Histone H1 and the Evolution of the Nuclear Sperm-Specific Proteins

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ABSTRACT

The chromosomal proteins of the sperm exhibit an enormous structural variability. During the last ten years, important progress has been made in the chemical and physical characterization of these proteins over a wide range of organisms. The data emerging from these studies indicate that the nuclear sperm-specific proteins are not as heterogeneous as originally thought. Indeed, from the compositional point of view they can be arranged in a discrete number of basic types: H Type (histone), P Type (protamine) and PL Type (protamine-like) consisting of proteins with an intermediate composition between histones and protamines. Research on the PL Type has been carried out mainly in molluscs. This taxonomic group is of special interest because it contains organisms with nuclear sperm-specific proteins that can be considered representative of each of the three protein types. Work carried out with bivalve molluscs suggests the possibility that all members of the PL Type have evolved or are related in one way or another to a PL-I protein with structural characteristics of an H1 histone. PL-I proteins have been recently identified in both protostomes and deuterostomes. The structural links existing amongst PL proteins, their arginine-rich composition and their relationship to chromosomal proteins of the histone H1 family suggest an evolutionary relationship amongst the three basic protein types. On this basis, an evolutionary pathway starting from an early histone precursor and leading to the protamine type which is present in the most evolved organisms of the deuterostome and protostome branches is proposed.

RÉSUMÉ

Histone H1 et évolution des protéines nucléaires spécifique des spermatozoïdes

Les protéines chromosomiques des spermatozoïdes montrent une immense variabilité structurale. Pendant les dix dernières années, d'importants progrès ont été faits dans la caractérisation chimique et physique de ces protéines dans des organismes très variés. Les résultats émergeant de ces études montrent que les protéines spécifiques des spermatozoïdes ne sont pas aussi hétérogènes que ce que l'on pensait originellement. En fait, elles peuvent être classées par leur composition en un nombre fini de types: type H (histone), type P (protamine) et type PL (proche des protamines) correspondant à des protéines ayant une composition intermédiaire entre les histones et les protamines. La recherche sur les protéines proches des protamines a porté principalement sur les Mollusques. Ce groupe taxonomique est d'un intérêt particulier car il comprend des organismes avec des protéines spécifiques des spermatozoïdes qui peuvent être considérées comme représentant chacun des trois types. Les travaux sur les Mollusques Bivalves suggèrent la possibilité que toutes les protéines du type PL ont évolué ou sont reliées d'une manière ou d'une autre avec une protéine PL-I avec les caractéristiques structurales d'une histone H1. Les protéines PL-I ont été récemment identifiées chez les Protostomiens et les Deutérostomiens. Les liens structuraux existant entre les protéines PL, leur composition riche en arginine et leurs relations avec les protéines chromosomiques de la famille des histones H1 suggèrent une relation évolutive entre les trois types de protéines. Sur cette base, un chemin évolutif commençant avec un précurseur histone et menant au type protamine qui est présent chez les organismes les plus évolués des Deutérostomiens et des Protostomiens est proposé.

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During spermatogenesis, chromatin from the stem cells undergoes several dynamic transitions which are often associated with important changes in its composition and structure. In most instances, the composition of the chromosomal proteins at the onset and in the final stages of spermatogenesis is quite different. These compositional changes significantly alter the structure of chromatin. As a result, chromatin becomes highly compacted and gene expression is completely shut off in the spermatozoon. The ways in which all this is achieved can be mediated by a wide spectrum of apparently diverse chromosomal proteins [17, 40] which are mirrored by the morphological diversity of the mature sperm cell [15]. The structure of chromatin arising from the protein-DNA interactions in each particular situation is in most instances poorly understood and the evolutionary relationship amongst these proteins still remains obscure.

This chapter reviews the classification of the nuclear sperm-specific proteins in the light of new biochemical data that have been gathered in recent years. With the information on the chromosomal sperm-proteins expanding over a wider range of taxonomic groups from both the protostome and deuterostome branches as well as in lower phylogenetic taxa, it is also possible to envisage and outline an evolutionary relationship for these proteins.

RESULTS AND DISCUSSION

Structural variability and compositional homogeneity of the nuclear sperm-specific proteins. The classification of the nuclear sperm-specific proteins, 25 years after.

In 1969, David BLOCH [17] published the first comprehensive catalogue of the nuclear sperm-specific proteins. Despite the attempt at classification proposed in his paper (Fig. 1), the author concluded: "...the variability (non-conservatism) of the protein reflects an evolutionary indifference to a relatively unimportant protein in an inert nucleus". This somehow pessimistic comment reflects BLOCH's difficulty in finding a phylogenetic relationship among the different groups of chromosomal sperm proteins. This was mainly due to the fact that even though proteins belonging to the same group in the classification had a similar amino acid composition, they exhibited an enormous structural variability. In addition, the patchy cytochemical and biochemical information, available at that time, came from organisms usually belonging to phylogenetically distant groups.

The availability of techniques such as high performance liquid chromatography and protein microanalysis has allowed us to extend this information to a much broader spectrum of organisms in recent years. More importantly, it has been possible, for the first time, to gain information on organisms from phylogenetically relevant/related taxonomic groups [59, 60].

The global picture emerging from these studies is that despite their enormous structural variability, the nuclear sperm-specific proteins can be grouped, from the compositional point of view, into three basic categories (Fig. 1). Type H (Histone type) consists of proteins with amino acid compositions that, although specific for the germ line, are structurally and compositionally similar to those of the somatic histone type (Table 1). This grouping is equivalent to the *Rana* type of BLOCH's classification [17] (see also Fig. 1). At the other end of this classification, type P (protamine type) consists of proteins of low molecular mass (4000 to 10 000 daltons), that are arginine rich (arginine content $\geq 60\%$). The P type is defined here irrespective of the presence or absence of cysteine in the protein molecule. Thus it brings together under a common name the monoprotamine and stable protamine type of BLOCH's classification [17] (Fig. 1). In the course of spermatogenesis, these proteins almost completely replace the histone counterpart of the stem cells (see [53] for a recent review on these proteins, and Table 3). The PL type (protamine-like) consists of proteins with an amino acid composition intermediate between the previous types. The arginine and lysine content usually amounts to at least 35-50% of their amino acid composition, but occasionally it can be higher. Although the relative ratio of these two amino acids may vary over a wide range, it usually stays constant for a given taxonomic group (Table 2). In

