0.1% trifluoroacetic acid as eluant with varying acetonitrile gradients.

Preparation and Purification of the Trypsin-Resistant Core of Histone H1. The trypsin-resistant cores of the two histone H1 variants from the sperm of *U. crassicornis* were prepared as described in Ausio et al. (1987) and were purified by reverse-phase HPLC on a VYDAC C_{18} column as described above.

Amino Acid Analysis. Amino acid analysis was carried out as described elsewhere (Jutglar et al., 1990).

Protein Sequencing. Protein sequencing was performed as described in Jutglar et al. (1990).

Light Microscopy. Nomarsky and bright-field micrographs were taken in a Leitz aristoplan light microscope.

Electron Microscopy. Sperm suspensions collected as described above were pelleted on a bench-top centrifuge for 5 min at room temperature. Immediately thereafter the pellet was resuspended in a small volume of filter-sterilized seawater, and the cell suspension was then fixed for 2 h at room temperature. Primary fixative contained 1 part of a 5% glutaraldehyde solution in 0.27 M NaCl, and 1 part of Millonig's phosphate buffer (Millonig, 1961). The osmolarity of the fixative was 970 mOsm. Specimens were postfixed in 1% osmium tetroxide for 1 h at 4 °C. The postfixative solution was comprised of 1 part of a 4% solution of osmium tetroxide (Stevens Metallurgical Co.), 1 part of Millonig's phosphate buffer, and 2 parts of a 0.75 M solution of NaCl. The osmolarity of the postfixative was 960 mOsm.

Fixatives were removed by a series of washings with a buffer rinse (comprising 1 part of Millonig's phosphate buffer and 1 part of a 0.6 M solution of NaCl), and the specimens were then dehydrated in a graded series of ethanol (up to 100%) with a total dehydration time of 60 min. Specimens were embedded in EPON (TAAB 812 resin, Marivac Ltd.) and blocks hardened at 60 °C overnight. Sections for transmission electron microscopy (with a thickness of 60−90 nm) were cut in a Reichter Ultramicrotome, mounted on clean copper grids, and stained with 2% aqueous uranyl acetate, pH 4.5 and 0.2% neutralized lead citrate, following conventional methods. Thin sections were observed in a Hitachi 700 transmission electron microscope at an accelerating voltage of 70 kV.

Mass Spectrometry. Mass spectrometry analysis was carried out by matrix-assisted laser desorption ionization using a Kratos Kompact MALDI III V2.0.1 (Kratos Analytical) as described elsewhere (Ausio et al., 1993).

Protein−DNA Binding Studies. The binding of different H1 histone fractions to DNA (calf thymus from Sigma) was analyzed as a function of the ionic strength at a protein/DNA ratio equivalent to a nominal full neutralization of the DNA phosphates by the arginine and lysine side chains of the proteins ($r^{+/-} = 1$) (Ausio & van Holde, 1987). This ratio was established according to $r^{+/-} = c_P 331/c_D M_{PZ} = 1$, where c_P and c_D are the concentration of protein (P) and DNA (D) in the complex and M_{PZ} is the equivalent mass of a positive charge in the protein. $M_{PZ} = M_r/Z^+$ where M_r = molecular mass of the protein and Z^+ = number of lysine and arginine residues. Equal volumes of DNA and protein at the appropriate concentrations to provide $r^{+/-} = 1$, were mixed at room temperature at each salt concentration. The buffer was 20 mM Tris-HCl, 1 mM EDTA, pH 7.5. The final DNA concentration of the mixture was in all cases ≈40 μg/mL. The sample was then centrifuged at 12000*g* for 10 min at 4 °C, and the A_{260} of the supernatant was measured and plotted against the NaCl concentration (see Figure 5A).

The concentration of DNA (c_D) was calculated using $A_{260}^{0.1\%} = 20$, and the protein concentrations were estimated using $A_{230}^{0.1\%} = 2.0$ as a first approximation. This latter value represents an average between the extinction coefficient of histone H5 ($A^{0.1\%} = 1.85$) (Camerini-Otero et al., 1976) and that of PL-I from *Spisula* ($A^{0.1\%} = 2.12$) (Ausio & van Holde, 1988). Histone concentrations were determined from electrophoretic analysis serial dilutions of the stock protein solution. The electrophoretic bands were scanned and calibrated against the same range of dilutions using histone H5 as a standard. In general, it was found that the estimate $A_{230}^{0.1\%} \approx 2$ remained fairly constant throughout all of the histone H1 related proteins used in this paper.

Binding of the Different Histone H1 Fractions to Nucleosome Core Particles. Unfractionated calf thymus histone H1 was obtained from Boehringer-Manheim. Histone H5 was purified from chicken erythrocytes as described previously (Garcia-Ramirez et al., 1990). Nucleosome core particles (consisting of 146 bp random sequence DNA) were obtained from chicken erythrocyte as described elsewhere (Ausio et al., 1989).

Nucleosome core particles at a starting $A_{260} \approx 0.5$ were titrated with increasing amounts of each of the different histone H1 fractions, and complex formation was measured by turbidimetry as described by Ali and Singh (1987). The histone H1 to nucleosome core particle ratio (*P/N*) (mol/mol) was determined from

$$P/N = \frac{M_D}{M_H} \times \frac{W_H}{W_D}$$

where M_D = molecular mass of 146 bp DNA, M_H = molecular mass of the histone H1 fraction, W_D = micrograms of DNA, and W_H = micrograms of histone. The M_H used for the different H1 fractions was 22 200 [unfractionated calf thymus H1 (De Lane, 1976)], 25 700 [*Spisula solidissima* (from mass spectroscopy unpublished results)], 20 850 [chicken erythrocyte H5, calculated from sequence (Briand et al., 1980)], and 24 600 and 22 800 for histone H1-I and H1-II fractions (*U. crassicornis* determined by mass spectroscopy, see Figure 2C).

