

Chromatin Organization During Spermiogenesis in *Octopus vulgaris*. II: DNA-Interacting Proteins

PEPITA GIMÉNEZ-BONAFÉ,¹ FINA MARTÍNEZ SOLER,¹ CARLOS BUESA,² PIERRE-ERIC SAUTIÈRE,^{3*} JUAN AUSIÓ,⁴ MOSTAFA KOUACH,³ HAROLD E. KASINSKY,^{1,5} AND MANEL CHIVA¹

¹Departament Ciències Fisiològiques II, Facultat Medicina, Universitat Barcelona, Campus Bellvitge, Barcelona, Spain

²Unitat de Bioquímica, Facultat Farmàcia, Universitat Barcelona, Barcelona, Spain

³URA, CNRS 1309, Chimie des Biomolécules, Institut Pasteur, Lille, France

⁴Department of Biochemistry and Microbiology, University of Victoria, Victoria, B.C., Canada

⁵Department of Zoology, University of British Columbia, Vancouver, B.C., Canada

ABSTRACT In this article we study the proteins responsible for chromatin condensation during spermiogenesis in the cephalopod *Octopus vulgaris*. The DNA of ripe sperm nuclei in this species is condensed by a set of five different proteins. Four of these proteins are protamines. The main protamine (Po2), a protein of 44 amino acid residues, is extraordinarily simple (composed of only three different amino acid types: arginine (R), serine (S), and glycine (G)). It is a basic molecule consisting of 79.5 mol% arginine residues. The rest of the protamines (Po3, Po4, Po5) are smaller molecules (33, 28, and 30 amino acid residues, respectively) that are homologous among themselves and probably with the main Po2 protamine. The ripe sperm nucleus of *O. vulgaris* also contains a small quantity of a molecule (Po1) that is similar to Po2 protamine. This protein could represent a Po2 protamine-precursor in a very advanced step of its processing. We discuss the characteristics of these proteins, as well as the relation between the complexity of chromatin condensation and the transitions of nuclear proteins during spermiogenesis in *O. vulgaris*. *Mol. Reprod. Dev.* 68: 232–239, 2004.

© 2004 Wiley-Liss, Inc.

Key Words: cephalopoda; *Octopus*; spermiogenesis; nucleus; chromatin; protamines

INTRODUCTION

In the accompanying article (Ribes et al., 2003) we have presented morphological observations on the phenomenon of spermiogenic chromatin condensation in *O. vulgaris*, as well as evidence that the condensing chromatin is tightly attached to spermatidyl and sperm nuclear poles (basal and apical). The condensation of chromatin and its attachment to nuclear poles are two separate processes which lead to different levels of DNA organization in the nucleus of the sperm cell.

In the present article we study the basic proteins that interact with DNA in *O. vulgaris* sperm nuclei. In this species these are protamines, proteins that are more basic than histones, and specialized in the packaging of DNA during spermiogenesis. Among the proteins,

protamines show possibly the highest rate of evolutionary change (Oliva, 1995; Lewis et al., 2003). Consequently their primary structure exhibits an extraordinary variability in different animal species. This variability in protamine structure is, at least partially, in keeping with the variability of morphological patterns observed in spermiogenic chromatin condensation (Càceres et al., 2002).

Among cephalopod mollusks, protamines have been studied in two species of decapods (squid and cuttlefish) and in the octopod *Eledone cirrhosa*. In the case of decapods, protamines are synthesized as precursor molecules (Wouters-Tyrou et al., 1991, 1995). These molecules possess a non-basic amino-terminal end and a carboxy-terminal part that is very rich in arginine (77–79 mol%). During the complete process of spermiogenic chromatin condensation, two major transitions in the nuclear protein complement occur. First, the entire protamine precursor molecule replaces histones in the nucleus; subsequently, the precursor loses its amino-terminal part (probably by means of an enzymatic deletion). Thus, the carboxy-terminal zone (that is the mature protamine) is the only DNA associated protein found in ripe sperm nuclei of decapods. These changes in DNA interacting proteins promote a progressive condensation of spermiogenic chromatin, starting with 40 nm diameter fibers which fuse posteriorly among themselves to produce a highly condensed sperm chromatin (Maxwell, 1975). The studies of decapod

Octopus vulgaris protamine sequences. Accession no.: SWISS-PROT P83214 (Po1 & Po2); P83215 (Po3); P83216 (Po4); and P83217 (Po5).

Grant sponsor: Ministerio de Ciencia y Tecnología and FEDER, Spain (to M.C.); Grant number: BMC-2002-04081-C02-02; Grant sponsor: Canada (to J.A.); Grant number: OGP-0046399-02; Grant sponsor: NSERC Canada (to H.E.K.); Grant number: 585401.

*Correspondence to: Pierre-Eric Sautière, Laboratoire de Neuroimmunité des Annelides, CNRS 8017 SN3, Bd Paul Langevin, Université des Sciences et Technologies de Lille 59 655 Villeneuve d'Ascq, France. E-mail: Pierre-Eric.Sautiere@univ-lille1.fr

Received 6 October 2003; Accepted 20 November 2003

Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/mrd.20068

sperm chromatin by X-ray diffraction indicate that these protamines are extremely efficient in DNA packaging. Just like the chromatin fibers, the strands of DNA in the ripe sperm nucleus are disposed in parallel, following the direction of the acrosome-centriole axis. They display a hexagonal arrangement with a distance of 20–22 Å between axes of the neighboring DNA strands (Suau and Subirana, 1977).