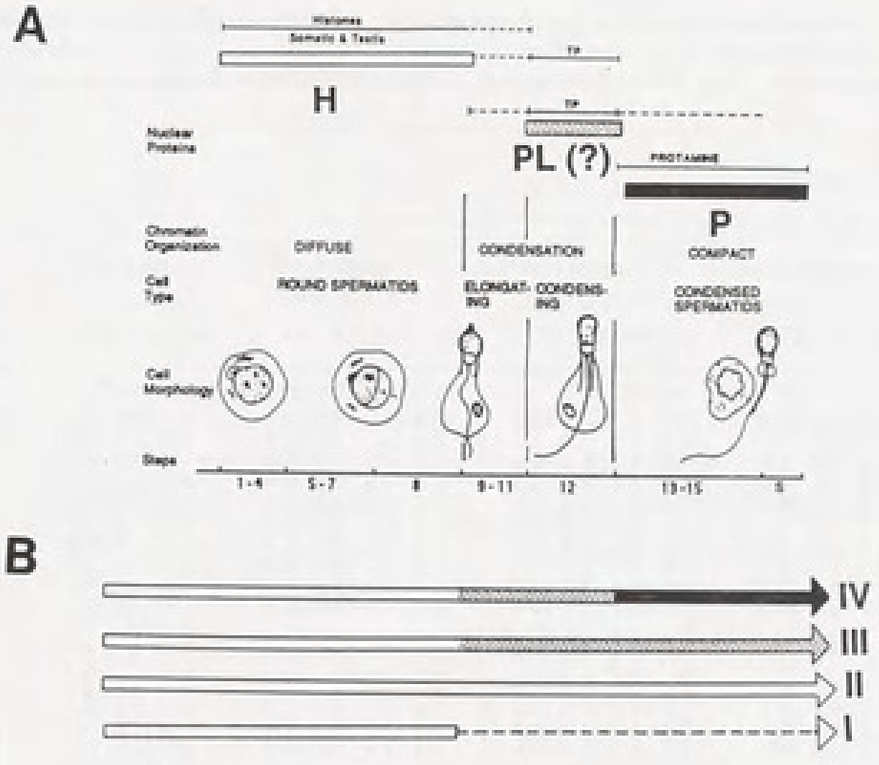

FIG. 1. — **A:** Schematic representation of the changes in the nucleus in ram spermiogenesis (modified from [42]). TP = transition proteins. **B:** Nuclear protein transitions in the four basic types of spermiogenesis. **C:** Classification of the nuclear sperm-proteins. The classification used in this paper is compared to that proposed by BLOCH [17].

most instances, these proteins can be structurally related to histone H1 (as will be discussed later) and as in the case of the P type these proteins replace to a large extent (≥80%) the somatic histones during spermiogenesis. In the final stages of spermatogenesis, PL proteins exhibit a significant degree of structural heterogeneity, with molecular masses in the 5 000-30 000 dalton range. This group corresponds to the type of intermediate proteins (*Mytilus* type) of BLOCH's classification [17] (see also Fig. 1).

The phylogenetic relevance of this later group of proteins was already anticipated by BLOCH [18]. He noted "... the similarities between the 'evolutionary intermediate' proteins of the mature sperm of mussel and the 'developmentally intermediate' proteins of the spermatids of *Loligo*

TABLE 1. — Amino acid composition (mol %) of the H-type proteins from the sperm of several representative organisms in comparison with somatic type H proteins from calf thymus.
LP = *Limulus polyphemus* [48], PM = *Petromyzon marinus* [60], CA = *Carassius auratus* [49] and CT = calf thymus [45].
tr = trace amounts.

	H1						H2A				H2B				H3			H4			
	LPa &b	LPa	LPc	PM	CA	CT	LP	PM	CA	CT	LP	PM	CA	CT	PM	CA	CT	LP	PM	CA	CT
Lys	28.7	24.1	20.0	27.6	29.2	**26.8**	10.2	9.7	10.5	**10.2**	14.2	14.1	14.0	**14.1**	8.3	10.0	**10.0**	10.1	10.4	10.1	**11.4**
His	-	-	-	-	0.5	-	1.8	1.6	2.0	**3.1**	2.6	3.3	2.0	**2.3**	1.7	1.4	**1.7**	2.3	1.8	2.0	**2.2**
Arg	3.2	2.7	6.7	2.2	2.1	**1.8**	8.6	9.8	8.6	**9.4**	6.8	8.2	7.8	**6.9**	13.8	13.2	**13.0**	12.1	13.4	12.5	**12.8**
Asx	2.5	3.5	6.1	3.9	2.6	**2.5**	6.6	6.3	6.1	**6.2**	5.8	4.8	5.2	**5.0**	4.2	4.4	**4.2**	5.6	4.8	5.3	**5.2**
Thr	6.4	7.4	6.0	3.6	4.0	**5.6**	2.6	3.2	5.2	**3.9**	6.9	6.6	6.2	**6.4**	7.5	6.4	**6.8**	6.3	6.7	6.5	**6.3**
Ser	6.5	8.8	9.4	7.3	6.0	**5.6**	4.3	5.2	5.0	**3.4**	12.5	10.3	7.0	**10.4**	4.5	5.1	**3.6**	4.6	2.0	2.7	**2.2**
Glx	5.3	7.9	10.1	4.7	4.0	**3.7**	10.8	9.4	9.5	**9.8**	9.2	6.4	8.6	**8.7**	12.0	11.7	**11.6**	8.1	6.5	6.8	**6.9**
Pro	11.2	8.8	8.7	9.7	9.3	**9.2**	4.9	4.1	5.7	**4.1**	2.2	6.2	4.8	**4.9**	4.5	5.0	**4.6**	1.6	1.4	1.6	**1.5**
Gly	5.5	6.8	5.8	4.0	5.4	**7.2**	11.2	11.4	10.2	**10.8**	5.1	5.9	7.9	**5.4**	5.6	6.3	**5.4**	16.4	16.8	14.7	**14.9**
Ala	15.4	15.8	10.0	21.8	22.6	**24.3**	12.7	13.1	13.7	**12.9**	12.3	11.6	11.6	**10.8**	13.3	13.0	**13.3**	7.4	7.9	8.0	**7.7**
1/2 Cys	-	-	-	-	-	-	tr.	-	-	-	-	-	-	-	0.6	-	**1.0**	tr.	-	-	-
Val	5.8	5.6	4.6	6.8	4.4	**5.4**	6.6	7.2	6.2	**6.3**	6.3	6.0	6.7	**7.5**	4.2	4.6	**4.4**	7.0	8.1	8.4	**8.2**
Met	-	-	1.2	-	-	-	-	0.5	-	-	1.4	2.1	-	**1.5**	0.8	-	**1.1**	tr.	0.2	-	**1.0**
Ile	2.8	3.1	3.8	1.1	2.0	**1.5**	4.5	3.7	3.3	**3.9**	4.6	5.8	4.7	**5.1**	4.7	5.0	**5.3**	5.1	5.5	5.4	**5.7**
Leu	3.0	3.3	5.2	5.3	4.7	**4.8**	12.1	11.9	11.4	**12.4**	4.6	4.5	6.5	**4.9**	8.9	8.8	**9.1**	7.4	8.6	8.5	**8.2**
Tyr	2.5	1.0	1.5	0.8	0.9	**0.9**	2.0	2.3	1.8	**2.2**	2.8	2.8	3.8	**4.0**	2.2	2.0	**2.2**	2.9	3.6	3.6	**3.8**
Phe	1.2	1.0	1.3	1.1	0.8	**0.9**	1.0	0.9	0.7	**0.9**	2.5	1.6	1.6	**1.6**	3.3	2.7	**3.1**	2.0	2.1	2.1	**2.1**
Trp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(squid) [16] and salmon"... (which both contain proteins of the P type in their mature sperm)... "suggest a relationship between ontogeny and phylogeny". Although the precise relationship (if any) between the "developmentally intermediate" proteins and PL proteins has yet to be established, BLOCH's hypothesis provides an excellent model for the biochemical classification of spermatogenesis within a phylogenetic context (Fig. 1B). Thus, from the point of view of the nuclear sperm-specific proteins which are present in the mature sperm and the protein transitions undergone by chromatin during the differentiation process, four basic types can be defined. The first group (type I) of this classification consists of those organisms which lose their chromosomal protein complement (histones) during spermiogenesis. As a result, DNA appears essentially naked in the mature sperm cell, which exhibits an uncondensed nucleus. In Type II, the somatic type (H type) of histones is maintained throughout the whole spermatogenesis. In most instances histones undergo an important turnover during which the somatic histones from the stem cells at the onset of spermatogenesis are partially or completely replaced by sperm-specific histones (Table 1). In the third group of this classification, the somatic and/or the germinal histone type of proteins (H type), is replaced to a large extent by proteins of the PL type. These are the proteins that are found in the mature spermatozoa. Finally, in group IV, at the onset of spermiogenesis, the histones of somatic and/or germinal type are initially displaced by a set of intermediate proteins "transition proteins" and finally replaced by arginine rich proteins of the protamine type.

