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Evolution of vertebrate chromosomal sperm proteins: implications for fertility and sperm competition

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The three major types of sperm nuclear basic proteins (SNBPs), histone (H type), protamine-like (PL type) and protamine (P type), are well represented in vertebrates. The three groups are evolutionarily related through a vertical evolutionary process (H → PL → P) that involves a transition from lysine to arginine-rich proteins and results in a sporadic but non-random distribution that can be phylogenetically traced. The arginine-rich P type has been selected in the course of evolution of the vertebrates, probably due to constraints imposed by internal fertilisation. Protamines are subject to a positive Darwinian selection process that results in the characteristic fast evolutionary rate shown by these proteins. This makes their use very suitable for the reconstruction of phylogenies of the different vertebrate groups. In mammals, two different types of protamines (P1 and P2) are present which, in the course of the evolution of this vertebrate group, have undergone a further transition to cysteine-rich proteins which further enhanced their DNA packing efficiency. From a functional perspective, protamines provide the most efficient packaging of sperm chromatin and can probably influence the shape of the sperm nucleus and chromatin stability, both of which have direct implications for fertility. In mammals, alterations of the ratio between P1 and P2 protamines as well as the ratio between histones and protamines are important determinants of sperm fertility. All of this suggests a potential involvement of protamines in sperm competition which is discussed in this paper.

"[Ramon y Cajal] would envisage the sperm-cells as activated by a sort of passionate urge in their rivalry for penetration into the ovum cell."
(Sherrington, 1949)

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Evolution of vertebrate sperm nuclear basic proteins

Chromatin, the complex of histones, non-histone proteins and DNA, is a dynamic structure. The core subunit of chromatin, known as the nucleosome core particle, is composed of 146 base pairs of DNA wrapped around an octamer of core histones (H3, H4, H2A and H2B). A linker histone (H1) protects an additional 20 base pairs to form the chromatosome (van Holde, 1988). Altering or remodeling of this structure is important for regulation of many nuclear metabolic events such as, regulation of gene expression, DNA replication and DNA repair. One of the most dramatic examples of chromatin remodeling occurs within male germ cells, where changes in both the protein composition and compaction level of the chromatin take place (Caron *et al.*, 2005). During the postmeiotic maturation of sperm (spermiogenesis), chromatin becomes supercondensed and transcriptionally inert. High compaction of the sperm haploid genome is needed to allow for a more hydrodynamic sperm head and may also protect the DNA from physical and chemical damage (Braun, 2001).

The chromosomal proteins involved in the organization of the mature sperm chromatin are generically known with the name of sperm nuclear basic proteins (SNBPs). They can be grouped into three categories: protamine (P type), protamine-like (PL type) and histones (H type) (Ausió, 1995, 1999). All three types of SNBPs are structurally analogous and condense DNA into chromatin fibers of 300-500 Å (Casas *et al.*, 1993), regardless of the structure of the individual nucleoprotein complexes (Eirín-López *et al.*, 2006a).

Organisms containing SNBPs of the P type are well represented amongst vertebrates and have been found in all classes with the exception of Agnatha (see Fig. 1). In these organisms the histones from the progenitor germ cells at the onset of spermatogenesis are replaced by protamine in the late stages of spermiogenesis (Oliva and Dixon, 1991). In mammals, somatic histones undergo post-translational modifications as well as replacement with specialised histone variants during meiotic prophase [reviewed in Lewis *et al.* (2003a); Govin *et al.* (2004); Kimmins and Sassone-Corsi (2005)]. Immediately after meiosis, histones are replaced by transition proteins (TNPs) (Meistrich *et al.*, 1978, 2003), which are unique to mammals. During spermiogenesis, the transition proteins are replaced by protamines, which vary greatly in amino acid sequence, but are all small and have a very high arginine content, which is often much greater than 30%. The high charge density of these arginine-rich proteins allows them to bind DNA with high affinity and to more efficiently shield the charges on the DNA phosphate backbone, compared to somatic histones, resulting in maximal compaction of the genome. Many mammalian protamines have cysteine residues which are involved in disulfide crosslinking that further contributes to the compaction of chromatin fibers (Lewis *et al.*, 2003b).

Some amphibians and fish have structurally intermediate protamine-like proteins in their sperm (PL type) (Fig. 1). These are highly heterogeneous but generally have a high combined arginine + lysine content (35-50%) and are closely related to histone H1 (Ausió, 1995, 1999). Alternatively, some amphibians and fish retain histones in their mature sperm (H type). Often sperm-specific histone variants of either linker histones or core histones or both are present in these instances. For example, frogs of the genus *Rana* have sperm containing sperm-specific histone H1s, which have higher lysine contents than the *Rana* somatic H1s, as well as a full somatic type histone complement (Itoh, Ausió and Katagiri, 1997).

From the examination of SNBPs from many invertebrates and vertebrates it was proposed that SNBPs had undergone a vertical evolution H → PL → P (Saperas *et al.*, 1994; Ausió, 1995, 1999). Although a link between PL proteins and linker histone H1 had been proposed (Ausió, 1995, 1999), it remained unclear how a high lysine content, which is characteristic of the members of the histone H1 family, could have given rise to arginine rich PL and P proteins.

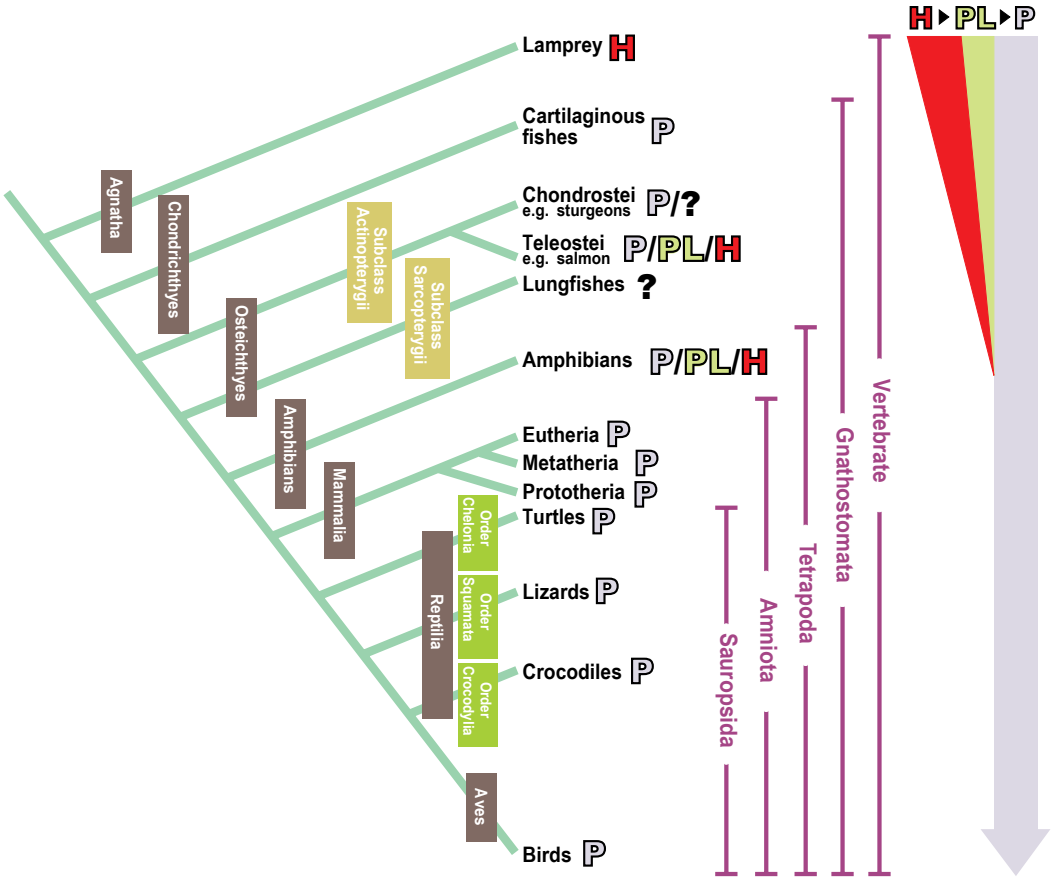

Figure 1. Cladogram of the subphylum Vertebrata showing the currently accepted relationships of monophyletic groups making up the subphylum. Each branch represents a monophyletic group. The arrow shown on the right hand side depicts the direction and approximate distribution of the three main types of SNBPs (in different colours) during the course of evolution of this group. H: histone type; PL: protamine-like type; P: protamine type. It has been proposed that protamines (P type) may have evolved from a histone H1-related protein as indicated at the base of the large arrow (Ausió, 1999; Lewis *et al.*, 2004; Eirin-Lopez *et al.*, 2006).