RESULTS

In this paper, we have studied for the first time the chromosomal protein composition of the sperm of a sea anemone (*Urticina crassicornis*). Although we were not able to induce spawning, samples highly enriched in motile sperm could be obtained from small incisions made on the outer body wall. Figure 1A shows a light microscope analysis

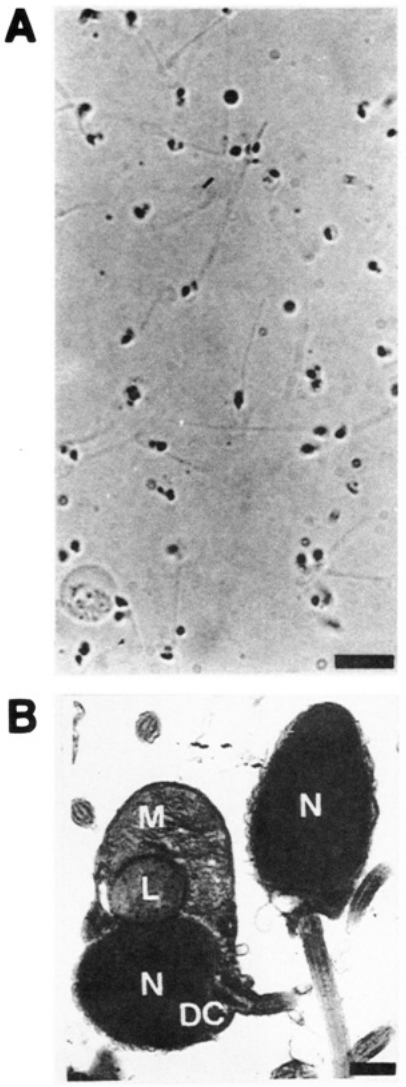

FIGURE 1: (A) Light microscope phase contrast micrograph of a sperm suspension of *Urticina crassicornis*. The bar is 5 μm. (B) Electron micrograph of a sample from the same sperm suspension showing details of the nuclei (N), the mitochondria (M), the distal centriole (DC), and the lipid inclusion (L). The bar is 0.5 μm.

which is representative of the average cell suspension sample used for the protein analysis. As can be seen, the sperm cells exhibit a variety of shapes (see also Figure 1B), all of them corresponding to a primitive sperm (Baccetti & Afzelius, 1976). Such morphological heterogeneity has also been detected in sperm samples expelled during spawning (Chia & Spaulding, 1972) and is the result of the presence of different extents of cytoplasm (Chia & Spaulding, 1972).

Nevertheless, more than 90% of the cells in our suspensions were of noncontaminating origin. Of these, more than 80% had all the structural features (see Figure 1B) that have been described in the mature sperm of other Cnidaria (Dewel & Clark; 1972, Clark, 1974) and, in most cases, exhibited nuclei with highly condensed chromatin.

Figure 2A,B shows the electrophoretic analysis of the sperm basic proteins present in the 0.4 N HCl nuclear extracts. As seen in Figure 2A (lane 1) and Figure 2B, two major proteins are present with electrophoretic mobility similar to that of somatic H1 histones (Figure 2A, lane 2). These proteins coexist with about a 30−40% histone core complement (Figure 2B). This amount was found to vary slightly within this range among the different sperm samples analyzed.

Like histone H1 proteins, the two major SNBP from *U. crassicornis* can be selectively extracted from the nucleus by dilute perchloric acid (5%). Figure 2C shows a MALDI analysis of one such perchloric acid extract. The two proteins with M_r 24 550 ± 70 and 22 730 ± 10 can be clearly distinguished in this spectrum. These two proteins were next purified by HPLC (see Figure 3), and their amino acid composition was determined (see Table 1).

Trypsin digestion of each of these proteins in the presence of 2 M NaCl (Ausio et al., 1987) led in both cases to trypsin-resistant peptides of molecular masses 7600 (for the protein fraction with lowest electrophoretic mobility) and 5800 Da (for the second protein fraction) as determined by SDS gel electrophoresis (see Figure 4A). The trypsin-resistant peptides were purified by reverse-phase HPLC, and the primary structure of these peptides was determined (see Figure 4B).

The amino acid sequence analysis of these peptides clearly revealed the H1 nature of the two major SNBP of *U. crassicornis*, which we labeled as H1-I and H1-II in order of their increasing electrophoretic mobility in urea−acetic acid gels. H1-II exhibits a reduced solubility in SDS and in most instances does not migrate under these electrophoretic conditions or it simply stacks on the interface between the stacking and separating gel (see Figure 2B).