In the case of the octopod *E. cirrhosa*, the histones of early spermatidyl nuclei are replaced by a single protamine which is not synthesized in the cell as a precursor molecule. Taking into account its primary structure (Giménez-Bonafé et al., 2002), the protamine of *E. cirrhosa* does not seem to be homologous to decapod protamines. *E. cirrhosa* protamine, moderately rich in lysine and arginine residues, stands out by being the most cysteine-rich protamine known to date. The establishment of interprotamine disulphide bonds by cysteines plays an important role during the last steps of chromatin condensation (Giménez-Bonafé et al., 2002). The complete process of spermiogenic chromatin condensation is much more complex in *E. cirrhosa* than in decapods. In *E. cirrhosa*, the genetic material begins to condense in supercoiled fibers that grow by coalescence. Later, the major fibers fuse, forming a three-dimensional network of chromatin which undergoes additional complex development. This leads to the final state of highly condensed chromatin in the spiralized nucleus of the mature sperm (for details, see Giménez-Bonafé et al., 2002).

Taking into account these antecedents, and given the complexity in the pattern of *O. vulgaris* spermiogenic chromatin condensation (Ribes et al., 2003), we have undertaken the study of DNA-interacting proteins in *O. vulgaris* sperm nuclei. The only previous information that is available on this subject comes from the recent studies of Mennella et al. (2002) describing a gonadal-specific, H1-like histone present in spermatidyl (but not in ripe sperm) nuclei, and two estimations of the amino acid composition of *Octopus* protamines (Subirana et al., 1973; Giménez-Bonafé et al., 1999).

MATERIALS AND METHODS

Living Organisms

Male specimens of the cephalopods *O. vulgaris* (octopoda) and *Loligo vulgaris* (decapoda) were collected on the Mediterranean coast of Spain and moved alive to the laboratory in cold seawater.

Nuclear Purification and Protein Extraction

Gonadal tissues were homogenized in ice-cold buffer (0.25 M sucrose, 10 mM MgCl₂, 3 mM CaCl₂, 10 mM Tris-HCl pH 7.0, 0.1% triton X-100) containing 50 mM benzamidine chloride as protease inhibitor, and centrifuged at 5,000g for 5 min. Crude nuclear pellets were homogenized again and sedimented three-times in the same buffer. Sperm cells obtained from spermatophores were submitted to the same procedure with minor modifications (Giménez-Bonafé et al., 1999).

Basic proteins were extracted from purified nuclei with diluted 0.4N HCl, precipitated with six volumes of cold acetone, washed three-times with cold acetone, and dried in vacuum.

Purification of Proteins

Proteins were usually purified by reverse phase HPLC using a C4, 300-A Delta-Pack column (25 × 0.46 cm²). The elution of proteins was carried out by applying a linear gradient of acetonitrile (0–60%) in 0.05% trifluoroacetic acid (Càceres et al., 1999). In one experiment, proteins were first separated by ion-exchange columns in CM-52 cellulose (Chiva et al., 1987, 1988). The peak containing both Po4 and Po5 protamines was then submitted to additional reverse phase HPLC purification.

Analytical Methodology

To determine the molecular mass of proteins, ion spray mass spectrometry (ISMS) was performed on a VG Bio-Q quadrupole mass spectrometer with a mass range of 3,000 Da. The electrospray ion source operated at atmospheric pressure. Calibration was done using charged ions from poly(ethyleneglycol)800, which were introduced separately. The electrospray was emitted at 3,000 V and the extraction cone voltage was adjusted to 150 V. The samples were dissolved in 1% acetic acid in water and afterwards, an equivalent volume of methanol was added. The sample concentration used was between 20 and 30 pmol/μl.

For sequence analysis, proteins were submitted to automated Edman degradation on an Applied Biosystems 470 gas-phase sequencer using the ORPTH program, slightly modified to ensure a better extraction of the 2-anilino-5-thiazolinone derivative of arginine (O3C Arg program). Identification of residues was performed as described in Sautière et al. (1988).

For analysis of the carboxy-terminal sequence of protamine Po2, the protein was digested by exopeptidase (PE Biosystems, CPP enzyme kit). After each cycle of digestion the remaining protein was analyzed by mass spectrometry and the amino acid excised was identified by the loss of molecular mass of protein.

Amino acid analyses were performed on a Beckman 6300 amino acid analyzer after hydrolysis in vacuum in 6 M HCl at 110°C for 24 hr.

Electrophoretic analyses of proteins were carried out in 15% (w/v) polyacrylamide-acetic acid-urea gels (PAGE) according to the procedure of Hurley (1977).

RESULTS

General Changes in Nuclear Protein Content During Spermiogenesis of *O. vulgaris*

Before purifying the basic proteins that interact with DNA in *O. vulgaris* mature sperm nuclei, we have studied the general change of nuclear protein content in gonads of this species in comparison with that of the decapod *Loligo vulgaris*. For this purpose we have purified the whole set of nuclei from very immature

gonads, mature gonads, and from ripe sperm spermatozoon contained in spermatophores both in *O. vulgaris* and *L. vulgaris*. Afterwards, the nuclear basic proteins were extracted by solubilization in 0.4N HCl and analyzed by electrophoresis on a 15% polyacrylamide minigel.

In agreement with the results obtained on the squids *Ilex argentinus* (Kadura and Khrapunov, 1988) and *Loligo pealeii* (Wouters-Tyrou et al., 1995), histones constitute the major basic protein component associated with DNA in immature gonads of *L. vulgaris* (Fig. 1, lane a). Figure 1 also shows that during gonadal development nuclear histones tend to disappear, being substituted by a protein with intermediate electrophoretic mobility (lane b). In ripe sperm nuclei only a protein with high electrophoretic mobility (protamine) is found (lane c). Wouters-Tyrou et al. (1995) demonstrated that the protein with intermediate electrophoretic mobility (lane b) is the precursor form of ripe protamine (lane c).