Two recent studies have shed light on the possible mechanisms underlying this change, lending direct support for a link between H1s, PLs, and protamines (Lewis *et al.*, 2004; Eirín-López *et al.*, 2006b). In the first study, examination of the SNBPs of two closely related species of tunicates, *Styela montereyensis* and *Ciona intestinalis*, revealed the presence of proteins of the PL type in their sperm (Lewis *et al.*, 2004). However, while in *S. montereyensis* the PLs were arginine rich, in *C. intestinalis* the PLs were lysine-rich. Detailed analyses of the coding nucleotide sequences suggested that a single frameshift mutation may have allowed lysine-rich clusters in the C-terminal tail of *C. intestinalis* to convert to arginine-rich clusters in *S. montereyensis*, establishing the evolutionary link between PL and protamine type SNBPs (Lewis *et al.*, 2004). A second report, focused on the evolutionary relationships shown by members of

the histone H1 family and the SNBPs of the PL type, revealed the presence of a common origin for both groups of proteins (Eirín-López *et al.*, 2006b). In this regard, it now seems clear that H1 histones and PLs are descendants of an ancient group of 'orphon' H1 replication-dependent histones, which were excluded to solitary genomic regions of the genome as early in metazoan evolution as before the differentiation of bilaterians. This 'orphon' lineage was ultimately responsible for the origin of the replication-independent somatic H1 lineage (as histone H5 and H1°), as well as SNBP lineage. Both of these lineages were subject to a purifying process, that shifted towards adaptive selection in the case of the SNBP lineage at the time of the lysine to arginine transition (Eirín-López *et al.*, 2006b). Due to the more efficient DNA condensation properties of arginine (Puigdoménech *et al.*, 1976; Hélène and Lancelot, 1982; Ausió *et al.*, 1984), proteins with a high global content of this amino acid would have been positively selected for during the course of evolution.

The evolution of SNBPs through a H $\rightarrow$ PL $\rightarrow$ P process involving a primordial replication independent histone H1 (Ausió, 1995, 1999; Eirín-López *et al.*, 2006a; Eirín-López *et al.*, 2006b) lends support to the old concept of a potential relationship between ontogeny and phylogeny (Bloch, 1976; Ausió, 1995, 1999). Indeed, during mammalian spermiogenesis a gradual transition in the composition of chromosomal proteins is observed that involves highly specialized H1 histones [H1t (Seyedin and Kistler, 1980), HILS1 (Iguchi *et al.*, 2003; Yan *et al.*, 2003), Hanp1/H1T2 (Martianov *et al.*, 2005; Tanaka *et al.*, 2005)] that precede and/or coexist with TNPs (Meistrich, 1989; Meistrich *et al.*, 2003) which are ultimately replaced by protamines (Lewis *et al.*, 2003b) in the mature spermatozoa.

The different SNBP types display a heterogeneous distribution across the different vertebrate groups (Fig. 1). Nevertheless, as it was shown in fish, the sporadic appearance of the different types is not random and follows the vertical evolutionary phylogeny of the groups where these proteins are present (Saperas *et al.*, 1994). As can be seen from Figure 1, the occurrence of protamines is seen in vertebrates as ancient as sharks and other cartilaginous fishes. Agnatha, which are a superclass of primitive jawless fish (lamprey and hagfish) have sperm that only contain histones and thus are of the H type (Saperas *et al.*, 1994, 1997). In contrast, all the species examined within the class Chondrichthyes (cartilaginous fish), which includes the sharks, skates and rays, are of the P type. Within the subclass Actinopterygii, or ray-finned fish, representatives from all SNBP types are present (Chiva, 1995). The examined sturgeons and paddle fish which are within the subclass Chondrostei contain protamines, whereas the teleost fish or bony fish (the largest group of living fish) are more diverse containing organisms of the H, PL and P types, even though they are all within the subclass Sarcopterygii. A similar sporadic SNBP distribution is seen within the class Amphibia (Kasinsky, 1989). It is apparent from the analysis of the SNBP composition of both fishes and amphibians that a lysine to arginine transition (or divergence from H type to PL type to P type) likely occurred multiple times in the course of their evolution. It has been suggested that the driving force behind this evolution in fish and amphibians may be differing constraints placed on the sperm by internal versus external fertilisation (Kasinsky, 1989, 1995). There is a correlation that suggests that the harsh and viscous environment that sperm are subjected to within female reproductive tracts during internal fertilisation may select for protamines (Mann *et al.*, 1982; Kasinsky *et al.*, 1985; Kasinsky, 1989, 1995).

Following the appearance of Amniota, all organisms contain protamines in their sperm (P type) (Fig. 1). This may suggest an evolutionary trend towards the use of protamines to package sperm DNA in 'higher' vertebrate organisms. The replacement of histones with protamines is typical of taxa located at the uppermost tips of evolutionary branches (Ausió, 1999). In mammals, two types of protamines have been identified: protamine P1 and the protamine P2 family. For the P2 family, proteolytic cleavage of the N-terminus of the P2 precursor protein yields

the form of the protein that is present in the mature sperm (Sautiere *et al.*, 1988; Hecht, 1989; Lewis *et al.*, 2003b). Although P1 has been found in all species studied, P2 is exclusively expressed in only a few eutherian organisms, including human and mouse (Oliva, 2006; and references therein). An additional compositional transition in the course of vertebrate SNBP evolution took place in mammals where some of their protamines became rich in cysteine (Oliva and Dixon, 1991; Lewis *et al.*, 2003b). This residue is absent from metatherian protamines and is uncommon in other chromosomal proteins (van Holde, 1988). Cysteine first appears in the protamines of placental (eutherian) mammals and it is well established in both the P1 and P2 protamine lineages. Cysteine also appeared later in the marsupial P1 protamines of the genus *Planigales* through a process of convergent evolution (Retief *et al.*, 1995a). The acquisition of cysteine, which can form inter- and intramolecular disulfide bonds (Vilfan *et al.*, 2004), adds stability and increases the compaction of the nucleoprotamine complexes. The lysine to arginine conversion in the transition from H to PL SNBPs and the acquisition of cysteine by the P type during the course of evolution of vertebrate SNBPs is highly reminiscent of the similar compositional transitions that have been observed in the equivalent invertebrate SNBP types (Lewis *et al.*, 2003b).

The importance of being a protamine and the evolution of vertebrate protamines

Despite the continued effort to characterise protamines from different representative groups of vertebrates, there is still an important lack of information on these proteins. Although protamines appear to be compositionally simple proteins (small and arginine rich), the amino acid distribution in the primary structure of protamines does not seem to be random. In fact, as it will be described in the following sections, the amino acid sequences of these proteins appear to be critical for their function and can be used to trace their evolution.

Protamines exhibit a large structural heterogeneity, as can be seen from the large extent of variation observed in their electrophoretic mobility and primary structure (Fig. 2). Despite this diversity, their amino acid sequence stores very valuable phylogenetic information (Oliva, 1995; Lewis *et al.*, 2003b). Figure 2A provides an electrophoretic analysis of protamines of representative organisms from different vertebrate groups (see Fig. 1) and Figure 2B outlines their corresponding amino acid sequences. We have shown in the past that it is possible to use this kind of sequence information to produce phylogenetic trees that closely resemble those obtained using other molecular markers and/or those based on paleontological/anatomical traits (Lewis *et al.*, 2003b). For instance, we were able to generate a phylogeny for the closely related species of sticklebacks that was indistinguishable from that generated from morphological and behavioural analysis of this group of Gasterosteoidei fish (Giménez-Bonafé *et al.*, 2000). Using a broader range of teleost fish species, for which there is a relatively abundant amount of sequence information for protamines, it was possible to reconstruct a phylogenetic tree which was almost identical to that currently available from the use of conventional markers (Frehlick *et al.*, 2006b). The protamine sequence information has also been useful in confirming the close relationship between birds and reptiles (Sauropsida) (Hunt *et al.*, 1996; Ausió *et al.*, 1999) and has additionally provided support to the monophyletic origin of Paleognathae birds (ostriches, emus, tinamou) (Ausió *et al.*, 1999). Similar phylogenetic results have been obtained with different groups of mammals (also see below) (Retief *et al.*, 1993; Queralt *et al.*, 1995; Retief *et al.*, 1995c).

The fidelity with which protamines work as phylogenetic markers is primarily due to the rapid evolution of these proteins (Oliva and Dixon, 1991; Oliva, 1995; Lewis *et al.*, 2003b), a trait they share with other reproductive proteins (Swanson and Vacquier, 2002). This fast evolu-