With the exception of group I, which has been described so far only in some members of the decapod crustaceans [72], all the other types of this classification are widespread in both deuterostomes and protostomes.

TABLE 2. — Amino acid composition (mol %) of some representative proteins of the PL-type. tr = trace amounts.

Group	Molluscs			Tunicate	Cephalochordate
Species	*Ensis minor*	*Mytilus californianus*	*Macoma nasuta*	*Styela plicata*	*Branchiostoma floridae*
Reference	[33]	[47]	[3]	[59]	[60]
	PL-I (EM-6)	PL-III	PL-III	P1	
Lys	24.9	20.5	25.2	14.3	24.7
His	4.0	-	0.2	1.1	-
Arg	21.7	32.1	27.9	32.5	25.3
Asx	2.4	-	3.5	4.6	-
Thr	2.0	2.5	-	1.2	-
Ser	18.9	15.4	28.7	4.6	16.5
Glx	1.8	-	0.9	2.2	-
Pro	tr	5.1	0.6	1.4	5.6
Gly	2.6	7.5	1.8	13.2	6.1
Ala	12.7	15.9	9.5	7.1	21.7
1/2 Cys	-	-	-	0.7	-
Val	2.3	1.1	0.4	4.6	-
Met	0.9	-	0.1	1.4	-
Ile	1.2	-	0.5	2.4	-
Leu	2.9	-	0.5	4.5	-
Tyr	0.6	-	0.1	2.2	-
Phe	1.0	-	0.2	2.2	-
Trp	-	-	-	-	-

Presence of an H1-like PL-protein in the sperm of Molluscs

The study of the nuclear sperm-specific proteins from molluscs is of special interest for several reasons. First, this taxonomic group consists of organisms that can be considered representative of any of the protein types described in the previous section. Also, this is the group from which the organism *Mytilus* (mussel) was selected by BLOCH to name the protein type corresponding to PL-type of the protein classification described in the preceding section (Fig. 1).

With regard to the first point, all the organisms of the subclass Pteriomorphia (oysters and scallops) of the class Bivalvia analyzed to date belong to the H type [2, 4, 52, 76]. All the members of the class Cephalopoda seem to contain chromosomal proteins of the P-type (Table 3), some of which may even contain cysteine, as in the case of *Eledone* [68]. Cysteine is also present in the mammalian P-type proteins. However, the most common proteins present in the sperm of the organisms from this phylum are by far the proteins from the PL-type. From a pragmatic point of view, these latter proteins can be classified into two major categories: those PLs which have a molecular mass smaller than 15,000 daltons and those with higher molecular mass. Although this division may seem arbitrary, it has profound structural implications, as will be described next.