A BLAST search from a combined protein−DNA database indicated that the trypsin-resistant peptide of histone H1-I was closely related to the globular core of histone H5 from the nucleated erythrocytes of different birds (Yaguchi et al., 1979; Krieg et al., 1982, 1983; Doenecke & Toenjes, 1984; Ramakrishnan et al., 1993). In all these cases, the extent of sequence identity was 51−53% whereas the extent of similarity taking into account the conserved amino acid substitution was 74%. Other related proteins included histone H1-delta from the sea urchin *Strongylocentrotus purpuratus* (Lieber et al., 1988) (51% identity, 66% similarity); and mouse $H1^0$ (Alonso et al., 1988) (49% identity, 69% similarity), and histone H1-I from *Caenorhabditis elegans* (Vanfleteren et al., 1988) (50% identity, 64% similarity) among others with lower matching scores.

A similar analysis carried out on the trypsin-resistant peptide of H1-II gave the highest matching score for the histone H1-ike protein EM5 from the sperm of the razor clam *Ensis minor* (Giancotti et al., 1992) (48% identity over a 35 amino acid matching stretch). This was followed by histone H5B and H5A from nucleated amphibian erythrocytes (Rutledge et al., 1988) (34−32% identity and 69% similarity in both cases) and histone H1-delta from the sea urchin *Strongylocentrotus purpuratus* (Lieber et al., 1988) (32% identity, 65% similarity).

Lower matching scores were found for histone H5 from chicken erythrocytes and for mouse human $H1^0$, similar to what was found for H1-I but with lower matching scores (30% identity, 68% similarity).

Once it was clear that H1-I and H1-II were both members of the histone H1 family, we decided to analyze their binding characteristics with respect both to naked DNA and to nucleosome core particles. The analysis was carried out in comparison with unfractionated histone H1 from calf thymus, histone H5 from chicken erythrocytes, and a histone H1-related PL-I protein from the sperm of the clam *S. solidissima* (Ausio et al., 1987; Ausio, 1992). It should be noted here

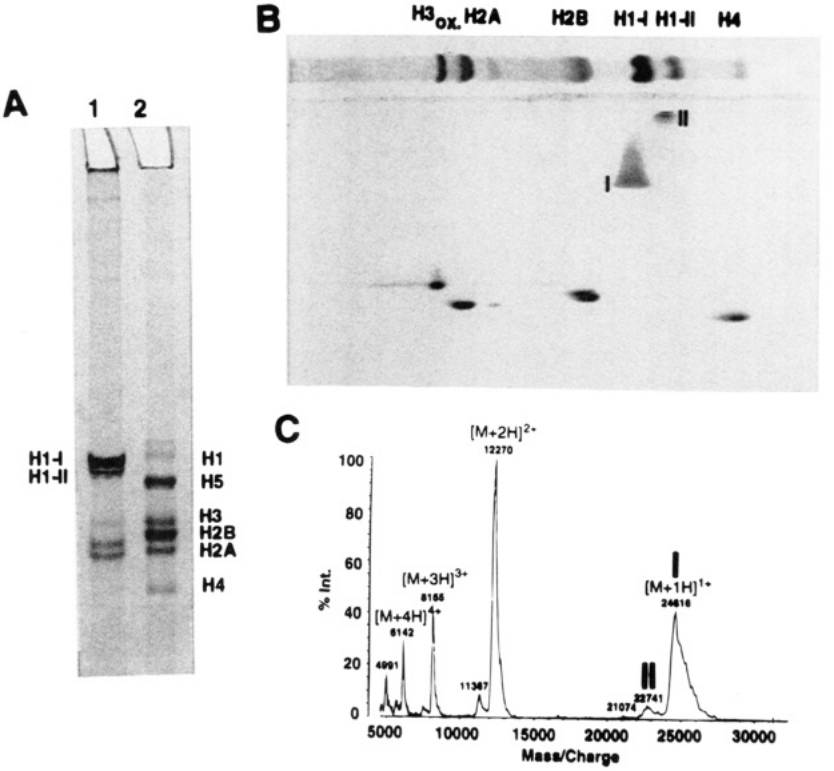

FIGURE 2: (A) Urea (2.5 M)−acetic acid (5%) PAGE of (1) SNBP from the sperm of *Urticina crassicornis* in comparison to (2) chicken erythrocyte histones. (B) Two-dimensional gel electrophoresis of the nuclear proteins from the sperm of *U. crassicornis*. The first dimension was on an acetic acid (5%)−urea (8 M)−Triton (0.5%) (AUT) PAGE prepared according to Bonner et al. (1980), and the second dimension was performed on an SDS−PAGE prepared according to Laemmli (1970). (C) Matrix-assisted laser desorption ionization spectrum of a 5% PCA soluble fraction from *U. crassicornis* SNBP.