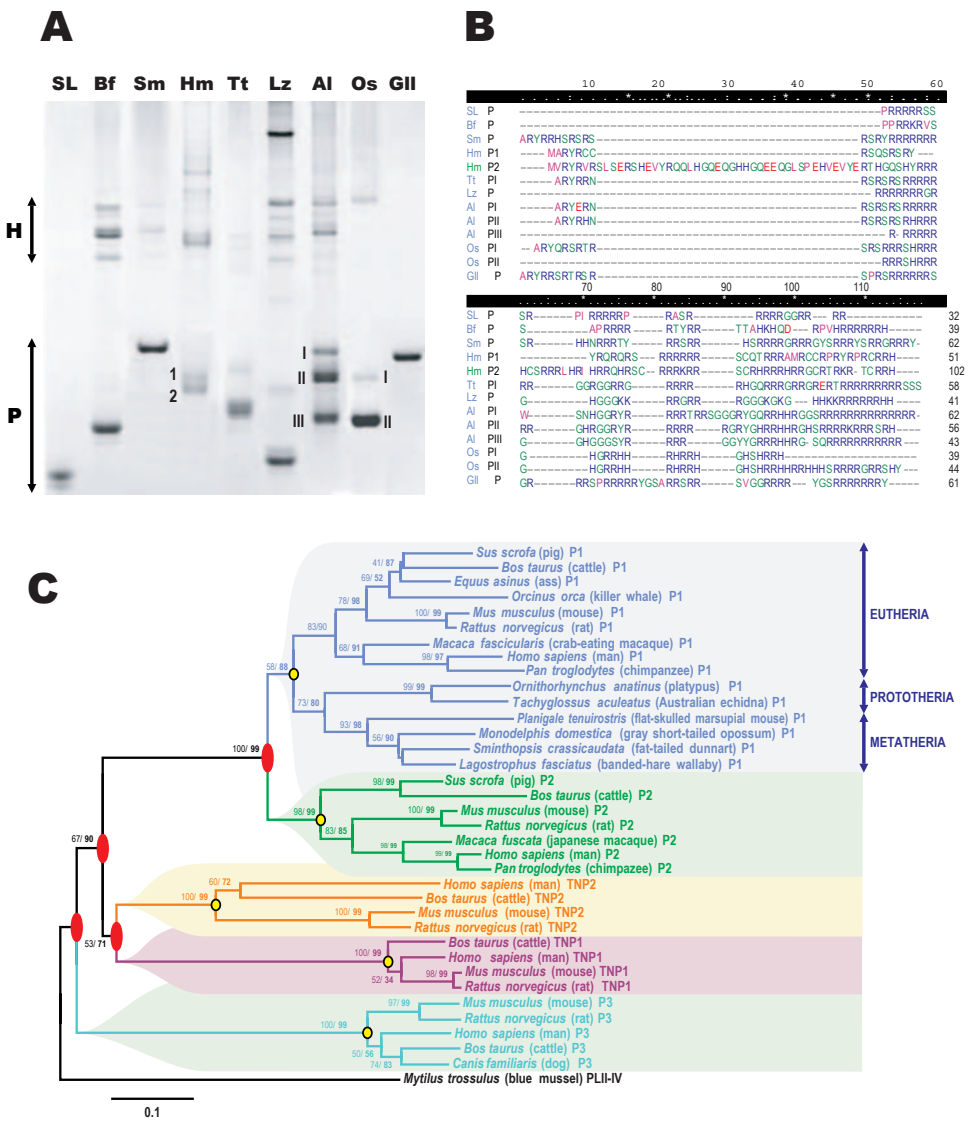

Figure 2. (**A**) Acetic acid-urea polyacrylamide gel electrophoresis (AU-PAGE) of protamines from several representative species of the main monophyletic groups of the subphylum Vertebrata shown in Figure 1. Al: *Alligator mississipiensis* (alligator); Bm: *Bufo marinus* (cane toad); Gll: *Gallus gallus* (rooster); Hm: *Homo sapiens* (human); Lz : *Anolis carolinensis* (green anole, lizard); Os: *Struthio camelus* (ostrich); SL *Oncorhyinchus keta* (chum salmon, salmine); Sm: *Sminthopsis crassicaudata* (fat-tailed dunnart); Tt: *Chrysemys picta* (painted turtle). H: histones; P: protamines. Amino acids are colour-coded according to their side-chain properties: Blue, basic; green, polar uncharged; pink, non-polar hydrophobic; red, acid. (**B**) Amino acid sequences of the protamines from the species shown in (A). The sequences have been aligned using the BIOEDIT (Hall, 1999) and CLUSTAL_X (Thompson *et al.*, 1997) programs. (**C**) Phylogenetic relationships between different mammalian chromosomal sperm proteins (P: protamines; TNP: transition proteins; PL: protamine-like proteins). The amino acid sequences were aligned as in B and the phylogenetic tree derived from this alignment was constructed as described elsewhere (Eirin-Lopez *et al.*, 2006). Numbers for the interior branches represent the BS (bootstrap probability) values followed by the CP (confidence probability, internal-branch test) values based on 1000 replications and are only shown when their value is higher than 50 % (Eirin-Lopez *et al.*, 2006).

tion has been ascribed to positive Darwinian selection (Rooney and Zhang, 1999; Clark and Civetta, 2000; Wyckoff *et al.*, 2000; Swanson and Vacquier, 2002) and involves an unusual form of purifying selection that is responsible for maintaining the overall high arginine composition of these proteins regardless of the specific position of the residues in the molecule (Rooney *et al.*, 2000).

We have taken advantage of all this and revisited here (Fig. 2C) the phylogenetic analysis of mammals using the SNBP sequence information available to date for this vertebrate group. The phylogenetic relationships between mammalian protamines and transition proteins bear an enormous resemblance to those obtained for these proteins when analysed within a broader phylogenetic tree that included SNBPs from all types belonging to both deuterostome and protostome organisms (Eirín-López *et al.*, 2006c). Accordingly, mammalian protamine P3 encoded by the *PRM3/GENE4* gene of the human protamine locus (Nelson and Krawetz, 1994; Kramer and Krawetz, 1998) appears to be more closely related to invertebrate PL proteins than to any other protamines (including mammalian P1 and P2 protamines encoded by *PRM1/PRM2* genes) (Eirín-López *et al.*, 2006b), a fact that could be taken as indicative of the more primitive origin of this mammalian protamine type. As pointed out by Oliva (2006) the name protamine for the protein encoded by *PRM3* may be misleading as it contains a large amount of glutamic acid which is uncharacteristic of a DNA binding protein. Interestingly however, the phylogenetic analysis suggests that a P3 ancestor may have been the precursor of mammalian TNP and P1 and P2 protamines (see Fig. 2C), in contrast to earlier propositions which suggested that *PRM3* was derived from *PRM1* (Kramer and Krawetz, 1998). It is then not surprising that, *PRM3* (Schluter and Engel, 1995; Schluter *et al.*, 1996; Kramer and Krawetz, 1998) and the *PRM1*, *PRM2* and *TNP2* genes are found in the same vicinity within the same protamine locus (Nelson and Krawetz, 1993).

As can be seen in Figure 2C, the analysis not only provides useful information about the potential evolutionary links between the SNBPs of mammals but their protamines (Oliva, 1995), in particular protamine P1, accurately define the phylogeny of the group. The Metatheria (Marsupialia) (Retief *et al.*, 1995a,b), Prototheria (Monotremata) (Retief *et al.*, 1993) and Eutheria (Oliva, 1995; Queralt *et al.*, 1995) appear well defined, thus providing yet another example of the usefulness of protamines as highly reliable phylogenetic markers.

In addition to all this, the tree shown in Figure 2C also suggests that transition proteins arose earlier in evolution than mammalian P1/P2 protamines and that P1 and P2 are closely related, with the slightly more recent appearance of the P2 type likely arising by gene duplication of a P1 precursor (Krawetz and Dixon, 1988). The evolutionary path followed by these mammalian SNBPs is reminiscent of the sequential order in which these proteins appear during spermatogenesis. Together with the close relation of TNPs to invertebrate PLs (Eirín-López *et al.*, 2006c) all this lends additional support to the notion of a relationship between the ontogeny and phylogeny of the three main SNBP types (Bloch, 1976; Ausió, 1995, 1999).

Do SNBPs affect sperm shape?

Amphibians provide an excellent system for a first approximation to the answer of this question as this vertebrate group contain species that are representative of each of the three main SNBP types (Frehlick *et al.*, 2006a) (Fig. 3).

A few preliminary considerations about chromatin organization need to be introduced here before further discussion on the topic. The association of histones, PL proteins and protamines with DNA results in nucleoprotein complexes with a fiber-like organization of 300-500 Å (van Holde, 1988; Casas *et al.*, 1993; Saperas *et al.*, 2006) that is ultimately determined by an

overall energy minimization of the complex and not by the specific nature of the protein–DNA interactions described next (Subirana, 1992). In the H type, such as in *Rana*, the chromatin is organised in a 300 Å fiber consisting of approximately six nucleosomes per turn (see Fig. 3D, H). In contrast, the invertebrate (Ausió and Subirana, 1982) and vertebrate (Saperas *et al.*, 2006) PL type chromatin consists of irregular parallel DNA bundles. In mammalian protamines, chromatin loops are organised in toroidal structures containing similar parallel nucleoprotamine bundles (Hud *et al.*, 1995; Ward and Zalensky, 1996; Balhorn, 1999; Brewer *et al.*, 1999) (see Fig. 3D PL/P).