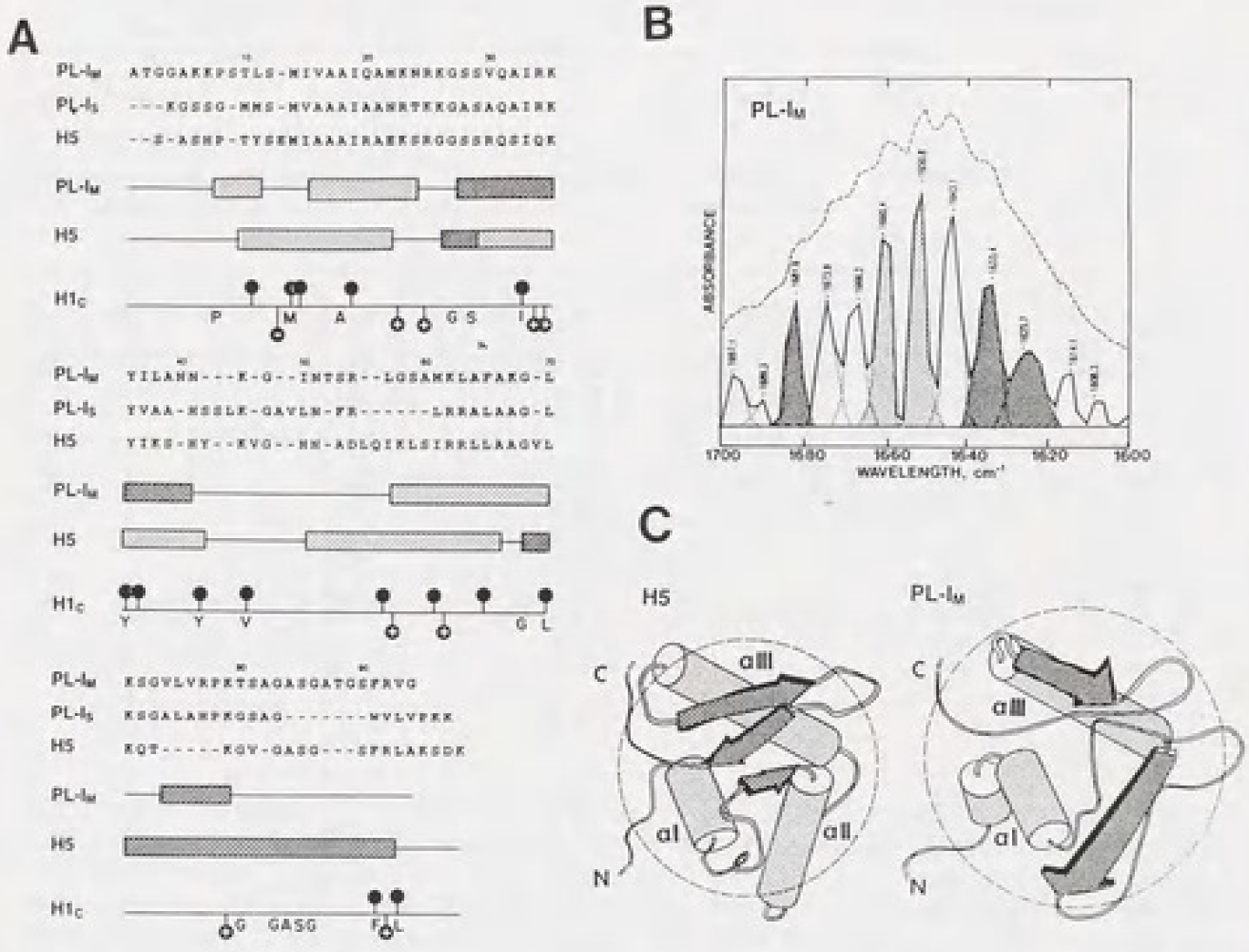

FIG. 2.— **A:** Sequence comparison between the trypsin-resistant cores of two PL-I proteins from bivalve molluscs (PL-I_M from *Mytilus californianus*, PL-I_S from *Spisula solidissima*) and the core histone H5 from chicken erythrocyte. The shaded boxes shown below identify the α-helix (light) and ß-sheet domains as determined by combination of secondary structure prediction and the experimental analysis shown in B [39]. Also shown are the major conserved features for the core of the histones of the H1 family [27]. In this representation, the black dots correspond to conserved hydrophobic residues. Conserved positively and negatively charged amino acids are also identified. **B:** Fourier-transform Infrared Spectroscopy (FTIR) analysis of the trypsin-resistant core of PL-I from *M. californianus*. The deconvolution and curve-fitting of the region corresponding to the amide I band of the infrared spectrum is shown. The different frequencies (peaks) of the spectrum can be assigned to different secondary structures [39]. α-helix (lightly shaded), ß-sheet (darkly shaded), other (not shaded). The areas under the different frequencies (peaks) are proportional to the amount of the corresponding secondary structure. C. Tertiary structure organization of the trypsin-resistant globular core of histone H5 determined by X-ray crystallography [55]. By comparison, an idealized hypothetical model for the trypsin-resistant core of PL-I from *M. californianus* is also shown. The model is based on the conservation of the primary structure and on the secondary structure assignments shown in A.

In order to account for the large molecular mass of some of the PL-proteins found in molluscs such as *Spisula solidissima* (surf clam) (which has a PL protein with Mr ≈ 27 000), SUBIRANA *et al.* [68] had earlier proposed a mechanism of "gene polymerization" from a PL precursor gene of lower molecular mass. In 1986 we decided to test this hypothesis [12] by

TABLE 3. — Amino acid composition (mol %) of several representative proteins of the protamine type P.
* average composition from the P1 and P2 components from [70]
tr = trace amounts.

Group	Molluscs	Molluscs	Fish	Amphibians	Reptiles	Birds	Mammals
Species	*Loligo pealeii*	*Gibbula divaricata*	*Oncorhyncus keta*	*Bufo japonicus*	*Chrysemys picta*	*Gallus domesticus*	*Bos taurus*
Reference	[9]	[68]	[1]	[70] *	[23]	[50]	[24]
			Salmine				P1
Lys	-	5.8	-	5.4	1.0	-	-
His	-	-	-	9.6	4.0	-	2.1
Arg	78.0	56.3	67.2	42.1	65.3	58.5	50.0
Asx	-	-	-	2.0	-	-	-
Thr	-	2.0	-	6.9	2.5	1.6	6.3
Ser	12.2	17.1	9.9	5.7	7.0	17.2	4.2
Glx	-	-	-	4.7	4.4	-	2.1
Pro	2.4	-	9.1	9.7	tr	3.5	-
Gly	-	4.9	6.5	0.8	13.2	8.6	4.2
Ala	-	9.6	1.3	4.9	1.3	3.2	2.1
1/2 Cys	-	-	-	0.3	-	-	12.5
Val	-	3.5	4.7	5.3	-	1.7	6.3
Met	-	-	-	-	-	-	-
Ile	-	-	1.3	-	-	-	2.1
Leu	-	-	-	-	-	-	2.1
Tyr	7.3	-	-	2.7	-	6.2	4.2
Phe	-	-	-	-	-	-	2.1
Trp	-	-	-	-	-	-	-

conducting a detailed structural and biochemical analysis of the PL protein of *Spisula*. The results surprisingly showed that, like somatic histone H1, the major PL protein component from *Spisula* sperm: 1) was *not insoluble* in dilute PCA (5%) [4], 2) could be digested with trypsin to produce a 75 amino acid trypsin-resistant core, 3) this trypsin-resistant core had a globular conformation, 4) the primary structure of this globular core exhibited a high degree of sequence similarity with the globular domain of somatic histone H1 and it fulfilled the constraints imposed by the consensus sequence established for the globular trypsin-resistant domain of this histone [27] (Fig. 2). When this was taken together with the fact that the trypsin-resistant core of histone H1 was the most conserved region of this molecule [26], it was clear that, despite the high lysine and arginine content of *Spisula* PL (Table 4), this molecule was a member of the histone H1 family.