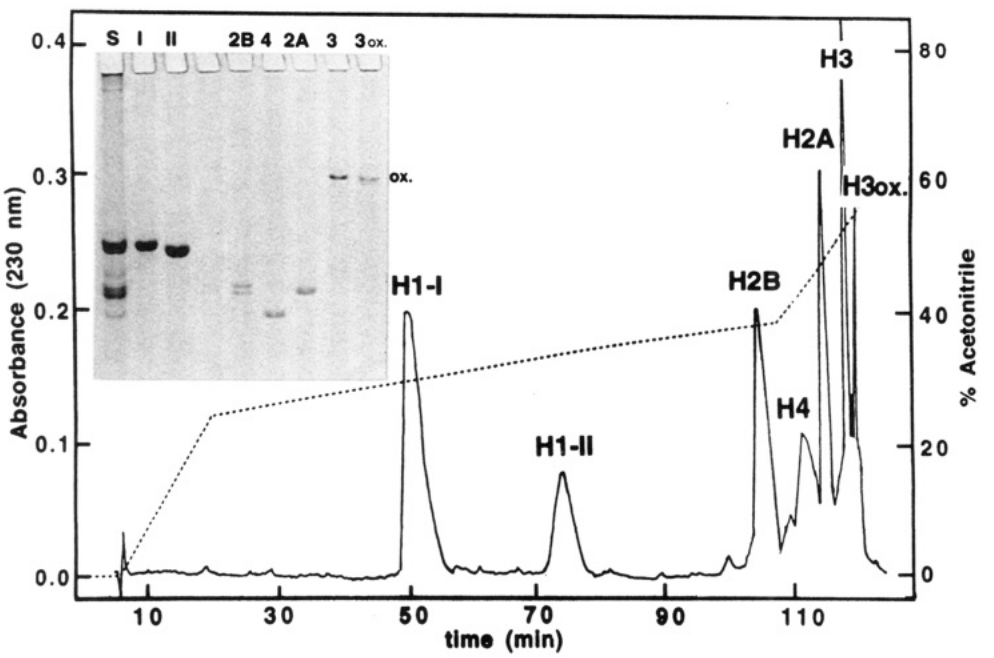

FIGURE 3: Reverse-phase HPLC fractionation of a 0.4 N HCl nuclear extract from the sperm of *U. crassicornis*. The fractionation was carried out on a (0.46 × 25 cm) Vydac C_4 column at a flow rate of 1 mL/min eluted with an acetonitrile gradient in the presence of 0.1% TFA. The inset shows a urea (2.5 M)−acetic acid (5%) PAGE of the different fractions eluted from the column.

that this characterization was merely for the purpose of comparison and not designed to gain any further insight into chromatin structure. Thus, the use of DNA size (146 bp) defined nucleosome core particles can be justified by the fact that linker histones bind to these particles with a similar binding affinity and stoichiometry as they do to higher nucleosomal structures (Ali & Singh, 1987). The results of this analysis are shown in Figure 5A−C. The results from DNA binding as a function of the ionic strength show DNA binding affinity increasing in the order PL-I > H1-II > H1-I ≥ H5 > H1. Whereas H1-I exhibits a similar behavior to histone H5, H1-II has a stronger binding affinity intermediate between histone H5 and the highly sperm-specific PL-I from the molluscs. From a comparison of the amino acid analysis

Table 1: Amino Acid Composition (mol %) of H1.1, H1.2, and H1.S_m in Comparison to Histones H1, H5, and Protein PL-I

	Urticina crassicornis			*Rhizostoma pulmo*		chicken erythrocyte	calf thymus	*Spisula solidissima*
amino acid	H1-I	H1-II	H1.S_m[d]	$H1_s$[a]	$H1_f$[a]	H5[b]	H1[b]	PL-I[c]
Lys	27.3	14.5	32.4	26.1	25.7	23.6	26.8	24.8
His	0.9	1.1	1.6	0.8	1.9	1.9		
Arg	10.3	12.4	3.1	2.8	2.3	12.4	1.8	23.1
Asx	1.5	4.5	5.5	4.7	5.3	1.7	2.5	0.6
Thr	4.0	3.6	3.0	3.2	3.2	3.2	5.6	4.3
Ser	9.3	12.0	6.6	10.6	6.9	11.9	5.6	21.7
Glx	4.6	6.7	5.3	4.1	4.6	4.3	3.7	0.6
Pro	7.5	2.9	11.8	8.3	12.3	4.7	9.2	2.4
Gly	6.9	13.2	4.5	4.9	7.0	5.3	7.2	3.0
Ala	16.9	12.7	15.4	19.9	17.3	16.3	24.3	14.2
half-Cys								
Val	2.1	5.2	3.5	4.6	5.2	4.2	5.4	2.3
Met	0.9	0.6		nd[e]	nd	0.4		0.4
Ile	2.0	1.8	2.4	2.1	2.6	3.2	1.5	0.5
Leu	3.2	5.5	2.6	5.7	3.0	4.7	4.5	1.7
Tyr	0.9	1.0	1.2	1.5	1.7	1.2	0.9	0.3
Phe	0.9	2.3	1.3	0.5	0.9	0.6	0.9	0.3
Trp								0.3

[a] Rozov et al. (1986). [b] From Mayes and Johns (1982). [c] From Ausio and Subirana (1982). [d] S_m = somatic. [e] nd = not determined.