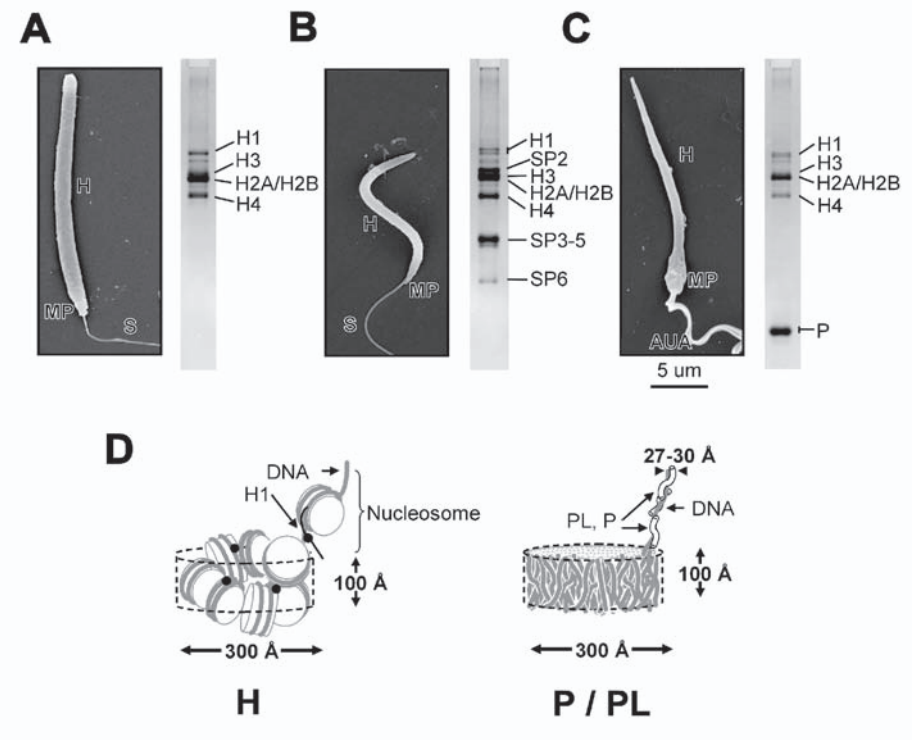

Figure 3. Scanning electron microscopy micrographs of the sperm heads (lanes 1) and AU-PAGE analysis of the SNBPs (lanes 2) of three different species of amphibians: (**A**) *R. catesbeiana* (bullfrog); (**B**) *X. laevis* (African clawed frog); (**C**) *B. marinus* (Cane toad). AUA: axoneme-undulating membrane-axial rod; H: head, MP: midpiece and S: single flagellum only with axoneme. (**D**) Schematic representation of a 100 Å cross-section of a 300 Å fiber of a histone-containing (H) or a P/PL-containing sperm chromatin fiber (see text for more details).

With the structural information currently available for these different types of chromatin organizations, it is possible to theoretically calculate the extent of DNA compaction achieved by each of them (see Fig. 3D). If we consider a 110 Å thick section and the DNA retains its B conformation (Ausió and Subirana, 1982; van Holde, 1988) with an average raise of 3.4 Å per base pair, it is possible to fit 100 nucleoprotein (P/PL) bundles (of approximately 30 Å in diameter) into a PL/P chromatin fiber with a 300 Å diameter. If the DNA was fully stretched this would amount to approximately (100 / 3.4) x 100 (complexes) = 2900 base pairs of DNA compacted within this cross section. In the H type, chromatin is organized in discrete subunits (nucleosomes) each consisting of approximately 210 base pairs of DNA. In the presence of histone H1, the nucleosome

arrays can fold into a higher order structure consisting of approximately 6 nucleosomes per turn and a 300 Å diameter. Since the nucleosome is approximately 100 Å tall, this implies that about 1300 base pairs of DNA are compacted within a 100 Å by 300 Å section. Thus, the PL/P– DNA complexes can compact DNA about twice (2.2) as densely as the complexes of the H type.

We have used several scanning electron microscopy (SEM) pictures of *Rana catesbeiana* (H type), *Xenopus laevis* (PL type) and *Bufo marinus* (P type) sperm whose haploid C-value ranges are: 6.63-9.00, 3.00-3.85 and 3.98-5.65 picograms respectively (Gregory, 2006) to calculate the approximate sperm head volume/C-value ratios (v/C) and in doing so we observed that both *X. laevis* and *B. marinus* apparently exhibit the same v/C ratio, whereas the v/C ratio is approximately 1.7 times larger in *R. catesbeiana* (Fig. 3A-C). This higher value for the H type chromatin is in good agreement with the value theoretically calculated from the corresponding chromatin conformations, although it is a bit lower (1.7 vs. 2.2). The lower than expected compaction value could be attributed to the fact that the 300 Å nucleosomally organised chromatin fibers (Fig. 3 D, H) can interdigitate to some extent (Daban, 2003; Robinson *et al.*, 2006) increasing the theoretically calculated value.

While all this does not answer completely the question raised at the beginning of this section, it does demonstrate that, in general, histones are less efficient in packaging sperm chromatin than P and PL proteins. In addition to better compacting the sperm chromatin P and PL proteins erase the epigenetic contribution of histones (Caron *et al.*, 2005; Rousseaux *et al.*, 2005). These are most likely two of the important reasons why these protein types, especially protamines, have been selected throughout evolution (see Fig.1). In terms of sperm head shape it is difficult from just the three amphibian examples shown in Figure 3 to make any predictions other than the observation that while PL/P types seem to lead to more conical streamlined head shapes (Fig. 3 B,C), histones appear to result in rounder shapes (Fig. 3A). The morphology of the sperm head for those organisms containing exclusively protamines can be extremely heterogeneous across different taxa (Baccetti and Afzelius, 1976; Oliva and Dixon, 1991) and no SNBP-related rule appears to exist other than the previous generalization for H versus PL/P type.

It is very likely that SNBPs are not the sole determinants of the sperm nuclear shape. Mouse sperm co-expressing different amounts of a heterologous rooster protamine does not exhibit significant changes in its gross morphology (Maleszewski *et al.*, 1998). It has been shown that SNBP composition and regulation of the contraction of the perinuclear microtubule matrix may have co-evolved to produce the highly unique structural characteristics of the sperm nucleus and head, such as occurs with the cysteine-rich protamines of the nucleus of the sperm of the marine octopod *Eledone cirrhosa* (Giménez-Bonafé *et al.*, 2002). A synergistic effect between multiple protein components would explain the enormous sperm head shape diversity, when compared to the three main groups of SNBPs. However, at the level of closely related species or taxa is possible that protamines and their relative composition (when several molecular species are present, as is the case in mammals) may result in alterations of the overall sperm architecture, as has been observed in protamine deficient human spermatozoa (Blanchard *et al.*, 1990) and in protamine 2 deficient mice (Cho *et al.*, 2003). As the shape of the head may play an important role in sperm competition (Roldan, Gomendio and Vitullo, 1992) it would be worth analyzing the protamine composition of closely related species in relation to the sperm head morphology to determine the existence of any potential correlation.

Mammalian SNBPs and infertility

The multiplicity of SNBPs involved in mammalian spermiogenesis [H1t (Seyedin and Kistler, 1980), HILS1 (Iguchi *et al.*, 2003; Yan *et al.*, 2003), Hanp1/H1T2 (Martianov *et al.*, 2005;

Tanaka *et al.*, 2005), TNPs (Meistrich, 1989; Meistrich *et al.*, 2003) and P1/P2 protamines (Lewis *et al.*, 2003b)] suggests that alterations in any of these proteins could affect male fertility. In this regard, homozygous *Hanp1/H1T2* disrupted male mice are infertile (Tanaka *et al.*, 2005), *Tnp1/Tnp2* deficient mice exhibit an altered chromatin structure and reduced fertility (Yu *et al.*, 2000; Zhao *et al.*, 2001; Zhao *et al.*, 2004) and haplo-insufficiency of P1 or P2 causes infertility in the same organism (Cho *et al.*, 2001, 2003). In the last example, the effect is system-dependent as bull and boar lack protamine P2 as a result of mutations on this gene (Maier *et al.*, 1990) and, in contrast to mice, P2 expression is suppressed in rats (Bunick *et al.*, 1990).

The role of vertebrate SNBPs and protamines in male infertility has been the subject of two recent extensive reviews (Nishimune and Tanaka, 2006; Oliva, 2006). In humans, which retain some (*ca.* 15%) sperm-specific residual histones in the mature sperm (Gatewood *et al.*, 1987; Wykes and Krawetz, 2003; Churikov *et al.*, 2004), three main types of alterations that have been described so far which can affect fertility are: altered histone/protamine ratio, altered P1/P2 ratio and mutated P1or P2 (Fig. 4 A, B).

Alterations in the relative amount of histones with respect to the protamine content can alter the sperm morphology (Blanchard *et al.*, 1990) and have been described in infertile men (Hofmann and Hilscher, 1991; Foresta *et al.*, 1992; de Yebra *et al.*, 1993; Carrell and Liu, 2001; Zhang *et al.*, 2006). Although this type of protein imbalance is usually related to protamine deficiency (Zhang *et al.*, 2006), in some instances it may alternatively be the result of an incomplete replacement of histones during spermiogenesis (Oliva, 2006), as is observed in males with antecedents of cryptorchidism (Fernandez Valades *et al.*, 2001).

An increase in the P1/P2 ratios as a source of infertility has long been recognized (Chevaillier *et al.*, 1987; Balhorn *et al.*, 1988; Bach *et al.*, 1990; Blanchard *et al.*, 1990; Belokopytova *et al.*, 1993; de Yebra *et al.*, 1993; Bench *et al.*, 1998; de Yebra *et al.*, 1998; Mengual *et al.*, 2003; Aoki *et al.*, 2005). Interestingly, an increased level of P2 precursors has also been described, suggesting a potential role of protamine processing in infertility (Bench *et al.*, 1998; de Yebra *et al.*, 1998; Torregrosa *et al.*, 2006).