When we study the nuclear protein transitions during the development of gonads of *O. vulgaris*, we find a qualitatively similar situation, but one that is quantitatively more complex (Fig. 1, lanes d–f). In this case the histones of nuclei from unripe gonads (lane d) are replaced by a collection of proteins of intermediate electrophoretic mobilities (lane e), and these in turn by four to five protamines in mature sperm nuclei (Po1–Po5) (lane f). Protein Po2 is the main *O. vulgaris* protamine and Po4/Po5 always appear in a significant quantity. On the other hand, Po1 and Po3 are minor proteins appearing in slightly variable amounts depending on the individual analyzed (compare lanes f and g). The results presented in Figure 1 suggest that during the spermiogenesis of *O. vulgaris*, nuclear histones are replaced by a group of protamine precursors which will become ripe protamines in the sperm nucleus (see

below). In comparison with decapods, the complexity in the change of nuclear proteins of spermiogenesis in *O. vulgaris* correlates well with the complexity of the pattern of chromatin condensation presented in the accompanying article (Ribes et al., 2003).

It is important to point out that nuclear protein transitions during *O. vulgaris* spermiogenesis can be still much more complex because gonadal-specific forms of histones probably appear at the initial phases of spermiogenesis. In the electrophoretic analysis presented in Figure 1, we have chosen the precise concentration of acrylamide and urea to separate histones from intermediate proteins and the latter from protamines. In particular, the testis-specific histone H1-like protein described by Mennella et al. (2002) cannot be resolved electrophoretically in a 15% polyacrylamide minigel.

Characterization of Po1–Po5 Protamines

In this section we study the primary structure of Po1–Po5 protamines. With this objective, protamines have been purified both from gonadal and sperm nuclei by ion-exchange and reverse-phase HPLC chromatographic methods (see “Materials and Methods”). Figure 2 shows a representative elution profile when

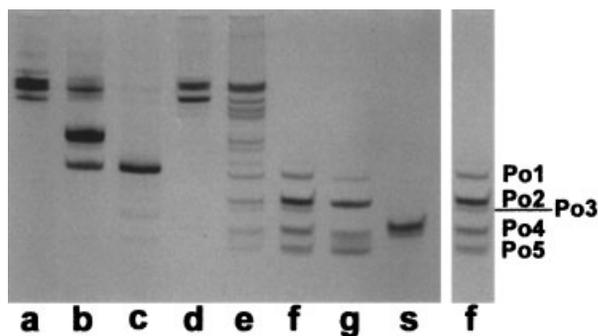


Fig. 1. Change in nuclear proteins during spermiogenesis of the octopod *O. vulgaris* and the decapod *L. vulgaris*. Electrophoretic pattern of nuclear proteins contained in nuclei from immature gonads (lanes a and d), mature gonads (lanes b and e), and spermatozoa (lanes c and f) belonging to the decapod *L. vulgaris* (a, b, c) and to the octopod *O. vulgaris* (d, e, f). Lanes f and g compare two extractions from ripe sperm nuclei belonging to two different individuals of *O. vulgaris*. Direction of electrophoresis is from the top (+) to the bottom (-). The mobility of proteins is compared with that of a standard of salmine protamine that has been loaded in lane s.

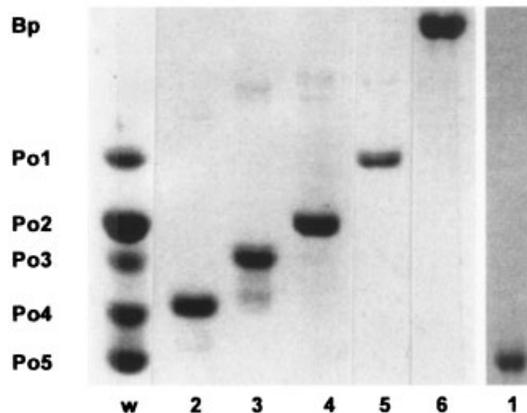
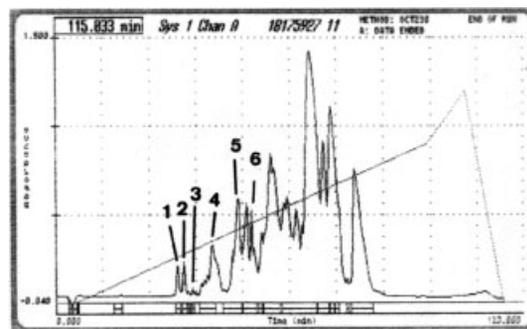


Fig. 2. Purification of *O. vulgaris* protamines. Reverse-phase HPLC elution profile (top) of the whole nuclear proteins (w) obtained from ripe gonads and electrophoretic controls (bottom) of the purified protamines. The first six peaks (top) contain protamines (Po2–Po5), a protamine precursor (Po1), and a larger gonadal nuclear protein (Bp), probably a large protamine precursor. The peaks eluted in the last part of the acetonitrile gradient contain a mixture of intermediate proteins and histones (not shown). Electrophoresis was performed in a high resolution gel of 15% (w/v) polyacrylamide-acetic acid-urea.

TABLE 1. Amino Acid Composition (mol%) of Protamines

	Bp	Po1		Po2 ^a		Po3	Po4		Po5		P _L	P _S
		a ^b	b	a	b		a	b	a	b		
lys	2.5	0.5	—	—	—	—	—	—	—	—	—	—
his	1.5	—	—	—	—	1.8	2.9	3.6	3.6	3.3	—	—
Arg	45.2	68.6	69.6	78.7	79.5	62.1	63.3	64.3	65.6	66.7	78.0	77.1
Asx ^c	2.8	—	—	—	—	—	—	—	—	—	—	—
Thr	—	—	—	—	—	—	—	—	—	—	—	—
Ser	11.5	4.8	5.4	2.0	2.3	10.3	3.5	3.6	2.8	3.3	12.1	11.5
Glx ^c	4.1	—	—	—	—	—	—	—	—	—	—	—
Pro	—	—	—	—	—	—	—	—	—	—	2.4	2.2
Gly	15.5	19.0	17.9	19.2	18.2	18.4	23.2	21.4	21.3	20.0	—	—
Ala	2.7	—	—	—	—	2.8	6.9	7.1	3.5	3.3	—	—
Cys	—	—	—	—	—	1.9	—	—	—	—	—	—
Val	4.2	1.7	1.8	—	—	—	—	—	—	—	—	—
Met	—	—	—	—	—	—	—	—	—	—	—	—
Ile	3.1	1.9	1.8	—	—	—	—	—	—	—	—	—
Leu	4.7	1.6	1.8	—	—	tr	—	—	—	—	—	—
Tyr	1.2	1.7	1.8	—	—	2.3	tr	—	3.0	3.3	7.3	9.1
Phe	1.0	—	—	—	—	—	—	—	—	—	—	—