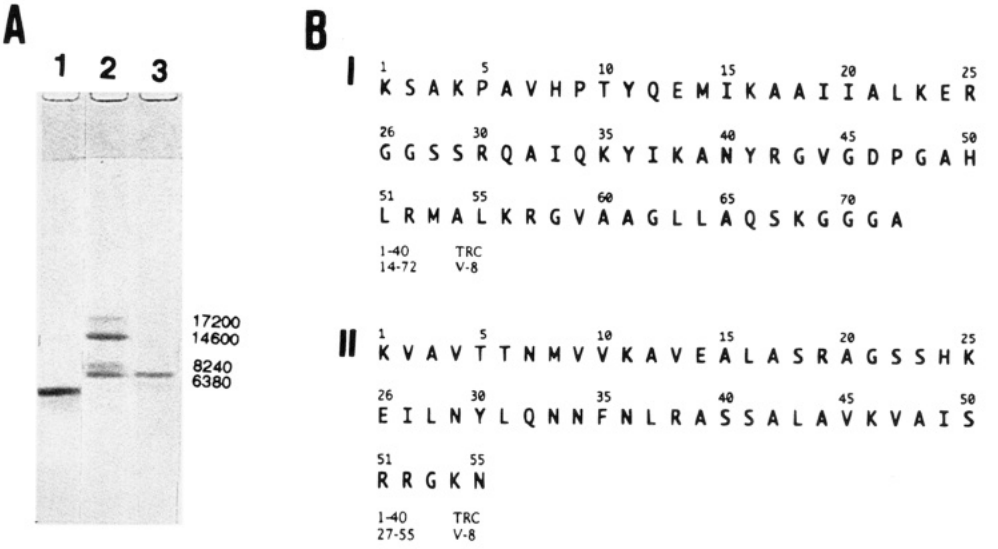

FIGURE 4: (A) Electrophoretic analysis (SDS−PAGE) of the trypsin-resistant peptides obtained from (1) fraction H1-II and (3) fraction H1-I from the sperm of *U. crassicornis*. Lane 2 is a low molecular weight protein marker consisting of horse myoglobin peptides (Pharmacia Inc.). (B) Primary structure of the trypsin-resistant peptides from (I) histone H1-I and (II) histone H1-II.

data shown in Table 1 (see also Z^+, Figure 5C), it is obvious that this difference in binding affinity cannot merely be explained by the extent of electrostatic interaction between these proteins and DNA. Factors other than the overall positive charge of these proteins such as the charge distribution and the secondary and/or tertiary structure of these molecules (Libertini et al., 1988) must play a very important role.

Because of the H1 nature of proteins H1-I and H1-II, we also decided to study their ability to bind to nucleosome core particles. The results of this analysis are shown in Figure 5B,C. For this analysis, we used the same turbidimetric approach that was used by Ali and Singh (1987) to study the binding of linker histones (histones of the H1 histone family) to nucleosome core particles.

Our results indicate that under approximate physiological conditions (100 mM NaCl, Figure 5B) all the proteins analyzed behave in a similar way with a midpoint transition corresponding to the binding of 1.3 ± 0.3 histone molecules per nucleosome under these conditions (see Figure 5C). These results are in contrast with the binding observed at very low ionic strength (in the absence of NaCl, 0 mM) where the extent of molecules bound shows a strong inverse dependence with the increase in the overall positive charge of the protein (see Figure 5B,C, 0 mM). Under these low-salt conditions, these proteins most likely lose the tripartite structural organization which is characteristic of the protein members of the histone H1 family. In this kind of organization, the trypsin-resistant core which has a globular conformation is flanked by two N- and C-terminal regions which lack any tertiary structure organization. The presence of this globular core has been held responsible for the specificity of H1 binding to nucleosome (Allan et al., 1980) such as in the case of our 100 mM conditions. At low salt, the proteins most likely adopt a rather extended conformation which allows for their electrostatic interaction with the negatively charged phosphate backbone in the nucleosome.

In addition to all these binding studies, we also made an attempt to characterize the chromatin organization of the nucleus of mature sperm as visualized from thin sections in the electron microscope (see Figure 6). Under these condi-