The potential role of SNBP mutations in infertility has been recently reviewed (Nishimune and Tanaka, 2006). Although this does not appear to be a common source of the problem, a few mutations have been described in both the *PRM1* and *PRM2* genes of infertile males. A single nucleotide polymorphism (SNP) (*c248t*) in the *PRM2* gene that results in early mRNA termination has been detected in an infertile patient that presumably results in haplo-insufficiency of P2 and is responsible for infertility (Tanaka *et al.*, 2003). More recently, an amino acid transition from R to S at position 34 of human P1 (see Fig. 4B) in a group of infertile patients has been described (Iguchi *et al.*, 2006). This mutation was shown to be the result of a heterozygous single nucleotide polymorphism and there are reasons to believe that it may be involved in infertility. The replacement of arginine by a serine in the context of the region of P1 where the transition takes place is interesting as it generates an RSR motif amenable to phosphorylation by SR-protein-specific kinase 1 (Papoutsopoulou *et al.*, 1999). Phosphorylation of the repetitive N-terminal "RS" domains and of protamine serine residues in general has been shown to play a critical role in the proper interaction and deposition of these proteins onto DNA (Bode *et al.*, 1977; also see Oliva and Dixon, 1991, and Raukas and Mikelsaar, 1999, for reviews) and hence are important for the proper organization of sperm chromatin. Thus, the appearance of an new internal serine in P1 could potentially have a very disruptive effect. In this regard, and as shown in Figure 4B, phosphorylation of S(34) introduces two negative charges that have the potential to neutralise the adjacent positive charge of the surrounding R(33)/R(35) leading to a significant modification of the protamine-DNA interaction and/or to changes in the local secondary

structure of the protein, both of which can result in an anomalous chromatin organization. Alterations of sperm chromatin structure have the potential to lead to infertility and altered binding of protamines to DNA may affect the proper formation of a male pronucleus after fertilisation.

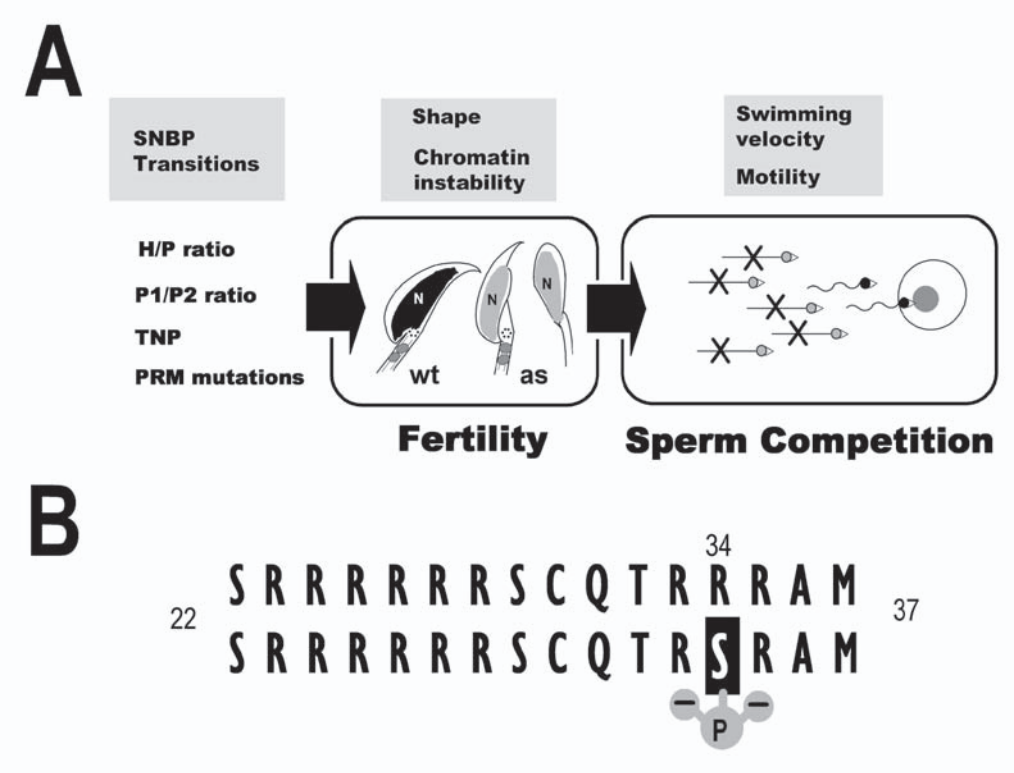

Figure 4. (**A**) Transitions in SNBPs can affect sperm morphology (shape) and chromatin instability, affecting fertility. Schematically shown is a representation of mouse sperm heads of the wild type (wt) or exhibiting an altered shape (as) resulting from a protamine 2 deficiency (Cho *et al.*, 2003) [redrawn from pictures shown in Fig. 2 from Cho *et al.* (2003)]. In conjunction with other cellular changes it is possible that the SNBP-mediated conformational changes may have a significant effect on sperm mobility (in this representation X indicates reduced mobility) which may play an important role in sperm competition (see text for a detailed discussion). H: histones; N: nucleus, P: protamines; TNP: transition proteins. (**B**) A recently discovered single nucleotide polymorphism leads to an amino acid transition (R to S) at position 34 of human protamine 1 and may participate in infertility (Iguchi *et al.*, 2006).

Can protamines play a role in sperm competition?

Support for the notion that protamines may have a role in sperm competition comes from evolutionary studies carried out with these proteins. It has been proposed that the positive Darwinian selection of protamine P1 in primates may have been driven by sperm competition (Rooney and Zhang, 1999; Wyckoff *et al.*, 2000; Swanson and Vacquier, 2002). However, the structural determinants involved in the process are not known. If protamines can have some direct or indirect effect on sperm shape and chromatin stability (Fig. 4A), altering the overall mobility

and having implications for infertility (Cho *et al.*, 2003), the exciting possibility exists that protamines may also play a role in sperm competition (Fig. 4A). It has also been shown that expression of a heterologous avian protamine from rooster in transgenic mice (Rhim *et al.*, 1995) resulted in reduced mobility and lower fertility (Maleszewski *et al.*, 1998). Therefore, it appears that protamines may have a significant effect in sperm competition, however further research in this area is still needed.

Recent work with Iberian red deer (*Cervus elaphus hispanicus*) has shown that the hydrodynamic shape of the sperm head in conjunction with the force generated by the flagellum play a critical role in determining sperm swimming velocity (Malo *et al.*, 2006). Also, a unique morphological conformation has been described for the falciform head of the wood mouse (*Apodemus sylvaticus*) sperm which results in sperm cooperation where distinctive aggregations of genetically related sperm results in the increase of the sperm's progressive motility (Moore *et al.*, 2002). As sperm swimming velocity and motility are very important determinants of male fertilisation success (Birkhead *et al.*, 1999; Froman *et al.*, 1999; Levitan, 2000; Gage *et al.*, 2004) the shape of the sperm head may be a component in sperm competition (Roldan *et al.*, 1992). In this hypothetical setting the influence of the protamine P1/P2 composition in the shape of the mice sperm heads is quite revealing and lends further support to the notion that protamines may play a role in sperm competition (Fig. 4A).

It has been pointed out that sperm competition is a potent evolutionary force among mammals (Roldan *et al.*, 1992) which influences sperm morphology (Gomendio and Roldan, 1991). It could be that the selection for protamines in the course of SNBP evolution was not exclusively determined by the transition from external to internal fertilisation but may have also been influenced by sperm competition.

Concluding remarks

The rapid evolution of sperm proteins, in particular protamines, is allowing us to observe the evolution of proteins at its best. The transition from the H type to the P type in the course of evolution may have ultimately been driven by the enhanced ability of these proteins to compact the genome, while efficiently erasing the epigenetic histone component inherited from the stem cells at the onset of spermatogenesis.

In general, the sperm head shape appears to be highly variable and independent of the SNBP composition. However, protamines and PL proteins compact DNA much more efficiently and the possibility exists that protamine variation may affect the shape of the sperm head in relatively closely related species and hence have a role in fertility and even perhaps in sperm competition. Increased chromatin compaction by protamines decreases the volume and streamlines the shape of the sperm head, providing better protection against externally damaging agents and enhancing sperm mobility. Ultimately, differences in sperm swimming velocity in internally fertilising organisms are an important component of sperm competition. Also, there is now a significant amount of accumulated data that indicates that protamines play an important direct role in fertility. This involvement suggests again that the SNBP transitions responsible may also affect sperm competition.

In closing, we would like to go back to Sherrington's quote at the beginning of this chapter on Cajal's anthropomorphic views of cell systems (Sherrington, 1949). Evolution of SNBPs together with other male reproductive proteins appears to have an important role in optimizing the cell parameters that allow sperm to accomplish its "passionate urge" (fertility) and successful "rivalry" (competition). This later issue, sperm competition, is an important selective force whose role in the evolution of SNBPs has yet to be explored.