Bp, Po1–Po5. Proteins purified from gonadal nuclei of *O. vulgaris*. For Po1, Po2, Po4, and Po5 the amino acid content determined (a) is shown together with the composition calculated from the sequence (b). In the right two columns of the table the composition of protamines from the decapods *Loligo pealii* (P_L) and *Sepia officinalis* (P_S) are shown (Wouters-Tyrou et al., 1991, 1995, respectively).

^aMost abundant protamine in *O. vulgaris*.

^bEstimated under the assumption that Po1 is a precursor of Po2.

^casx = asp + asn; glx = glu + gln; tr = trace.

HPLC columns are loaded with the extracts of proteins obtained from nuclei of the ripe gonad, as well as an electrophoretic control. The protein designated as Bp in Figure 2 (lane 6) only appears in gonadal, but not in sperm nuclei. Once purified, each one of the Po1–Po5 protamines has been analyzed by ISMS, amino acid composition, and amino acid sequence. The amino acid composition of these proteins is given in Table 1.

Protamine Po2. The complete characterization of the main protamine Po2 is presented in Figures 3 and 6 and in Tables 1 and 2. Analyzed by ISMS this protein displays a mass of 6,028 Da when purified from ripe sperm nuclei (Fig. 3A). When protamine Po2 has been obtained from nuclei of gonads, a small fraction of the molecule appears with a mass excess of 80 Da, indicating that part of the population of the protein is found mono-

phosphorylated in spermatidyl nuclei (arrow in Fig. 3B). The complete sequence of Po2 was determined by automated Edman degradation of the intact protein (Fig. 6) and by enzymatic digestion with exopeptidase (PE Biosystems, CPP enzyme kit) followed by ISMS mass analysis of released products (Fig. 3C). The automated Edman degradation allowed us to determine the amino-terminal part from residues 1 to 40. On the other hand, the exopeptidase carboxy-terminal sequencing identified the last five amino acid residues (all of them arginines). Table 2 shows that the molecular mass observed by ISMS and that estimated from sequence are in complete agreement. Amino acid composition (Table 1) is also coincident with the sequence results obtained. Thus, protamine Po2 is a very simple and specialized protein containing 44 amino acid residues which belong to only three amino acid types: arginine (R), glycine (G), and serine (S). This molecule contains a single serine residue in position 9, which is in agreement with the presence of a mono-phosphorylated form of Po2 in spermatids. The internal organization of *O. vulgaris* protamine molecules in comparison with the known cephalopod protamines will be detailed in the "Discussion."

Protamines Po3, Po4, and Po5. Figures 4 and 6 and Tables 1 and 2 present the characterization of protamines Po3, Po4, and Po5. By ISMS analysis, these proteins exhibit masses of 4,389, 3,536, and 3,941 Da, respectively (Fig. 4). As occurs in the case of Po2, they appear to be partially mono-phosphorylated when purified from spermatidyl nuclei. The complete amino acid sequence was obtained for Po4 and Po5 by automated Edman degradation (Fig. 6). Both of them are

TABLE 2. Molecular Mass (Da) of Protamines

	A		B
	Gonad	Sperm	
Po1	7,427	7,428	7,429.0 ^a
Po2	6,027	6,028	6,029.6
Po3	4,389	4,389 ^b	—
Po4	3,536	3,537 ^b	3,538.0
Po5	3,941	3,941	3,942.5

A. Molecular mass (Da) determined by ISMS when proteins are obtained from gonads or sperm. **B.** Theoretical molecular mass calculated from the sequences of the protamines obtained. Phosphorylated forms do not appear in the table.

^aEstimated under the assumption that this molecule is a precursor of Po2 protamine.

^bNot shown in figures.