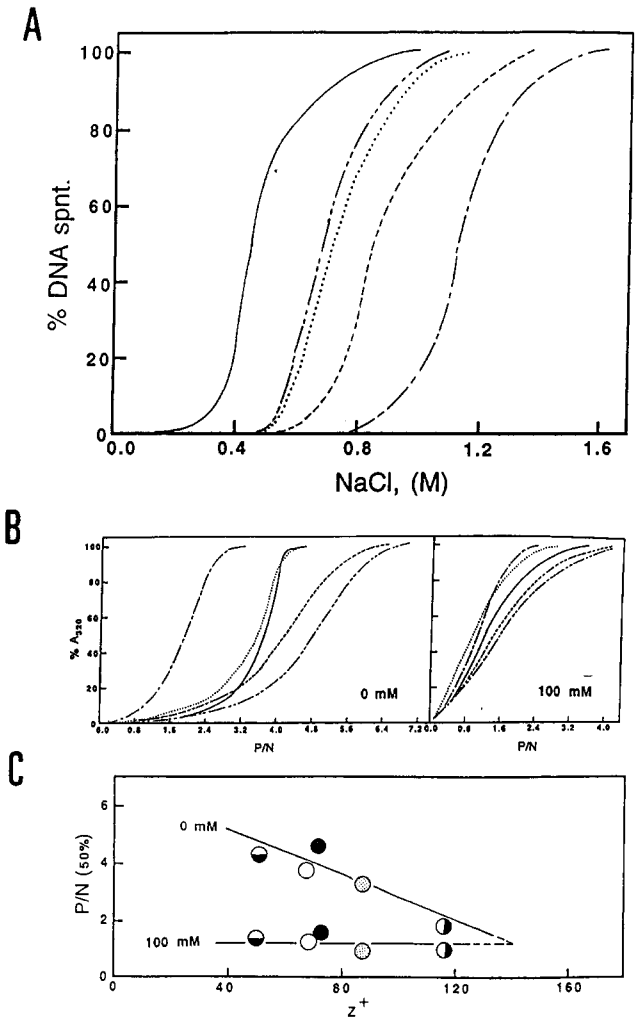

FIGURE 5: (A) [NaCl] dependence of the binding of different histone H1-related proteins to DNA. (B) Nucleosome−histone H1 complex formation (in the absence, 0 mM, or in the presence of NaCl, 100 mM) as determined by the turbidity (absorbance at 320 nm, A_{320}) dependence on *P/N* (protein/nucleosome ratio) defined as described under Materials and Methods. For the purpose of comparison of the complexing ability of the different H1-related proteins, the apparent absorbances (A_{320}) for each histone were normalized with respect to the highest A_{320} corresponding to the saturation plateau for each histone. The percentile (% A_{320}) thus represents the relative ratio of the A_{320} at a given *P/N* relative to the maximum A_{320} achieved at saturation. The buffer conditions were 10 mM Tris-HCl, 0.5 mM EDTA, pH 7.5, with (100 mM) or without (0 mM) NaCl. (C) Variation of the *P/N* corresponding to the middle point of the transitions shown in panel B, as a function of the absolute number of positively charged residues (Z^+) present in the histone H1-related proteins analyzed in panels A and B. (—, ○) histone H1 from calf thymus; (-- -- —, ●) histone H5 from chicken erythrocyte; (— - —, ◑) PL-I from *S. solidissima*; (•••, speckled white circles) histone H1-I from the sperm of *U. crassicornis*; and (---, ◒) histone H1-II from the sperm of *U. crassicornis*.

tions, it is possible to ascertain the presence of granular structures of about 40−50 Å in diameter (Figure 6D) homogeneously distributed throughout the nucleus.

DISCUSSION

The biochemical analysis of the sperm nucleus of the sea anemone *U. crassicornis* reveals the presence of two major sperm nuclear basic proteins. These proteins represent about 60−70% of the chromosomal proteins and coexist with a full complement of core histones. Whether or not this remaining core histone complement is due to the presence of contaminating immature sperm cells in the cell suspensions used remains yet to be determined. However, as pointed out by Chia and Spaulding (1972), the sperm of this anemone are unique in that a significant amount of immature cells are present even in discharged samples as ascertained by morphological criteria. In fact, as we have stated previously, more than 90% of the cells in our samples were of noncontaminating origin, of which more than 80% had the structural features of mature sperm. Therefore, the possibility that the core histone component comes wholly from contaminant immature cells cannot be dismissed.

The two major proteins (H1-I and H1-II) present in the chromatin of the sperm of *U. crassicornis* exhibit a distinct lysine-rich amino acid composition (see Table 1) which allows us to classify them in a generic way as members of the histone H1 family. Their relation to histone H1 is further emphasized by the presence of a trypsin-resistant core (see Figure 4A) and by their solubility in 5% perchloric acid. However, their composition is different from that of most somatic H1 histones and different from the somatic H1 extracted from somatic tissues of the same organism (see Table 1). Their amino acid composition is also different from that of the H1 histones previously isolated from the sperm of the jellyfish *Rhizostoma pulmo* (Rozov et al., 1986). From comparison of the amino acid analysis compositions shown in Table 1, these two proteins appear to be more closely related to erythrocyte histone H5. Like histone H5, they have a higher arginine and serine composition and they also contain methionine which is absent in most somatic histone H1 fractions.

The relatedness of these proteins to the histone H1 family and more specifically to histone H5 from the nucleated erythrocytes of chicken and amphibians can be firmly established from the primary structure of their trypsin-resistant cores (Figure 4B and see also Figure 6D for a comparison of the amino acid sequences) and from the extent of their sequence similarity described under Results. This is a strikingly similar situation to what had been previously described for PL-I, a highly specialized chromosomal protein which replaces most of the core histones during spermiogenesis of several bivalve molluscs (Ausio et al., 1987; Ausio, 1992). In this context, the fact that erythropoiesis and spermiogenesis are both terminally differentiated systems might have played an important role in the course of the evolutionary selection of these H5-like proteins.

The presence of this kind of protein in Cnidaria supports the notion that a histone H5 (highly modified member of the histone H1 family) ancestor may have already existed during the Precambrian in the ancestors of all modern animals (Rozov et al., 1986). This in turn provides support to the idea that protamines (the small arginine-rich proteins found in the sperm of some vertebrates) as well as all the sperm proteins may have evolved from histone precursors (Subirana et al., 1973), the latter involving the existence of an early highly specialized molecule of the histone H1 family (Ausio, 1995).

From the structural point of view, the primary structure similarity between H1-I and H1-II from *U. crassicornis* to histone H1 and histone H5 (see Figure 7D) points to a similar extent of secondary structure similarity. As can be seen in Figure 7, all the conserved residues of the consensus histone