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References

Aoki, V.W., Liu, L. and Carrell, D.T. (2005). Identification and evaluation of a novel sperm protamine abnormality in a population of infertile males. *Human Reproduction* **20:** 1298-1306

Ausió J. (1995). Histone H1 and the evolution of the nuclear sperm specific proteins. In: *Advances in Spermatozoal Phylogeny and Taxonomy*, Volume 166. Edited by Jamieson, B.G.M., Ausió, J. and Justine, J.L., Memoires de Museum National d'Histoire Naturelle, Paris, pp 501-514

Ausió, J. (1999). Histone H1 and evolution of sperm nuclear basic proteins. *Journal of Biological Chemistry* **274:** 31115-31118

Ausió, J., Greulich, K.O., Haas, E. and Wachtel, E. (1984). Characterization of the fluorescence of the protamine thynnine and studies of binding to double-stranded DNA. *Biopolymers* **23:** 2559-2571

Ausió, J., Soley, J.T., Burger, W., Lewis, J.D., Barreda, D. and Cheng, K.M. (1999). The histidine-rich protamine from ostrich and tinamou sperm. A link between reptile and bird protamines. *Biochemistry* **38:** 180-184

Ausió, J. and Subirana, J.A. (1982). Nuclear proteins and the organization of chromatin in spermatozoa of *Mytilus edulis*. *Experimental Cell Research* **141:** 39-45

Baccetti, B. and Afzelius, B.A. (1976). *The Biology of the Sperm Cell*. Monographs in developmental biology, Volume 10, Karger, Basel, Switzerland

Bach, O., Glander, H.J., Scholz, G. and Schwarz, J. (1990). Electrophoretic patterns of spermatozoal nucleoproteins (NP) in fertile men and infertility patients and comparison with NP of somatic cells. *Andrologia* **22:** 217-224

Balhorn, R., Cosman, M., Thornton, K., Krishnan, V.V., Corzett, M., Bench, G., Kramer, C., Lee IV, J., Hud, N.V., Allen, M., Prieto, M., Meyer-Ilse, W., Brown, T.J., Kirz, J., Zhang, X., Bradbury, E.M., Maki, G., Braun, R.E. and Breed, W. (1999). Protamine mediated condensation of DNA in mammalian sperm. *In*: *The Male Gamete: From Basic Knowledge to Clinical Applications*. Edited by Gagnon, C., Cache River Press, Vienna , IL , pp. 55-70

Balhorn, R., Reed, S. and Tanphaichitr, N. (1988). Aberrant protamine 1/protamine 2 ratios in sperm of infertile human males. *Experientia* **44:** 52-55

Belokopytova, I.A., Kostyleva, E.I., Tomilin, A.N. and Vorob'ev, V.I. (1993). Human male infertility may be due to a decrease of the protamine P2 content in sperm chromatin. *Molecular Reproduction and Development* **34:** 53-57

Bench, G., Corzett, M.H., De Yebra, L., Oliva, R. and Balhorn, R. (1998). Protein and DNA contents in sperm from an infertile human male possessing protamine defects that vary over time. *Molecular Reproduction and Development* **50:** 345-353

Birkhead, T.R., Martinez, J.G., Burke, T. and Froman, D.P. (1999). Sperm mobility determines the outcome of sperm competition in the domestic fowl. *Proceedings of the Royal Society of London, series B* **266:** 1759-1764

Blanchard, Y., Lescoat, D. and Le Lannou, D. (1990). Anomalous distribution of nuclear basic proteins in round-headed human spermatozoa. *Andrologia* **22:** 549-555

Bloch, D.P. (1976). Histones of sperm. In: *Handbook of Genetics*. Edited by King, R.C., Plenum Press. New York, NY, pp 139-167

Bode, J., Willmitzer, L. and Opatz, K. (1977). On the competition between protamines and histones: studies directed towards the understanding of spermiogenesis. *European Journal of Biochemistry* **72:** 393-403

Braun, R.E. (2001). Packaging paternal chromosomes with protamine. *Nature Genetics* **28:** 10-12

Brewer, L.R., Corzett, M. and Balhorn, R. (1999). Protamine-induced condensation and decondensation of the same DNA molecule. *Science* **286:** 120-123

Bunick, D., Balhorn, R., Stanker, L.H., and Hecht, N.B. (1990). Expression of the rat protamine 2 gene is suppressed at the level of transcription and translation. *Experimental Cell Research* **188:** 147-152

Caron, C., Govin, J., Rousseaux, S., and Khochbin, S. (2005). How to pack the genome for a safe trip. *Progress in Molecular and Subcellular Biology* **38:** 65-89

Carrell, D.T. and Liu, L. (2001). Altered protamine 2 expression is uncommon in donors of known fertility, but common among men with poor fertilizing capacity, and may reflect other abnormalities of spermiogenesis. *Journal of Andrology* **22:** 604-610

Casas, M.T., Ausió, J. and Subirana, J.A. (1993). Chromatin fibers with different protamine and histone compositions. *Experimental Cell Research* **204:** 192-197

Chevaillier, P., Mauro, N., Feneux, D., Jouannet, P. and David, G. (1987). Anomalous protein complement of sperm nuclei in some infertile men. *The Lancet* **2:** 806-807

Chiva, M., Saperas, N., Caceres, C. and Ausió, J. (1995). Nuclear basic proteins from the sperm of tunicates, cephalochordates, agnathans and fish. In: *Advances in Spermatozoal Phylogeny and Taxonomy*, Volume 166. Edited by Jamieson, B.G.M., Ausió, J. and Justine J.L., Memoires de Museum National d'Histoire Naturelle, Paris, pp. 501-514

Cho, C., Jung-Ha, H., Willis, W.D., Goulding, E.H., Stein, P., Xu, Z., Schultz, R.M., Hecht, N.B. and Eddy, E. M. (2003). Protamine 2 deficiency leads to sperm DNA damage and embryo death in mice. *Biology of Reprodution* **69:** 211-217

Cho, C., Willis, W.D., Goulding, E.H., Jung_Ha, H., Choi, Y. C., Hecht, N.B. and Eddy, E.M. (2001). Haploinsufficiency of protamine-1 or -2 causes infertility in mice. *Nature Genetics* **28:** 82-86

Churikov, D., Zalenskaya, I.A. and Zalensky, A.O. (2004). Male germline-specific histones in mouse and man. *Cytogenetics and Genome Research* **105:** 203-214

Clark, A.G. and Civetta, A. (2000). Evolutionary biology. Protamine wars. *Nature* **403:** 261-263

Daban, J.R. (2003). High concentration of DNA in condensed chromatin. *Biochemistry and Cell Biology* **81:** 91-99

de Yebra, L., Ballesca, J.L., Vanrell, J.A., Bassas, L. and Oliva, R. (1993). Complete selective absence of protamine P2 in humans. *Journal of Biological Chemistry* **268:** 10553-10557

de Yebra, L., Ballesca, J.L., Vanrell, J.A., Corzett, M., Balhorn, R. and Oliva, R. (1998). Detection of P2 precursors in the sperm cells of infertile patients who have reduced protamine P2 levels. *Fertility and Sterility* **69:** 755-759

Dixson, A.F. and Anderson, M.J. (2004). Sexual behavior, reproductive physiology and sperm competition in male mammals. *Physiology and Behavior* **83:** 361-371

Eirín-López, J.M., Frehlick, L.J. and Ausió, J. (2006a). Protamines, in the footsteps of linker histone evolution. *Journal of Biological Chemistry* **281:** 1-4

Eirín-López, J.M., Lewis, J.D., Howe, L.A. and Ausió, J. (2006b). Common phylogenetic origin of protamine-like (PL) proteins and histone H1: Evidence from bivalve PL genes. *Molecular Biology and Evolution* **23:** 1304-1317

Fernandez Valades, R., Hortas Nieto, M.L., Castilla, J.A., Lopez de la Torre Casares, M., Valladares Mendias, J.C., Alaminos Mingorance, M., Ruiz Montes, A., Castejon Casado, F.J. and Sanchez Lopez-Tello, C. (2001). [Alterations of nuclear maturity in spermatozoids of males with antecedents of cryptorchidism]. *Cirugía pediátrica: organo oficial de la Sociedad Española de Cirugía Pediátrica* **14:** 95-97

Foresta, C., Zorzi, M., Rossato, M. and Varotto, A. (1992). Sperm nuclear instability and staining with aniline blue: abnormal persistence of histones in spermatozoa in infertile men. *International Journal of Andrology* **15:** 330-337

Frehlick, L.J., Eirín-López, J.M., Field, E.D., Hunt, D.F. and Ausió, J. (2006a). The characterization of amphibian nucleoplasmins yields new insight into their role in sperm chromatin remodeling. *BMC Genomics* **7:** 99

Frehlick, L.J., Eirín-López, J.M., Prado, A., Su, H.W., Kasinsky, H.E. and Ausió, J. (2006b). Sperm nuclear basic proteins of two closely related species of scorpaeniform fish (*Sebastes maliger, Sebastolobus* sp.) with different sexual reproduction and the evolution of fish protamines. *Journal of Experimental Zoology A Comparative Experimental Biology* **305:** 277-287

Froman, D.P., Feltmann, A.J., Rhoads, M.L. and Kirby, J.D. (1999). Sperm mobility: A primary determinant of fertility in the domestic fowl (*Gallus domesticus*). *Biology of Reprodution* **61:** 400-405

Gage, M.J., Macfarlane, C.P., Yeates, S., Ward, R.G., Searle, J.B. and Parker, G.A. (2004). Spermatozoal traits and sperm competition in Atlantic salmon: relative sperm velocity is the primary determinant of fertilization success. *Current Biology* **14:** 44-47

Gatewood, J.M., Cook, G.R., Balhorn, R., Bradbury, E.M. and Schmid, C.W. (1987). Sequence-specific packaging of DNA in human sperm chromatin. *Science* **236:** 962-964