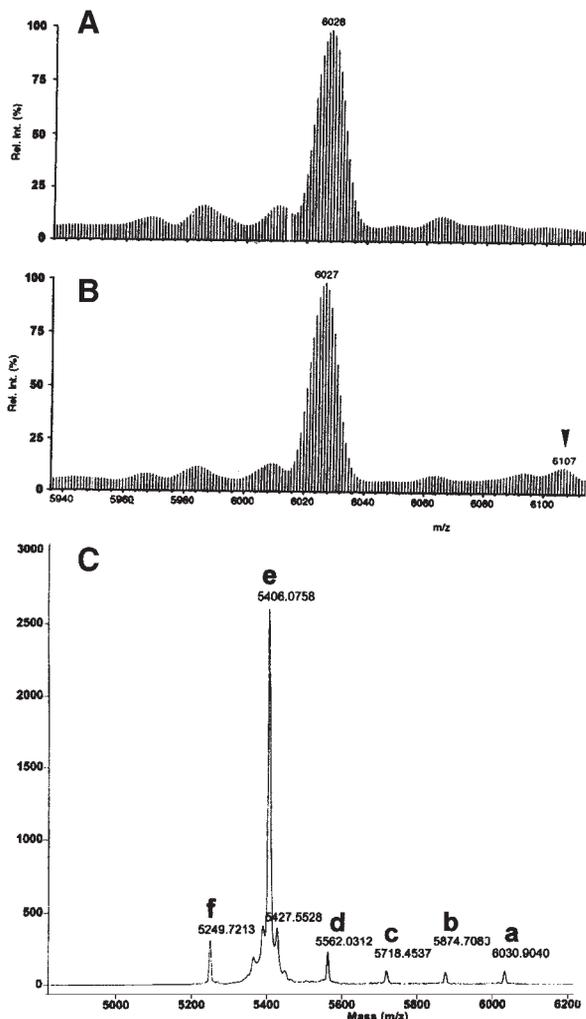


Fig. 3. Characterization of Po2, the main *O. vulgaris* protamine. **A, B:** Ion spray mass spectrometry (ISMS) analysis of protamine Po2 purified from ripe sperm nuclei (A) and nuclei of mature gonads (B). The coincidence between molecular masses observed is practically perfect (6,027–6,028 Da). Note that a fraction of Po2 protamine obtained from gonadal nuclei behaves with a molecular mass of (6,027 + 80) Da (arrow). **C:** Carboxy-terminal analysis of the masses of the entire Po2 protamine (a) and the successive forms lacking 1st, 2nd, 3rd, 4th, and 5th carboxy-terminal amino acid residues (b, c, d, e, and f, respectively). Each new C-terminal deletion generates a mass diminution of 156.4 ± 0.2 Da corresponding to one arginine residue (156.2 Da).

short molecules (28 and 30 amino acid residues, respectively), composed mainly (but not exclusively) of arginine and glycine residues, and with only a single residue of serine. Tables 1 and 2 show the coincidence between results observed and estimated. Figure 6 also shows the alignment of Po4 and Po5 sequences, clearly indicating that these proteins are homologous, with the identities between amino acid positions being denoted by the symbol “+.”

Protamine Po3 is a very minor component and we could only obtain a small quantity of this molecule in

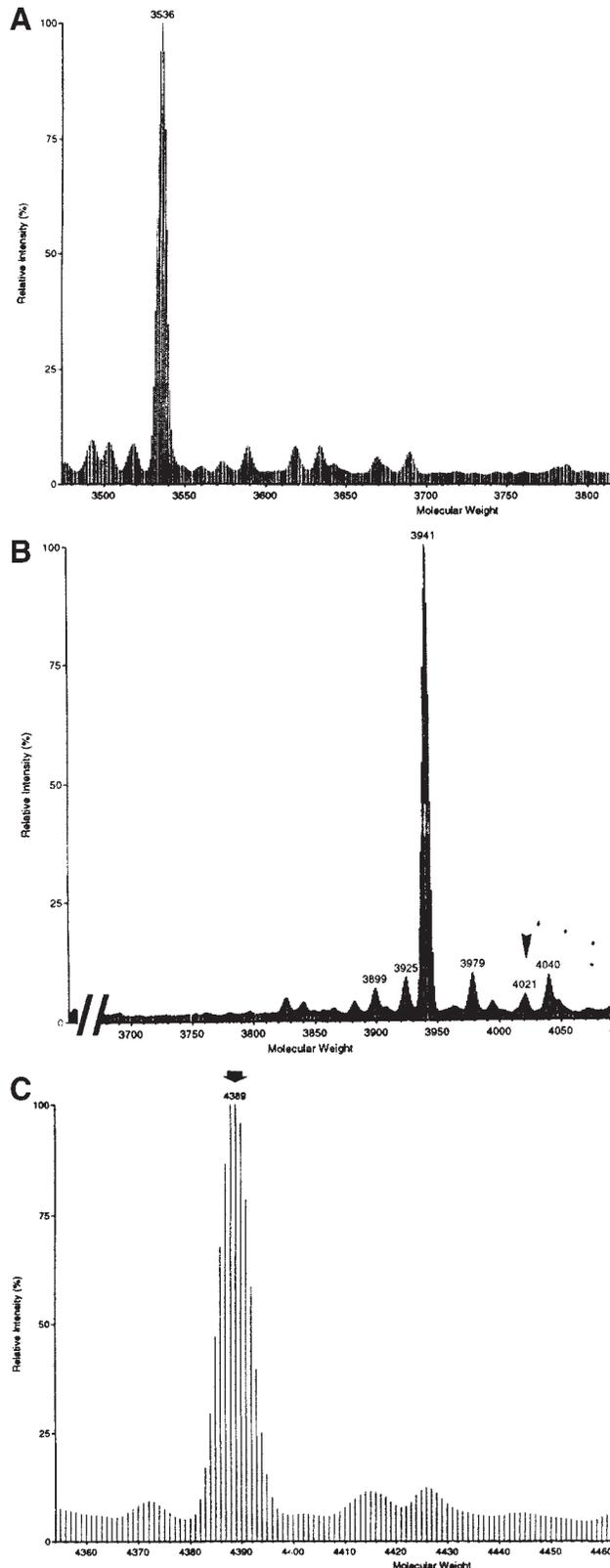


Fig. 4. Molecular mass of protamines Po4 and Po5, and a minor protamine Po3. ISMS of **A:** Protamine Po4 from ripe sperm nuclei. **B:** Protamine Po5 from nuclei of ripe gonads. **C:** Protamine Po3 from ripe sperm nuclei. The arrowhead in (B) indicates the mono-phosphorylated fraction. Molecular mass of proteins are: Po3 = 4,389, Po4 = 3,536, and Po5 = 3,941 Da, respectively.