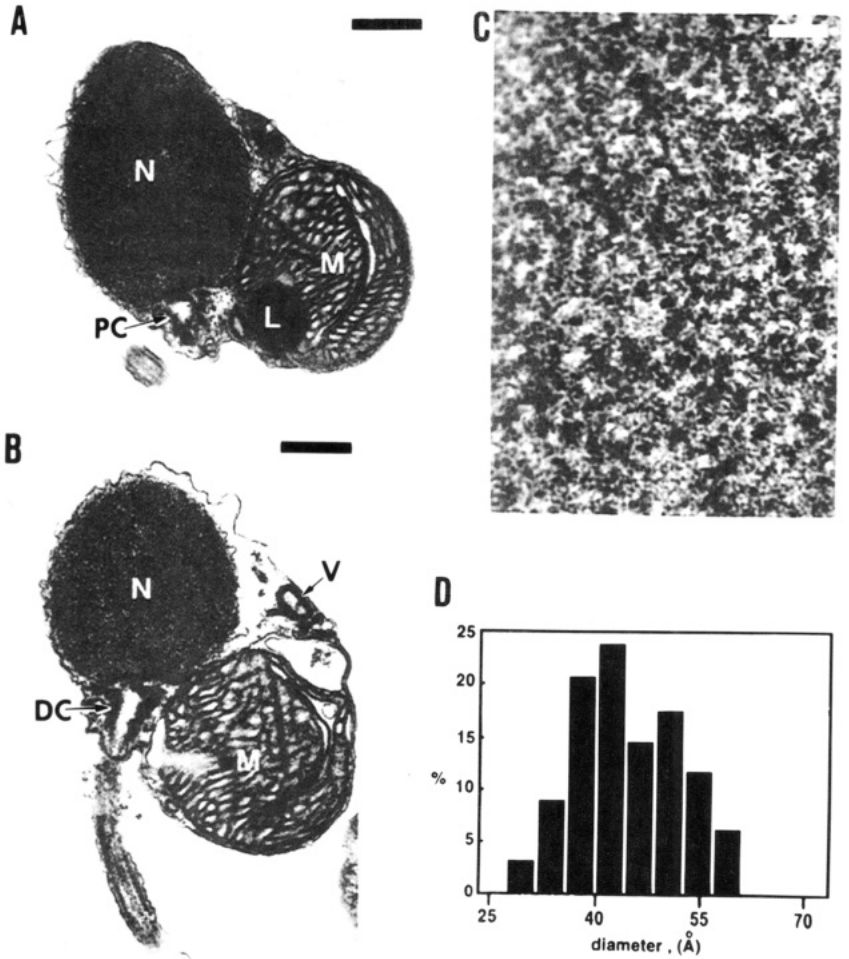

FIGURE 6: (A, B) Electron micrographs of thin sections of sperm from *U. crassicornis* to show the granular appearance of the nuclear contents. N, nucleus; M, mitochondria; L, lipid inclusion; PC, proximal centriole; DC, distal centriole; V, vesicles. The bar in (A) and (B) corresponds to 500 nm. (C) A higher magnification detail of the nucleus to show the granular nucleoprotein organization. The bar is 50 nm. (D) Size distribution of the globular structures seen in (C).

H1 sequence (Crane-Robinson & Ptitsyn, 1989) are also conserved in these molecules, particularly the regions corresponding to the three α-helices which are characteristic of the trypsin-resistant core of members of the histone H1 family (Crane-Robinson & Ptitsyn, 1989). The amphipathic nature of these helices is extremely conserved (see Figure 7A,B,C) which is indicative of a similarly conserved tertiary structure. In fact, under near-physiological conditions, all these molecules can bind to nucleosomal DNA in a similar way (see Figure 5B,C) which is determined by the tertiary organization of the trypsin-resistant globular domain (Ali & Singh, 1987; Segers et al., 1991).

Despite all this, it is still not known to what extent histones H1-I and H1-II bind to nucleosomes in the sperm cells of *U. crassicornis*. As discussed earlier, the answer to this question mainly depends on whether or not the 40% of core histones that are found associated to them in the sperm are the result of contamination by immature cells, especially because, as it has been pointed out earlier, obtaining completely mature sperm in this organism is very difficult. Alternatively, the presence of histones in the sperm could be the result of an incomplete replacement of the histones of the stem cells as it occurs with the PL proteins of bivalve molluscs which coexist with 20−25% of histones in the mature sperm (Ausio, 1986; Ausio & van Holde, 1987). The more primitive nature of H1-I and H1-II and their lower DNA binding affinity (Figure 5A) compared to that of PL could then account for the lower extent of core histone replacement.

In an attempt to discern between these two possibilities, we analyzed the chromatin organization of the highly condensed nucleus of mature sperm using electron microscopy (see Figure 6). Careful analysis of previous electron microscopy work carried out on spermiogenesis of closely related species of anemone indicates the presence of 230−250 Å fibers in the early stages of spermiogenesis (Dewel & Clark, 1972) similarly to what has also been described in bivalve molluscs (Casas et al., 1993). As spermiogenesis proceeds, these fibers apparently coalesce, and in the final stages, the highly condensed nucleus shows a smaller granular organization (Dewel & Clark, 1972) similar to that shown in Figure 6. The relation of the 40−50 Å structures seen in Figure 6D to these fibers is unknown (although these latter ones could possibly represent some kind of internal organization of the former), yet they preclude any major kind of nucleosomal organization such as that observed in the cells of somatic tissues.

Nevertheless, it may be that both of the possibilities considered above are present to a certain extent. It is possible that the sperm of *U. crassicornis* consists of a heterogeneous mixture of different cells consisting of varying amounts of core histones.