Giménez-Bonafé, P., Ribes, E., Zamora, M.J., Kasinsky, H.E and Chiva, M. (2002). Evolution of octopod sperm I: Comparison of nuclear morphogenesis in Eledone and Octopus. *Molecular Reproduction and Development* **62:** 357-362

Giménez-Bonafé, P., Laszczak, M., Kasinsky, H.E., Lemke, M.J., Lewis, J.D., Iskandar, M., He, T., Ikonomou, M.G., White, F.M., Hunt, D.F., Chiva, M. and Ausió, J. (2000). Characterization and evolutionary relevance of the sperm nuclear basic proteins from stickleback fish. *Molecular Reproduction and Development* **57:** 185-193

Gomendio, M. and Roldan, E.R.S. (1991). Sperm competition influences sperm size in mammals. *Proceedings of the Royal Society of London, series B* **243**: 181-185

Govin, J., Caron, C., Lestrat, C., Rousseaux, S. and Khochbin, S. (2004). The role of histones in chromatin remodelling during mammalian spermiogenesis. *European Journal of Biochemistry* **271:** 3459-3469

Gregory, T.R. (2006). Animal Genome Size Database. http://www.genomesize.com

Hecht, N.B. (1989). Mammalian protamines and their expression. In: *Histones and Other Basic Nuclear Proteins*. Edited by Hnilica, L.S., Stein, G.S. and Stein J.L., CRC Press, Boca Raton, FL, pp. 347-373

Hélène, C. and Lancelot, G. (1982). Interactions between functional groups in protein-nucleic acid associations. *Progress in Biophysics & Molecular Biology* **39:** 1-68

Hofmann, N. and Hilscher, B. (1991). Use of aniline blue to assess chromatin condensation in morphologically normal spermatozoa in normal and infertile men. *Human Reproduction* **6:** 979-982

Hud, N.V., Downing, K.H. and Balhorn, R. (1995). A constant radius of curvature model for the organization of DNA in toroidal condensates. *Proceedings of*

the National Academy of Sciences USA **92:** 3581-3585

Hunt, J.G., Kasinsky, H.E., Elsey, R.M., Wright, C.L., Rice, P., Bell, J.E., Sharp, D.J., Kiss, A.J., Hunt, D.F., Arnott, D.P., Russ, M.M., Shabanowitz, J. and Ausió, J. (1996). Protamines of reptiles. *Journal of Biological Chemistry* **271:** 23547-23557

Iguchi, N., Tanaka, H., Yomogida, K. and Nishimune, Y. (2003). Isolation and characterization of a novel cDNA encoding a DNA-binding protein (Hils1) specifically expressed in testicular haploid germ cells. *International Journal of Andrology* **26:** 354-365

Iguchi, N., Yang, S., Lamb, D. J. and Hecht, N. B. (2006). An SNP in protamine 1: a possible genetic cause of male infertility? *Journal of Medical Genetics* **43:** 382-384

Itoh, T., Ausió, J. and Katagiri, C. (1997). Histone H1 variants as sperm-specific nuclear proteins of *Rana catesbeiana*, and their role in maintaining a unique condensed state of sperm chromatin. *Molecular Reproduction and Development* **47:** 181-190

Kasinsky, H.E. (1989). Specificity and distribution of sperm basic proteins. In: *Histones and Other Basic Nuclear Proteins*. Edited by Hnilica, L.S., Stein G.S. and Stein J.L., CRC Press Inc., Boca Raton, FL, p 73-163

Kasinsky, H.E. (1995). Evolution and origins of sperm basic proteins. In: *Advances in Spermatozoal Phylogeny and Taxonomy*, Volume 166. Edited by Jamieson, B.G.M., Ausió, J. and Justine, J.L., Memoires de Museum National d'Histoire Naturelle, Paris, pp. 447-462

Kasinsky, H.E., Mann, M., Lemke, M. and Huang, S.Y. (1985). Diversity of sperm basic chromosomal proteins in vertebrates: a phylogenetic point of view. In: *Chromosomal Proteins and Gene Expression*. Edited by Reeck, G.R., Goodwin, G.H. and Puigdomenech, P., Plenum Press, New York, NY, pp. 333-359

Kimmins, S. and Sassone-Corsi, P. (2005). Chromatin remodelling and epigenetic features of germ cells. *Nature* **434:** 583-589

Kramer, J.A. and Krawetz, S.A. (1998). Genesis of a novel human sequence from the protamine PRM1 gene. *Comparative Biochemistry and Physiology. C, Pharmacology, Toxicology & Endocrinology* **120:** 467-473

Krawetz, S.A. and Dixon, G.H. (1988). Sequence similarities of the protamine genes: implications for regulation and evolution. *Journal of Molecular Evolution* **27:** 291-297

Levitan, D.R. (2000). Sperm velocity and longevity trade off each other and influence fertilization in the sea urchin Lytechinus variegatus. *Proceedings of the Royal Society of London, series B* **267:** 531-534

Lewis, J.D., Abbott, D.W. and Ausió, J. (2003a). A haploid affair: core histone transitions during spermatogenesis. *Biochemistry and Cell Biology* **81:** 131-40

Lewis, J.D., Saperas, N., Song, Y., Zamora, M.J., Chiva, M. and Ausió, J. (2004). Histone H1 and the origin of protamines. *Proceedings of the National Academy of Sciences USA* **101:** 4148-4152

Lewis, J.D., Song, Y., de Jong, M.E., Bagha, S.M. and Ausió, J. (2003b). A walk though vertebrate and invertebrate protamines. *Chromosoma* **111:** 473-482

Maier, W.M., Nussbaum, G., Domenjoud, L., Klemm, U. and Engel, W. (1990). The lack of protamine 2 (P2) in boar and bull spermatozoa is due to mutations within the P2 gene. *Nucleic Acids Research (Online)* **18:** 1249-1254

Maleszewski, M., Kuretake, S., Evenson, D., Yanagimachi, H., Bjordahl, J. and Yanagimachi, R. (1998). Behavior of transgenic mouse spermatozoa with galline protamine. *Biology of Reproduction* **58:** 8-14

Malo, A.F., Gomendio, M., Garde, J., Lang-Lenton, B., Soler, A.J. and Roldan, E.R.S. (2006). Sperm design and sperm function. *Biology Letters* **2:** 246-249

Mann, M., Risley, M.S., Eckhardt, R.A. and Kasinsky, H.E. (1982). Characterization of spermatid/sperm basic chromosomal proteins in the genus *Xenopus* (Anura, Pipidae). *Journal of Experimental Zoology* **222:** 173-186

Martianov, I., Brancorsini, S., Catena, R., Gansmuller, A., Kotaja, N., Parvinen, M., Sassone-Corsi, P. and Davidson, I. (2005). Polar nuclear localization of H1T2, a histone H1 variant, required for spermatid elongation and DNA condensation during spermiogenesis. *Proceedings of the National Academy of Sciences USA* **102:** 2808-2813

Meistrich, M.L. (1989). Histone and basic nuclear prtein transitions in mammalian spermatogenesis. In: *Histones and Other Basic Nuclear Proteins*. Edited by Hnilica, L.S., Stein, G.S. and Stein, J.L., CRC Press Inc., Boca Raton, FL, pp. 165-182

Meistrich, M.L., Brock, W.A., Grimes, S.R., Platz, R.D. and Hnilica, L.S. (1978). Nuclear protein transitions during spermatogenesis. *Federation Proceedings* **37:** 2522-2525

Meistrich, M.L., Mohapatra, B., Shirley, C.R. and Zhao, M. (2003). Roles of transition nuclear proteins in spermiogenesis. *Chromosoma* **111:** 483-488

Mengual, L., Ballesca, J.L., Ascaso, C. and Oliva, R. (2003). Marked differences in protamine content and P1/P2 ratios in sperm cells from percoll fractions between patients and controls. *Journal of Andrology* **24:** 438-447

Moore, H., Dvorakova, K., Jenkins, N. and Breed, W. (2002). Exceptional sperm cooperation in the wood mouse. *Nature* **418:** 174-177

Nelson, J.E. and Krawetz, S.A. (1993). Linkage of human spermatid-specific basic nuclear protein genes. Definition and evolution of the P1—>P2—>TP2 locus. *Journal of Biological Chemistry* **268:** 2932-2936

Nelson, J.E. and Krawetz, S.A. (1994). Characterization of a human locus in transition. *Journal of Biological Chemistry* **269:** 31067-31073

Nishimune, Y. and Tanaka, H. (2006). Infertility caused by polymorphisms or mutations in spermatogenesis-specific genes. *Journal of Andrology* **27:** 326-334

Oliva, R. (1995). Sequence, evolution and transcrip-

tional regulation of avian-mammalian P1 type protamines. In: *Advances in Spermatozoal Phylogeny and Taxonomy*, Volume 166, Edited by Jamieson, B.G.M., Ausió, J. and Justine, J.L., Memoires du Museum national d'Histoire naturelle, Paris, pp. 537-548

Oliva, R. (2006). Protamines and male infertility. *Human Reproduction Update* **12:** 417-435

Oliva, R. and Dixon, G.H. (1991). Vertebrate protamine genes and the histone-to-protamine replacement reaction. *Progress in Nucleic Acid Research and Molecular Biology* **40:** 25-94

Papoutsopoulou, S., Nikolakaki, E., Chalepakis, G., Kruft, V., Chevaillier, P. and Giannakouros, T. (1999). SR protein-specific kinase 1 is highly expressed in testis and phosphorylates protamine 1. *Nucleic Acids Research* **27:** 2972-2980