Extension of these analyses to PL proteins from other bivalve molluscs [20, 33, 34, 39] indicates that the extent of sequence similarity of the globular domain of these molecules and that of other histone H1 members is in the 30-40% range, with higher values in the case of the sperm-specific histone H1 proteins. This value should be considered high, taking into account the high evolutionary variability of the histones of the H1 family [37]. We call these proteins PL-I proteins. We define them as PL proteins (Fig. 1C) which have an internal trypsin-resistant

TABLE 4. — Amino acid composition (mol %) of the PL-I proteins of different organisms.
tr = trace amounts.

Organism	*Metridium senile*	*Spisula solidissima*	*Chelysoma productum*	*Mullus barbatus*	Calf thymus histone H1	Chicken erythrocyte histone H5
Reference	Unpublished	[8]	Unpublished	[58]	[45]	[45]
Lys	11.0	24.8	17.3	24.2	26.8	23.6
His	0.5	-	1.8	-	-	1.9
Arg	25.4	23.1	20.8	22.1	1.8	12.4
Asx	2.8	0.6	5.0	5.1	2.5	1.7
Thr	4.3	4.3	2.1	4.5	5.6	3.2
Ser	11.2	21.7	6.8	9.2	5.6	11.9
Glx	5.4	0.6	4.0	tr	3.7	4.3
Pro	2.5	2.4	1.9	6.8	9.2	4.7
Gly	8.6	3.0	18.7	5.4	7.2	5.3
Ala	11.6	14.2	6.7	11.9	24.3	16.3
1/2 Cys	-	-	-	-	-	-
Val	7.6	2.3	2.9	4.9	5.4	4.2
Met	-	0.4	0.5	tr	-	0.4
Ile	2.3	0.5	3.4	tr	1.5	3.2
Leu	3.6	1.7	5.8	5.8	4.5	4.7
Tyr	1.4	0.3	1.6	tr	0.9	1.2
Phe	1.8	0.3	1.2	tr	0.9	0.6
Trp	-	0.3	-	-	-	-

globular core with structural and compositional similarity to the globular counterpart of the protein members of the histone H1 family. Unlike the sperm-specific H1 histones found in echinoderms or in the bivalve group Pteriomorphia [2, 54] (and like protamines), PL-I proteins replace most of the core and linker histones present at the onset of spermiogenesis. They account for ≥ 80% of the chromosomal protein of the mature spermatozoon.

The N and C terminal non-globular domains of these molecules are extremely variable. Most of the basic residues are found in these regions and in many instances the arginine residues are arranged in clusters similar to those found in the proteins of the protamine type [4].

All PL proteins with $M_r \geq 15{,}000$ analyzed to date are members of the PL-I protein group. When similar structural analyses as those carried out with these molecules were extended to other PL proteins with $M_r < 15{,}000$ [3], no trypsin-resistant peptide could ever be detected in these molecules, regardless of their overall compositional similarity to PL-I. This could already be anticipated from the complete absence of hydrophobic amino acids (Phe, Tyr, Leu...), which are usually present in low amounts in PL-I proteins.

Chromatin Structures

During the last twenty years important progress has been made in our knowledge of the chromatin organization of the nucleus of somatic cells [71]. In contrast, our knowledge of chromatin organization in the sperm cell has progressed at a slower pace but has benefited greatly from the information gained in the former as will be discussed later.