The cells representing the "mature sperm" could still retain some of the core histones which have been replaced by

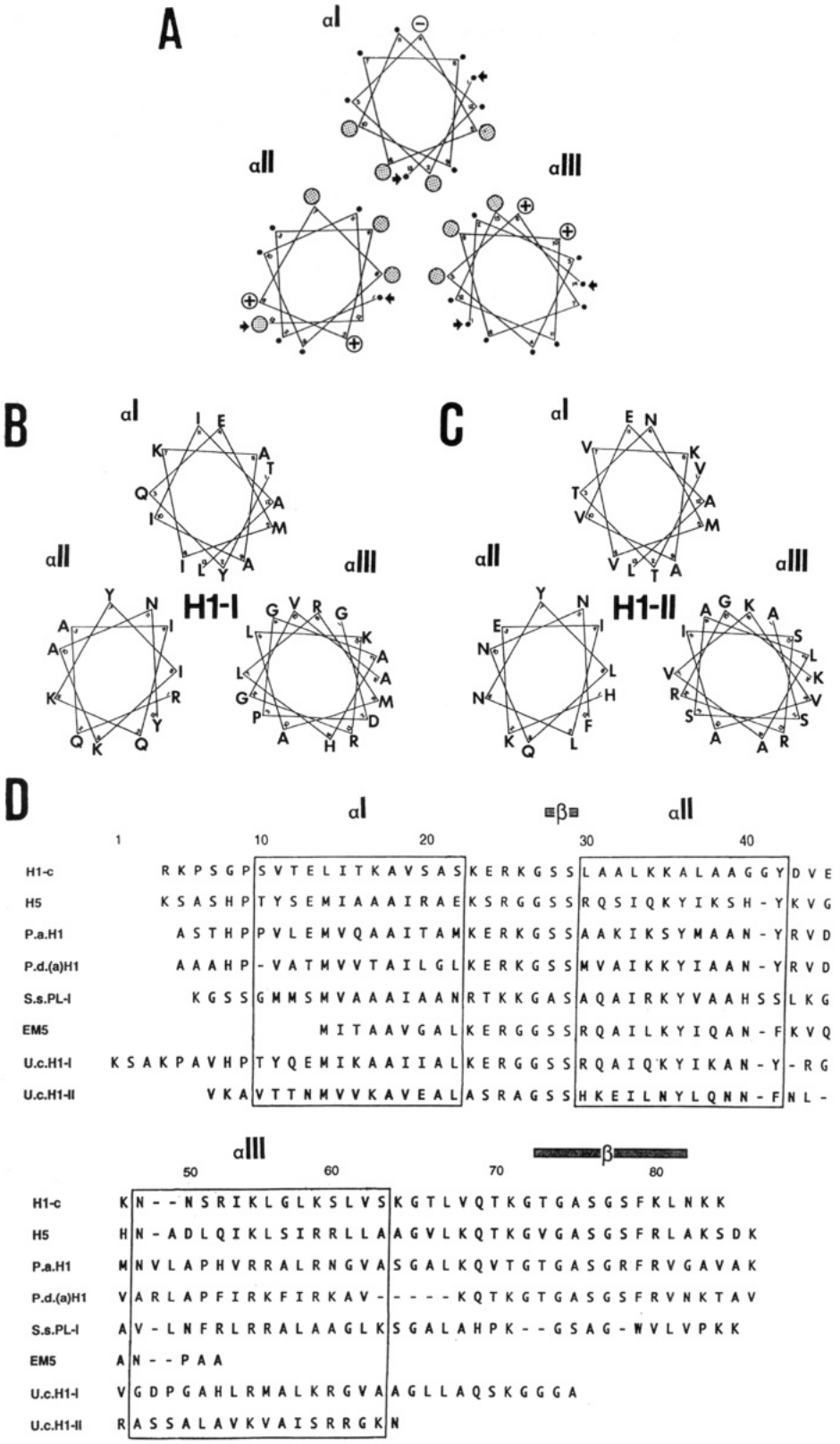

FIGURE 7: (A) Helical wheel representation of the three α-helices from the globular core of consensus histone H1 (see section D of this figure). The helical stretches shown correspond to those experimentally determined by X-ray crystallography of the histone H5 core (Ramakrishnan et al., 1993). The conserved polar and hydrophobic (the latter are represented by shaded circles) residues of the histone H1 core are also shown (Crane-Robinson & Ptitsyn, 1989). (B, C) Helical wheel depiction of the putative α-helix domains of the trypsin-resistant core of (B) histone H1-I and (C) histone H1-II from *Urticina crassicornis* (see Figure 4). (D) Sequence comparison of the trypsin-resistant peptides from different proteins of the histone H1 family. H1c, histone H1 consensus sequence (Wells & Brown, 1991); H5, chicken erythrocyte histone H5 (Briand et al., 1980); P.a.H1, *Parechinus angulosus* (sea urchin sperm) histone H1 (Strickland et al., 1980); P.d.(a)H1, *Platynereis dumerilii* (annelid sperm) histone H1 (fraction a) (Kmiecik et al., 1985); S.s.PL-I, *Spisula solidissima* (surf clam sperm) PL-I (Ausio et al., 1987); EM5, *Ensis minor* (razor clam sperm) histone H1-like protein EM5 (Giancotti et al., 1992); U.c.H1-I and U.c.H1-II, histones H1-I and H1-II from the sperm of the sea anemone *Urticina crassicornis*. The numbers start with the first amino acid of the trypsin-resistant peptide of histone H1-I from *U. crassicornis*. The boxes correspond to the α-helices, and the shaded regions represent the β motifs based on the crystallographic analysis of histone H5.

specific histones (H1-I and H1-II) to a lesser extent of what has been described during the replacement of histones by PL-I in bivalve molluscs (Ausio, 1995). All this may simply reflect the primitive evolutionary status of the sperm of these organisms (Baccetti & Afzelius, 1976) as witnessed by the presence of the two sperm-specific histones H1-I and H1-II which have been described in this paper.

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