Puigdoménech, P., Martinez, P., Palau, J., Bradbury, E. M. and Crane-Robinson, C. (1976). Studies on the role and mode of operation of the very-lysine-rich histones in eukaryote chromatin. Nuclear-magnetic-resonance studies on nucleoprotein and histone phi 1-DNA complexes from marine invertebrate sperm. *European Journal of Biochemistry* **65:** 357-363

Queralt, R., Adroer, R., Oliva, R., Winkfein, R.J., Retief, J.D. and Dixon, G.H. (1995). Evolution of protamine P1 genes in mammals. *Journal of Molecular Evolution* **40:** 601-607

Raukas, E. and Mikelsaar, R.H. (1999). Are there molecules of nucleoprotamine? *Bioessays: News and Reviews in Molecular, Cellular and Developmental Biology* **21:** 440-448

Retief, J.D., Krajewski, C., Westerman, M. and Dixon, G.H. (1995a). The evolution of protamine P1 genes in dasyurid marsupials. *Journal of Molecular Evolution* **41:** 549-555

Retief, J.D., Krajewski, C., Westerman, M., Winkfein, R.J. and Dixon, G.H. (1995b). Molecular phylogeny and evolution of marsupial protamine P1 genes. *Proceedings of the Royal Society of London, series B* **259:** 7-14

Retief, J.D., Rees, J.S., Westerman, M. and Dixon, G. H. (1995c). Convergent evolution of cysteine residues in sperm protamines of one genus of marsupials, the Planigales. *Molecular Biology and Evolution* **12:** 708-712

Retief, J.D., Winkfein, R.J. and Dixon, G.H. (1993). Evolution of the monotremes. The sequences of the protamine P1 genes of platypus and echidna. *European Journal of Biochemistry* **218:** 457-61

Retief, J.D., Winkfein, R.J., Dixon, G.H., Adroer, R., Queralt, R., Ballabriga, J. and Oliva, R. (1993). Evolution of protamine P1 genes in primates. *Journal of Molecular Evolution* **37:** 426-434

Rhim, J.A., Connor, W., Dixon, G.H., Harendza, C.J., Evenson, D.P., Palmiter, R.D. and Brinster, R.L. (1995). Expression of an avian protamine in transgenic mice disrupts chromatin structure in spermatozoa. *Biology of Reproduction* **52:** 20-32

Robinson, P.J., Fairall, L., Huynh, V.A. and Rhodes, D. (2006). EM measurements define the dimensions of the "30-nm" chromatin fiber: Evidence for a compact, interdigitated structure. *Proceedings of the National Academy of Sciences USA* **103:** 6506-6511

Roldan, E.R.S., Gomendio, M. and Vitullo, A.D. (1992). The evolution of eutherian spermatozoa and underlying selective forces: female selection and sperm competition. *Biological Reviews* **67:** 551-593

Rooney, A.P. and Zhang, J. (1999). Rapid evolution of a primate sperm protein: relaxation of functional constraint or positive Darwinian selection? *Molecular Biology and Evolution* **16:** 706-710

Rooney, A.P., Zhang, J. and Nei, M. (2000). An unusual form of purifying selection in a sperm protein. *Molecular Biology and Evolution* **17:** 278-283

Rousseaux, S., Caron, C., Govin, J., Lestrat, C., Faure, A. K. and Khochbin, S. (2005). Establishment of male-specific epigenetic information. *Gene* **345:** 139-153

Saperas, N., Ausió, J., Lloris, D. and Chiva, M. (1994). On the evolution of protamines in bony fish: alternatives to the "retroviral horizontal transmission" hypothesis. *Journal of Molecular Evolution* **39:** 282-295

Saperas, N., Chiva, M., Casas, M. T., Campos, L., Eirín-López, J. M., Prieto, C., Subirana, J. A. and Ausió, J. (2006). A unique vertebrate histone H1-related protamine-like (PL) protein results in an unusual sperm chromatin organization. *FEBS Letters* **273:** 9548-9561

Saperas, N., Chiva, M., Pfeiffer, D. C., Kasinsky, H. E. and Ausió, J. (1997). Sperm nuclear basic proteins (SNBPs) of agnathans and chondrichthyans: variability and evolution of sperm proteins in fish. *Journal of Molecular Evolution* **44:** 422-431

Sautiere, P., Martinage, A., Belaiche, D., Arkhis, A. and Chevaillier, P. (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. *Journal of Biological Chemistry* **263:** 11059-11062

Schluter, G., Celik, A., Obata, R., Schlicker, M., Hofferbert, S., Schlung, A., Adham, I. M. and Engel, W. (1996). Sequence analysis of the conserved protamine gene cluster shows that it contains a fourth expressed gene. *Molecular Reproduction and Development* **43:** 1-6

Schluter, G. and Engel, W. (1995). The rat Prm3 gene is an intronless member of the protamine gene cluster and is expressed in haploid male germ cells. *Cytogenetics and Cell Genetics* **71:** 352-355

Seyedin, S.M. and Kistler, W.S. (1980). Isolation and characterization of rat testis H1t. An H1 histone variant associated with spermatogenesis. *Journal of Biological Chemistry* **255:** 5949-5954

Sherrington, C. (1949). A memoir of Dr. Cajal. In: *Explorer of the Human Brain. The Life of Santiago Ramón y Cajal (1852-1934)*, Edited by Canon, D.F., H. Schuman, New York, NY. pp. 14

Subirana, J.A. (1992). Order and disorder in 30 nm chromatin fibers. *FEBS Letters* **302:** 105-107

Swanson, W.J. and Vacquier, V.D. (2002). The rapid evolution of reproductive proteins. *Nature Reviews.*

Genetics **3:** 137-144

Tanaka, H., Iguchi, N., Isotani, A., Kitamura, K., Toyama, Y., Matsuoka, Y., Onishi, M., Masai, K., Maekawa, M., Toshimori, K., Okabe, M. and Nishimune, Y. (2005). HANP1/H1T2, a novel histone H1-like protein involved in nuclear formation and sperm fertility. *Molecular and Cellular Biology* **25:** 7107-7119

Tanaka, H., Miyagawa, Y., Tsujimura, A., Matsumiya, K., Okuyama, A. and Nishimune, Y. (2003). Single nucleotide polymorphisms in the protamine-1 and -2 genes of fertile and infertile human male populations. *Molecular Human Reproduction* **9:** 69-73

Torregrosa, N., Dominguez-Fandos, D., Camejo, M.I., Shirley, C.R., Meistrich, M.L., Ballesca, J.L. and Oliva, R. (2006). Protamine 2 precursors, protamine 1/protamine 2 ratio, DNA integrity and other sperm parameters in infertile patients. *Human Reproduction* **21:** 2084-2089

van Holde, K.E. (1988). *Chromatin*. Springer-Verlag, NY.

Vilfan, I.D., Conwell, C.C. and Hud, N.V. (2004). Formation of native-like mammalian sperm cell chromatin with folded bull protamine. *Journal of Biological Chemistry* **279:** 20088-20095

Ward, W.S. and Zalensky, A.O. (1996). The unique, complex organization of the transcriptionally silent sperm chromatin. *Critical Reviews in Eukaryotic Gene Expression* **6:** 139-147

Wyckoff, G.J., Wang, W. and Wu, C.I. (2000). Rapid evolution of male reproductive genes in the descent of man. *Nature* **403:** 304-309

Wykes, S.M. and Krawetz, S.A. (2003). The structural organization of sperm chromatin. *Journal of Biological Chemistry* **278:** 29471-29477

Yan, W., Ma, L., Burns, K.H. and Matzuk, M.M. (2003). HILS1 is a spermatid-specific linker histone H1-like protein implicated in chromatin remodeling during mammalian spermiogenesis. *Proceedings of the National Academy of Sciences USA* **100:** 10546-105451

Yu, Y.E., Zhang, Y., Unni, E., Shirley, C.R., Deng, J.M., Russell, L.D., Weil, M.M., Behringer, R.R. and Meistrich, M. L. (2000). Abnormal spermatogenesis and reduced fertility in transition nuclear protein 1-deficient mice. *Proceedings of the National Academy of Sciences USA* **97:** 4683-4688

Zhang, X., San Gabriel, M and Zini, A. (2006). Sperm nuclear histone to protamine ratio in fertile and infertile men: evidence of heterogeneous subpopulations of spermatozoa in the ejaculate. *Journal of Andrology* **27:** 414-420

Zhao, M., Shirley, C.R., Hayashi, S., Marcon, L., Mohapatra, B., Suganuma, R., Behringer, R.R., Boissonneault, G., Yanagimachi, R. and Meistrich, M. L. (2004). Transition nuclear proteins are required for normal chromatin condensation and functional sperm development. *Genesis* **38:** 200-213

Zhao, M., Shirley, C.R., Yu, Y.E., Mohapatra, B., Zhang, Y., Unni, E., Deng, J.M., Arango, N.A., Terry, N.H., Weil, M.M., Russell, L.D., Behringer, R.R. and Meistrich, M.L. (2001). Targeted disruption of the transition protein 2 gene affects sperm chromatin structure and reduces fertility in mice. *Molecular and Cellular Biology* **21:** 7243-7255