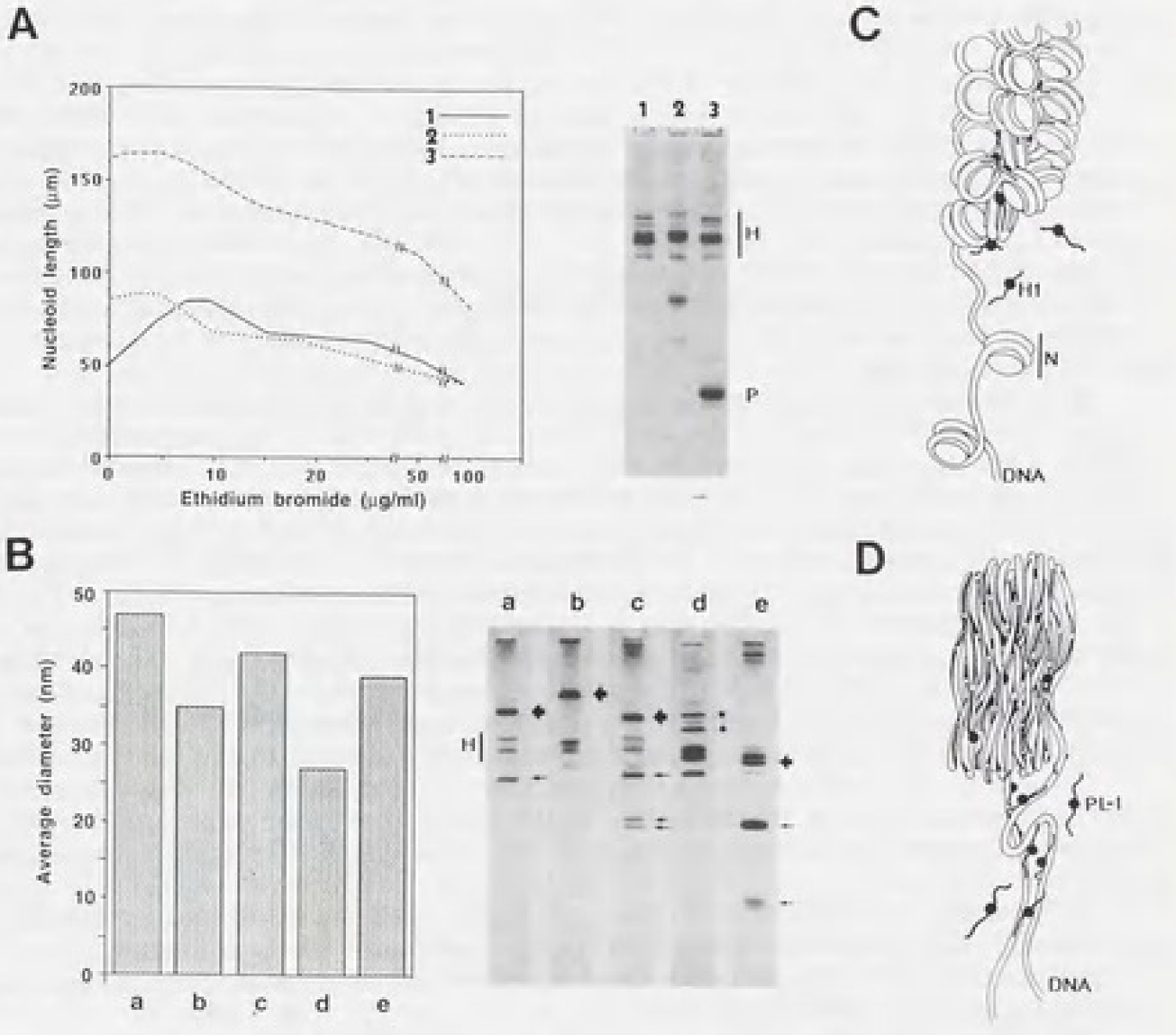

FIG. 3. — **A:** Change in the length of sperm nucleoids as a function of the ethidium bromide concentration. Figure redrawn from [56]. 1. Sperm nucleoids consisting of protein of the H type (*Rana catesbeiana*) exhibit a bipharic change. As a result of the interaction of the H type proteins (histones) with DNA, chromatin adopts a nucleosomal organization (see C). Nucleosomes stabilize DNA in a negatively supercoiled configuration. As the ethidium bromide concentration increases, the native negative superhelicity is increasingly lost (ethidium bromide intercalation twists DNA in a positive sense), DNA becomes more relaxed and the size of the nucleoid, therefore, keeps increasing until all negative supercoils have been removed (equivalence point ~8 µg/ml ethidium bromide). Further increase in the ethidium bromide beyond this point induces positive supercoiling. 2, 3. In contrast, sperm nucleoids consisting of protein of the PL (*Xenopus laevis*), 2, and P type (*Bufo fowleri*), 3, exhibit only a decrease in their dimensions as the concentration of ethidium bromide increases. This indicates that DNA has a relaxed conformation in the nucleoprotein structure arising from its interaction with these kinds of proteins. The 5% acetic acid, 2.5 M urea polyacrylamide gel electrophoresis (PAGE) shows the protein composition of the sperm nuclei of 1) *Rana catesbeiana*, 2) *Xenopus laevis*, 3) *Bufo* sp. **B:** Average diameter of the chromatin fibres from advanced spermatids of marine bivalve molluscs with different H- and PL-type protein compositions (modified from [22]). A 5% acetic acid, 2.5 M urea PAGE is also shown. a) *Ensis ensis*, b) *Callista chione*, c) *Donax trunculus*, d) *Pecten maximus*, and e) *Mytilus edulis*. The thick arrows point to the PL-I proteins and the thin arrows point to other PL protein components of smaller molecular mass. **C:** Upon interaction with histone H1, the polynucleosome filament folds into a higher order structure. This results in the 30 nm fibres observed in the sperm of organisms containing H-type chromosomal proteins. **D:** Hypothetical arrangement of the PL-DNA nucleofilaments in the (25-50 nm) chromatin fibres observed in advanced spermatids (as in B) of the organisms with a nuclear PL-type protein composition. H1, H2A, H2B, H3, H4, histone types, N, nucleosome, P, protamine.

Chromatin organization (somatic, H-type). Chromatin resulting from the interaction of DNA with proteins of the histone type (H-type) is organized in discrete nucleosome subunits. In the nucleosome, about 200 base pairs of DNA are wrapped in approximately two negative superhelical turns about a histone core octamer (consisting of "core histones" H2A, H2B, H3 and H4). Thus, DNA is stabilized by nucleosomes in a negative supercoiled state, which poises eukaryotic chromatin for genetic activity (replication, transcription). In addition to the "core histones", "linker histones" (proteins of the H1 histone family) bind to the linker DNA regions connecting the neighbouring nucleosome subunits and condense chromatin into higher order structure fibres of about 300 Å in diameter (Fig. 3C). Although electrostatic interactions play an important role in the maintenance of this organization, only about half of the negatively charged DNA phosphates are neutralized by the arginine/lysine side chains. This makes the nucleohistone complex very sensitive to environmental ionic conditions [65] and amenable for interaction with other regulatory proteins.

The realization that histones are not only passive structural blocks, but also functional elements [35] represents one of the most important landmarks in our understanding of the function-structure relationships of somatic chromatin. Despite the lack of DNA sequence specificity of the histone-DNA interactions, the resulting nucleosome structures may play, by themselves or in conjunction with other regulatory proteins [43, 75], a very important role in the modulation of the genetic activity of the chromatin complex [6]. The ability of different histone H1 (linker histones) subtypes to condense the chromatin fibre to different extents, and thereby possibly to be involved in its genetic activity, has also been postulated [25]. As will be discussed at the end of this chromatin section, the latter consideration is important in understanding the presence of proteins of the H type in the sperm of some organisms. All these functional and structural aspects of the H-type chromatin organization may explain why somatic histones have been conserved so invariably through evolution, in contrast with the proteins of the sperm.

Chromatin organization resulting from the interactions between the PL-P type proteins with DNA. I am next going to highlight briefly what I consider to be the most recent significant advances in chromatin organization resulting from the interaction of DNA with proteins of the PL and P type.

Although PL and P proteins usually coexist with a small amount of histones in the sperm nucleus [2, 14, 32], the structure of the nucleoprotein complexes arising from the interaction of these proteins with DNA lacks the nucleosomal organization of the somatic chromatin type, as can be envisaged by X-ray diffraction [7, 10, 63].

The overall negative superhelicity of DNA is lost [56] (Fig. 3A), most likely as a result of the topoisomerase II activity associated with the histone displacement/replacement by these PL or P proteins [46, 57]. Thus, the nucleohistone-nucleoprotamine (protamine-like) transition leads to a complete reorganization of chromatin, while possibly maintaining the specific three dimensional organization of DNA and its DNA loop domain structure [73].

The detailed molecular structures of the nucleoprotein (P, PL) complexes are still controversial. Both PL and P proteins interact electrostatically with DNA (which basically retains a B conformation [63]) to form fully saturated complexes, unlike the somatic nucleohistone [1, 10, 13]. In these complexes, the PL and P proteins have been postulated to adopt an α-helical like configuration [51, 66, 74]. The positioning of these proteins in the major or minor groove of DNA has not yet been experimentally settled [65]. Nevertheless, recent raman spectroscopy analysis suggest that fish protamine may adopt an unusual $1 \rightarrow 3$ γ turn (non α helical) structure interacting with the major groove of the DNA [36].