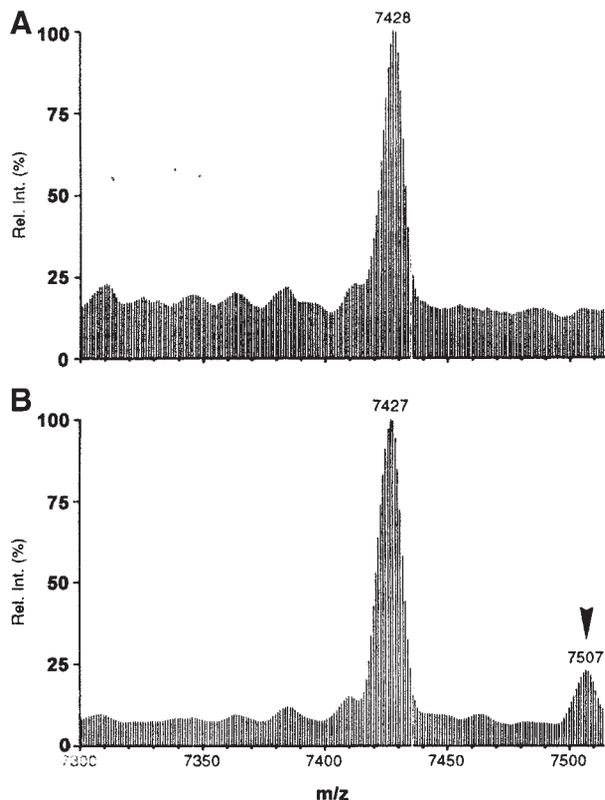


Fig. 5. Molecular mass of nuclear sperm protein Po1. ISMS of Po1 protein obtained from ripe sperm nuclei (A) and gonadal nuclei (B). The arrowhead in (B) points to the mono-phosphorylated form. Molecular mass = 7,427 Da.

pure form. Due to this fact, its sequence has only been determined unequivocally up to residue 24 (Fig. 6), whereas mass spectrometry (4,389 Da, Fig. 4) indicates that the entire protein should contain 33 ± 1 amino acid residues. Nevertheless, the comparison of the known part (24 amino-terminal residues) with protamines Po3 and Po4 allows us to conclude that Po3 is a minor protein closely related to Po4 protamine.

Protamine-precursor Po1. As has been noted previously, the extracts of protein Po1 appear in a variable proportion depending on the sample analyzed. Together with Po3, Po1 is only a minor protein present in ripe sperm nuclei of *O. vulgaris*. The analysis of its molecular mass and amino-terminal sequence (see Figs. 5 and 6 and Tables 1 and 2) demonstrates that this protein is a precursor of the main protamine Po2, which, like some protamine-precursors of mammals (Debarle et al., 1995), remains at low and variable proportions in ripe sperm nuclei. This fact agrees with the assumption that the protamines of *O. vulgaris* come from a larger precursor molecules. In this sense, the amino acid composition of Bp protein (Fig. 2 and Table 1) is typical for that of a large protamine precursor. Similar examples have been described in Càceres et al. (1999), and in Ribes et al. (2001).

DISCUSSION AND CONCLUSIONS

Molecular Characteristics of *O. vulgaris* DNA-Interacting Proteins in the Sperm Cell

In ripe sperm nuclei of *O. vulgaris*, four protamines can be found (Po2–Po5), and a minor amount of a molecule (Po1) that represents, in all probability, a precursor form of the main Po2 protamine. The amino acid sequence and composition of these molecules are shown in Figure 6 and Table 1, respectively.

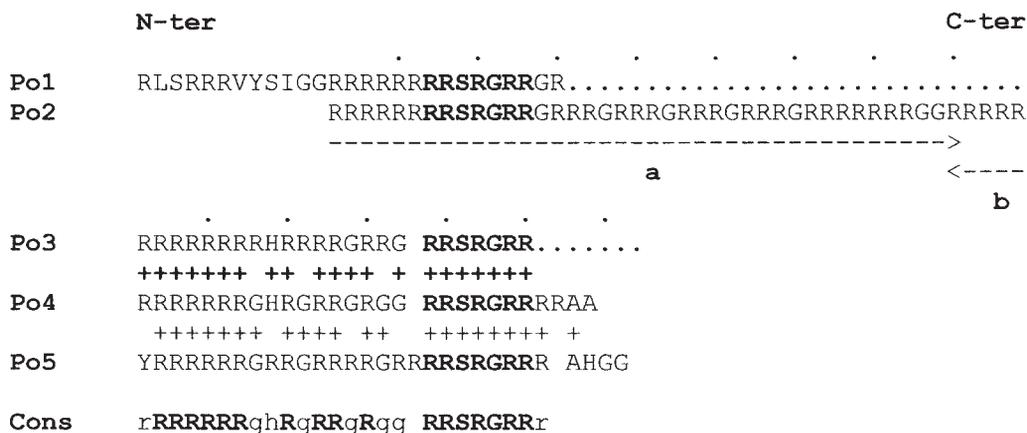


Fig. 6. Primary structures of *O. vulgaris* protamines. Po1. Partial sequence of putative precursor of the main protamine Po2. The complete identity of overlapped residues, the coincidence in the molecular mass that is observed and estimated (Table 2), as well as the lack of Po1 in the ripe sperm of some individuals (Fig. 1), strongly suggest that Po1 is a precursor of Po2 protamine. Po2. Complete sequence of the main protamine obtained from automated Edman degradation (a) and carboxy-terminal analysis (b). Po3. Minor

protamine (partial sequence). Po4 and Po5. Complete sequence of the protamines with the highest electrophoretic mobilities. The sign “+” indicates identity in the position of amino acid residues in the sequences compared. **Bottom:** Consensus sequence of Po3, Po4, and Po5 protamines. The RRSRGRR motif shared by all sperm protamines of *O. vulgaris* is shown in bold. N-ter: amino-terminus. C-ter: carboxyl-terminus. Black dots above the sequences indicate every fifth amino acid residue.

a) **RRRRRRSRRRRRRSRRRSRS** PYRRRY**RRRRRRRRRR**SRRRRY**RRRR**SY**RRRR**YRRRR
 b) **RRRRRRRRS**RGRR**GRRR**GRRR**GRRR**GRRR**GRRRRRRR**RG**GRRRRR**
 c) FCGSK**PRC**PR**CKPRC**SR**SKKRC**RR**CSRI**VKKCC**RRR**SKCCRR**RR**PCPC**PRK**KL**RCC**
 KRRPKRR**CPK**RRKK**KRC**RR**KC**

Fig. 7. Comparison of primary structure among ripe protamines of cephalopods. **a:** Sequence of *Sepia officinalis* ripe protamine Ps (Martin-Ponthieu et al., 1991); **b:** *O. vulgaris* ripe protamine Po2 (this study); **c:** Protamine P_E of *E. cirrhosa* (Giménez-Bonafé et al., 2002). Black dots above each sequence indicate every fifth amino acid residue. Similar sequences are shown in bold.