At the higher order level of organization, it has been shown recently [22] that PL proteins (Fig. 3B), like H type proteins, can organize the nucleoprotein complexes into 250-500 Å fibres regardless of the particular PL composition and the absence of nucleosome-like structures. This is an important finding because it indicates that the higher order structures of the nucleoprotein complexes are mainly determined by the ionic nature of the interactions involved [31, 67], rather

than the particular structure of the proteins (H, PL or P) itself. Intermediate 300-400 Å fibres have also been described during the process of chromatin condensation in the sperm of cephalopods [44], salmon [77], lizards [19] and humans [62], all containing P type proteins.

Sperm chromatin variability

After analyzing the different types of chromatin structures, the next question that arises is, what is the reason for so much chromatin variability in the sperm? Although there is no clear cut answer to this question, in what follows I will discuss several points which may provide a useful hint into the problem.

The only functional theme that all the sperm chromatin types I through IV, in Fig. 1B, have in common is the complete shut off of the genetic activity in the mature spermatozoon.

Since H type is the protein type which is always present at the onset of spermiogenesis in any of these groups, regardless of the final nuclear protein composition, it is clear from what has been mentioned earlier, that the silencing of the genetic activity at this point can be achieved in several different ways.

One possible way (type I of spermiogenesis) would be the complete erasing of the starting chromatin structure (including nucleosomes, and other chromatin associated regulatory proteins). An alternative way could be by increasing the ratio between linker histones and core histones or by using specific linker histones [54] that lock chromatin in a functionally inert structure (type II, Fig. 1B). A third possibility would be the replacement of the H type proteins by highly charged sperm-specific PL or P proteins (types III and IV of Fig. 1B) which remove the nucleosomal organization and negative superhelicity of DNA. Thus, in addition to the genetic repressive effect, types I, III and IV also have in common the erasing of the nucleosome imprinting of the stem cells. It is not clear yet whether or not in the case of type II a reorganization (randomization) of the nucleosome positioning takes place upon binding of the specific linker histones, which could produce a similar erasing effect.

While we do not know exactly why type P has been increasingly selected throughout evolution, several arguments can be made. Although the four types appear to be equally efficient from the two previous points of view, it is obvious that type I leaves the genome more exposed to possible damage by physical/chemical mutagenic agents. Types II, III and IV differ mainly in the extent of chromatin compaction achieved. Substitution of histone by PL (and removal of the otherwise unnecessary nucleosomes) and finally by P leads to an increasingly more compacted nucleus that may have finally been selected by the constraints imposed by the mechanisms of fertilization [40] or by other selective pressure mechanisms yet to be established.

Evolution of the nuclear sperm-specific proteins

From what has been discussed in the previous sections, it is clear that the PL-type represents an intermediate type both from the structural and functional point of view. Whereas protamines have only been found at the tips of the most evolved groups from both the protostome and deuterostome branches, PL proteins are already found in the eukaryote groups preceding this branching. A rather exhaustive analysis recently carried out in our laboratory on several organisms belonging to different groups of the phylum Cnidaria has revealed that a primitive H1-PL-I protein is the major chromosomal protein found in the sperm nucleus of these organisms (unpublished results, but see Fig. 4A). Evidently the intermediate structural features of the PL proteins, discussed earlier, must reflect their intermediate evolutionary position.

All this, provides support for the early hypothesis put forward by SUBIRANA several years ago that sperm-specific nuclear proteins, including protamines, may have evolved from a common histone ancestor [64]. In the light of the experimental information presently available, we propose the evolutionary pathway which is shown in Fig. 5A. Accordingly, all the chromosomal sperm

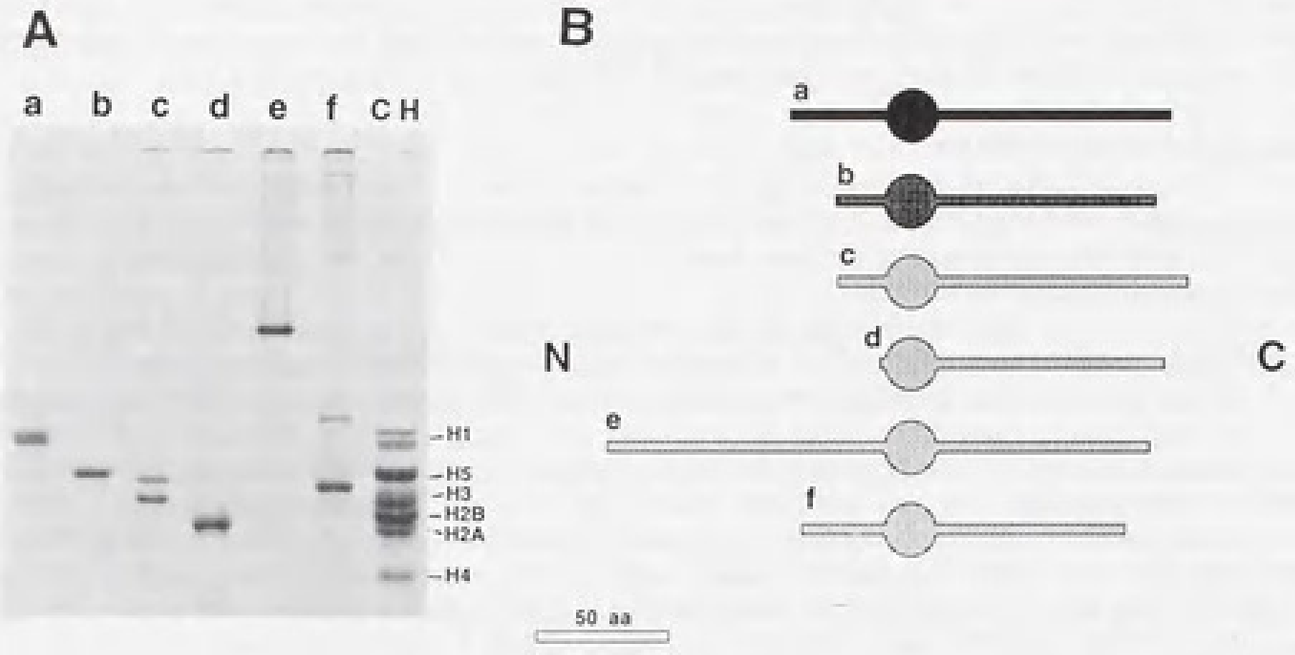

FIG. 4. — Widespread distribution of PL-I proteins in the eukaryotic kingdom. **A:** Urea acetic acid polyacrylamide gel electrophoresis of (a) calf thymus somatic histone H1, (b) chicken erythrocyte histone H5, (c-f) nuclear proteins from the sperm of (c) *Mullus surmuletus* (fish), (d) *Chelysoma productum* (tunicate), (e) *Spisula solidissima* (clam) and (f) *Metridium senile* (anemone). (The arrows point to the PL-I protein components.) **B:** Comparative schematic representation of the teriary structure (circle = globular core, linear regions = N, C terminal domains) of the protein PL-I from different organisms and somatic histones H1, H5. The letter symbols designate the same species as in (A). CH = chicken erythrocyte histone standard.