The main protamine Po2 is probably one of the simplest and most basic protamine molecules found in sperm nuclei of animals. Po2 protamine consists exclusively of three different amino acid types (R, G, S). The general organization of this molecule [R₈SR(GR_n)₇] consists of a long amino-terminal cluster of arginine residues followed by a SR motif and seven groups of GR_n (n being variable, although GR₃ appears four-times within the sequence). The highly basic chemical character of the molecule is given by the extraordinary proportion of arginine residues (35 R in a total of 44 residues; 79.5 mol%), which are accompanied by a single phosphorylable serine residue and by eight residues of glycine (structurally the simplest type of amino acid).

The simplicity of Po2 protamine can be understood if we consider that it comes from a precursor protein. In Figure 7 and Table 3 we show, for comparative purposes, the primary structure of protamine Po2 from *O. vulgaris* (Fig. 7b), protamine Ps from *Sepia officinalis* (Fig. 7a), and protamine Pe from *E. cirrhosa* (Fig. 7c). It is important to observe the contrast between the compositional simplicity of protamines coming from a precursor molecule (Fig. 7a,b), with the relative complexity of protamines that are synthesized as a final form (Fig. 7c). This fact is not unique to cephalopods, but has been observed in other kinds of mollusks that have appeared recently in evolution (Càceres et al., 1999, 2002).

We should consider that protamine molecules accomplish two main functions: (a) During the process of spermiogenesis the protamine must produce an ordered displacement of histones on DNA, causing a definite chromatin condensation pattern which must be reverted easily during gamete fertilization; and (b) once the process of spermiogenesis is completed, the protamine

should maintain the sperm DNA in a highly condensed state until the sperm nucleus penetrates the oocyte cytoplasm. Under this point of view, the molecules of protamine-precursors are highly adapted to these two functions. The precursors of protamines show two clearly defined and differentiated regions. The amino-terminal parts are relatively complex and rich in different amino acid types, whereas the carboxy-terminal zones are very simple and present a very basic chemical character. The amino-terminal zones, as well as their processing (that is their enzymatic deletions), may play an important role in the slow and organized displacement of histones during spermiogenesis, determining the morphological pattern of chromatin condensation. On the other hand, the carboxy-terminal regions of protamine-precursors are the mature protamines found in ripe sperm nuclei. These are simplified proteins specialized in maintaining the packaging of DNA.

From the evolutionary point of view it is also interesting to point out that *O. vulgaris* protamine Po2 displays a series of general similarities with the protamine molecules of decapods. First, the precursor is finally deleted at the GG dipeptide [precursor-GG-protamine → precursor-GG + protamine] (see Fig. 6 and compare with the results of Wouters-Tyrou et al., 1991, 1995). Second, their amino-terminal end is composed of a long cluster of arginines followed by a serine (Fig. 7). Finally, the rest of the molecule is composed of arginine clusters separated by a few non-basic amino acid residues. It seems to be that when natural selection acts on the protamines, it tends to maintain the global proportion of arginine rather than their position in the molecule (Rooney et al., 2000). In this regard, protamine

TABLE 3. Comparison of Amino Acid Composition Among Ripe Protamines of Cephalopods Based on Primary Structure

Protamine	Amino acid residues	Arginine residues	Lysine residues	Amino acid types	Presence of precursor
<i>S. officinalis</i> P _s ^a	57	44 (77.2 mol%)	0	4 (R, S, Y, P)	Yes
<i>O. vulgaris</i> Po2 ^b	44	35 (79.5 mol%)	0	3 (R, G, S)	Yes
<i>E. cirrhosa</i> P _E ^c	84	31 (36.9 mol%)	16 (19.0 mol%)	10 (R, K, C, P, G, S, I, F, L, V)	No

^aMartin-Ponthieu et al. (1991).

^bThis study.

^cGiménez-Bonafé et al. (2002).

Po2 belongs to the same category as that of decapod protamines (Fig. 7 and Table 3).

Protamines Po3, Po4, and Po5 are undoubtedly homologous among themselves. Their amino-terminal end possesses a long cluster of arginines and the rest of the molecules are coarsely arranged in GR_n groups. In spite of this, they contain several additional amino acid residues (H, A, Y). In all of them the single phosphorylatable serine residue is found in the carboxy-terminal part (position 20 or 21), being part of a sequence of seven amino acid residues [RRSRGRR] which is conserved in all of them (see Fig. 6). This heptapeptide sequence is also shared by the main Po2 protamine, so that it is likely that all protamines found in the sperm nucleus of *O. vulgaris* come from a single ancestral gene.

General Comment About the Complexity in Spermiogenic Chromatin Condensation of *O. Vulgaris*

Spermiogenic chromatin of *O. vulgaris* initiates its condensation in a pattern of fibres similar to decapods, but afterwards these fibres (25 nm diameter) form structures of progressively increasing size (40 nm → 60 nm → 80 nm), leading to a final homogeneous and compact thread-like appearance. The precise motifs of these complex structural transitions are still difficult to understand, but they should be correlated with the complexity of the nuclear protein transitions. The changes of nuclear proteins during spermiogenesis in *O. vulgaris* are much more complex than in decapods. In the mature sperm nucleus of *O. vulgaris* there are four protamines (Po2–Po5), each one perhaps coming from a separate precursor molecule. All these protamines are found to be (at least partially) phosphorylated during spermiogenesis. As it has been noted previously, the fact that only a small quantity of phosphorylated proteins is present may indicate that serines are dephosphorylated before the complete processing of the precursor.

ACKNOWLEDGMENTS

We are thankful to Dr. N. Cortadellas and Dr. J.A. Subirana for their scientific assistance.

REFERENCES

Chiva M, Kasinsky HE, Subirana JA. 1987. Characterization of protamines from four avian species. *FEBS Lett* 215:237–240.
 Chiva M, Kasinsky HE, Mann M, Subirana JA. 1988. On the diversity of sperm basic proteins in vertebrates. VI. Cytochemical and biochemical analysis in birds. *J Exp Zool* 245:304–317.
 Càceres C, Ribes E, Muller S, Cornudella L, Chiva M. 1994. Characterization of chromatin-condensing proteins during spermiogenesis in a neogastropod mollusc (*Murex brandaris*). *Mol Reprod Develop* 38:440–452.
 Càceres C, Giménez-Bonafé P, Ribes E, Wouters-Tyrou D, Martinage A, Kouach M, Sautière P, Muller S, Palau J, Subirana JA, Cornudella L, Chiva M. 1999. DNA-interacting proteins in the spermiogenesis of the mollusc *Murex brandaris*. *J Biol Chem* 274:649–656.

Càceres C, Giménez-Bonafé P, Zamora MJ, Ribes E, Saperas N, Kasinsky HE, Chiva M. 2002. Protamines in archaegastropod and cenogastropod molluscs: An example of a radical evolutionary change in a protein model. *Trends Comp Biochem Physiol* 7:75–82.
 Debarle M, Martinage A, Sautière P, Chevillier Ph. 1995. Persistence of protamine precursors in mature sperm nuclei of the mouse. *Mol Reprod Develop* 40:84–90.
 Giménez-Bonafé P, Ribes E, Kasinsky HE, Subirana JA, Chiva M. 1999. “Keratinous” state of *Eledone cirrhosa* sperm cells, and their special nuclear protein. *J Exp Zool* 283:580–589.
 Giménez-Bonafé P, Ribes E, Sautière P, Gonzalez A, Kasinsky HE, Kouach M, Sautière PE, Ausió J, Chiva M. 2002. Chromatin condensation, cysteine rich protamine, and establishment of disulphide interprotamine bonds during spermiogenesis of *Eledone cirrhosa* (Cephalopoda). *Eur J Cell Biol* 81:341–349.
 Hurley CK. 1977. Electrophoresis of histones: A modified Panyim and Chalkley system for slab gels. *Anal Biochem* 80:624–626.
 Kadura SN, Khrapunov SN. 1988. Displacement of histones by sperm-specific proteins at different stages of spermatogenesis of squid. *Eur J Biochem* 175:603–607.
 Lewis JD, Song Y, de Jong ME, Bagha SM, Ausió J. 2003. A walk through vertebrate and invertebrate protamines. *Chromosoma* 111:473–482.
 Martin-Ponthieu A, Wouters-Tyrou D, Belaiche D, Sautière P, Schlinder P, Van Dorsselaer A. 1991. Cuttlefish sperm protamines 1: Amino acid sequences of two distinct variants. *Eur J Biochem* 195:611–619.
 Maxwell WL. 1975. Spermiogenesis of *Eusepia officinalis* (L), *Loligo forbesi* (Streenstrup) and *Alloteuthis subulata* (L) (Cephalopoda, Decapoda). *Proc Roy Soc London B*191:527–535.
 Mennella MRF, Farina B, Irace MV, di Cristo C, di Cosmo A. 2002. Histone H1-like protein and a testis-specific variant in the reproductive tracts of *Octopus vulgaris*. *Mol Reprod Develop* 63:355–365.
 Oliva R. 1995. Sequence, evolution and transcriptional regulation of avian–mammalian P1 type protamines. In: Jamieson BMG, Ausió J, Justine JL, editors. *Advances in spermatozoal phylogeny and taxonomy*, 166. Paris: Mem Mus natn Hist nat. pp 537–548.
 Ribes E, Sanchez de Romero LD, Kasinsky HE, del Valle L, Giménez-Bonafé P, Chiva M. 2001. Chromatin reorganization during spermiogenesis of the mollusc *Thais hemostoma* (Muricidae): Implications for sperm nuclear morphogenesis in cenogastropods. *J Exp Zool* 289:304–316.
 Ribes E, Giménez-Bonafé P, Martínez-Soler F, Gonzalez A, Kasinsky HE, Chiva M. 2004. Chromatin organization during spermiogenesis in *Octopus vulgaris*. I Morphological structures. *Mol Reprod Develop* 68:223–231.
 Rooney AP, Zhang J, Nei M. 2000. An unusual form of purifying selection in a sperm protein. *Mol Biol Evol* 17:278–283.
 Sautière P, Martinage A, Belaiche D, Arkhis A, Chevillier Ph. 1988. Comparison of the amino acid sequences of human protamines HP2 and HP3 and of intermediate basic nuclear proteins HPS1 and HPS2. Structural evidence that HPS1 and HPS2 are pro-protamines. *J Biol Chem* 263:11059–11062.
 Suau P, Subirana JA. 1977. X-ray diffraction studies of nucleoprotamine structure. *J Mol Biol* 204:653–661.
 Subirana JA, Cozcolluela C, Palau J, Unzeta M. 1973. Protamines and other basic proteins from spermatozoa of molluscs. *Biochim Biophys Acta* 317:364–379.
 Wouters-Tyrou D, Chartier-Harlin MC, Martin-Ponthieu A, Boutillon C, Van Dorsselaer A, Sautière P. 1991. Cuttlefish spermatid-specific protein T: Molecular characterization of two variants T1 and T2, putative precursors of sperm variants Sp1 and Sp2. *J Biol Chem* 266:17388–17395.
 Wouters-Tyrou D, Martin-Ponthieu A, Ledoux-Andula N, Kouach M, Jaquind M, Subirana JA, Sautière P. 1995. Squid spermiogenesis: Molecular characterization of testis pro-protamines. *Biochem J* 309:529–534.