proteins would have arisen from a primitive histone precursor, presumably the same from which the somatic lysine-rich histone H1 lineage would have also originated. The enormous structural variability and the rapid evolution [37] of the proteins from the H1 family make it very difficult to trace the origin of such a precursor and its identification with any protein previous to the metazoan organization (see KASINSKY, this volume).

The next step in the evolution of these proteins in the case of the sperm lineage would involve an increase in the arginine contents of this originally lysine-rich precursor. In the early stages such protein would still coexist with a full complement of the somatic-type of histones (histone H type of spermiogenesis, see Fig. 1B). The sperm-specific H1 histones of the sperm of echinoderms provide a good example of such histones. A further increase in the arginine content would allow these molecules to displace and replace the somatic histones, as occurs with the PL-I molecules found in the sperm of many bivalve molluscs [2, 4, 5, 13]. It is important to point out here that PL-I is not only restricted to molluscs. As shown in Fig. 4A, PL-I proteins have been identified in many phylogenetic groups, including chordates [59] and vertebrates [61].

In the next evolutionary step the PL-I proteins would lose their globular core, giving rise to smaller PL proteins either by processes of post-translational cleavage (Fig. 5B), such as in *Mytilus* [21], or by other mechanisms yet to be described, such as alternative splicing. Evidence for alternative splicing has already been provided for the bovine protamine 2 gene [41].

The small PL proteins may have then evolved into the arginine-rich protamines, which are found in the upper phylogenetic levels of the deuterostome and protostome branches. In the process of selection, arginine may have been selected over lysine because of its higher potential to form hydrogen bonds [11].

The evolutionary pathway from histones to specialized sperm-specific histone H1, PL-I, PL

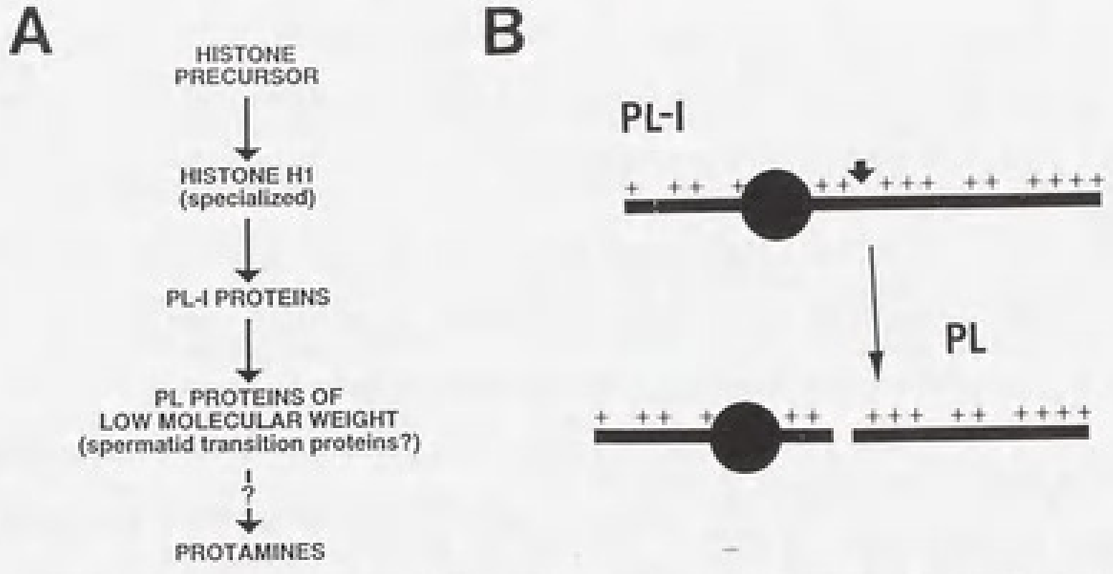

FIG. 5. — **A:** Proposed evolutionary relationship for the nuclear sperm-specific proteins. **B:** Some of the low molecular weight PL proteins (Mr < 15000 Da) might have arisen from post-translational cleavage of a larger PL-I precursor [21].

and protamines appears in repeated instances within different phyla both in protostomes [69] and in deuterostomes [60]. The presence of the most primitive protein types, H and PL, decreases as the P type increases in the most evolved groups of the phylogenetic tree. Thus, the scheme shown in Fig. 5A represents, in fact, the "mode" rather than the "tempo" [30] of evolution followed by the chromosomal sperm proteins.

The selection of an H1-related protein type at the base of this evolutionary pattern in each different phylum may have occurred initially by a process of evolutionary convergence due to the intrinsic ability of this molecule to condense chromatin and possibly to lock it in an "inert" structure. This could have happened at the expense of increasing the arginine content of this protein and/or the stoichiometric ratio of this molecule with respect to the nucleosome subunit. In this process the tripartite organization of histone H1 and most of the secondary structure of its globular core have been significantly conserved and therefore both functional and structural convergence most likely occurred in this case. At the other end of this evolutionary pattern, all protamines exhibit a highly arginine-rich composition with very similar primary sequences consisting of arginine clusters (see KASINSKY, in this volume). The possibility therefore exists that this could represent a convincing case of genuine sequence convergence [29] starting from different PL-I proteins. It should be pointed out, however, that such a possibility is hard to prove considering the small size (30-80 amino acids) of protamines, which excludes the possibility of long range cladistic analysis [28].

The evolutionary "mode" of the nuclear sperm-specific proteins presented here represents an alternative to the "retroviral hypothesis" proposed for the origin of protamines [38]. This hypothesis was initially put forward in order to account for the sporadic distribution of the protein types H and P in fishes. As a matter of fact, a recent thorough reexamination of the distribution of the three protein types H, PL, and P in the sperm of bony fish [61] has not been able to provide any support to the retroviral origin of protamines in this vertebrate class.

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