

Chromatin remodelling in vertebrate spermatozoa

by

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B.Sc, University of Victoria, 2001

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of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

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Abstract

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During spermatogenesis, one of the most drastic examples of chromatin remodelling takes place. In many organisms this coincides with drastic changes in chromatin composition, as histones are replaced by sperm nuclear basic proteins (SNBPs) of the protamine type (P-type). Due to their smaller size and higher charge, protamines compact sperm chromatin more efficiently. However, many organisms do not undergo this composition change and instead either retain histones similar to those in somatic cells in their sperm (H-type) or gain protamine-like proteins (PL-type), often in addition to histone. Fish and amphibian models are used in this thesis because they include genera with SNBPs representative of each of the three main types and provide a unique opportunity to study chromatin compaction. I focused on species that contain a partial or complete complement of histones in the sperm.

Chapter 1 of this thesis is a review of the SNBP evolution, distribution and roles in chromatin compaction. In Chapter 2, the complete cDNA sequence of *Xenopus laevis* sperm specific proteins SP1 and SP2 is determined. Structural and functional analyses show that SP1/SP2 proteins are related to proteins of the histone H1 family, particularly

to vertebrate histone H1x and are members of the protamine-like- I (PL-I) group of SNBPs.

In H-type organisms that retain histones in their sperm, a remodelling of chromatin and a reduction in nuclear volume still occur during spermiogenesis. However, the factors that lead to the condensation of chromatin in these organisms are unknown and are addressed in Chapter 3. *Ictalurus punctatus* is determined to have sperm chromatin of the H-type, which is maximally compacted and organized into a highly repetitive structure indicative of uniformly condensed chromatin. Several histone variants and post-translational modifications (PTMs) are examined as a preliminary survey of factors potentially responsible for this compaction. Of the PTMs present in catfish testes, the most significant were histone H3 trimethylated at lysine 27, which is a well known marker of facultative heterochromatin, and histone H4 phosphorylated at serine 1, which has been documented to affect nuclear size and may help stabilize chromatin compaction in mice and yeast.

A second extreme remodelling of the paternal pronucleus occurs following fertilization in order to convert the highly compacted, transcriptionally inert chromatin of the sperm into a substrate that is recognizable by the transcription and replication machinery of the zygote. Nucleoplasmin, a nuclear chaperone, participates in this remodelling in amphibians by displacing the specialized P-type and PL-type proteins from the sperm chromatin and by the transfer of H2A/H2B dimers. Nucleoplasmin was originally isolated from *Xenopus* (PL-type) and belongs to the nucleophosmin/nucleoplasmin (NPM) family of proteins, which have diverse functions in the cell (Reviewed in Chapter 4). The existence of H-type sperm raises uncertainty about

the need for a nucleoplasmin-mediated removal process in these organisms. In Chapter 5, the presence of nucleoplasmin in *Rana catesbeiana* (H-type) and *Bufo marinus* (P-type) is assessed. The amphibian nucleoplasmins are shown to phylogenetically group with mammalian NPM2 proteins and the implications suggested by the presence of nucleoplasmin in organisms of all three SNBP-types are discussed.

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List of Abbreviations

Å	Angstrom
aa	Amino acid
Ac	Acetylated
ACN	Acetonitrile
AUT	Acetic acid-Urea-Triton X-100
BLAST	Basic local alignment search tool
bp	Base pair
BRDT	Bromodomain, testis-specific protein
CD	Circular dichroism
cDNA	Complementary DNA
C-terminus	Carboxyl terminus
Da	Dalton
DAPI	4,6-diamidino-2-phenylindole;
DNA	Deoxyribonucleic acid
dNPL	<i>Drosophila</i> nucleoplasmin-like protein
DPI	Dots per inch
DTT	Dithiothreitol
EDTA	Ethylenedinitrilo-tetraacetic acid
EM	Electron microscopy
H	Histone
Hanp1	Haploid germ cell-specific nuclear protein 1
HAP	Hydroxyapatite
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
Hex	Hexamer
HILS1	Histone H1-like sperm specific protein 1
HP1 α	Heterochromatin protein 1 alpha
HP1 β	Heterochromatin protein 1 beta
HPLC	High performance liquid chromatography

K	Lysine
LTQ-FTMS	Linear ion trap quadrupole – Fourier transformation mass spectrometry
MALDI-TOF	Matrix assisted laser desorption ionization-time-of-flight
MBT	Mid blastula transition
me2	Dimethylated
me3	Trimethylated
MENT	Myeloid and erythroid nuclear termination protein
MNase	Micrococcal nuclease
mRNA	Messenger RNA
MRW	Mean (amino acid) residue weight
MS	Mass spectrometry
MW	Molecular weight
NA	Nucleic acid
NASP	Nuclear autoantigenic sperm protein
NCP	Nucleosome core particle
NES	Nuclear export signal
NLS	Nuclear localization signals
NoLS	Nucleolar localization signal
NPM	Nucleophosmin/nucleoplasmin family
N-terminus	Amino terminus
P	Protamine
PAGE	Polyacrylamide gel electrophoresis
PARP-1	Poly(ADP-ribose) polymerase1
PBS	Phosphate buffered saline
PcG	Polycomb group
PCR	Polymerase chain reaction
phos	Phosphorylated
PL	Protamine-like
PolyCAT	Polyaspartic acid cation-exchange
RACE	Rapid amplification of cDNA ends

RD	Replication dependent
RI	Replication independent
RT	Reverse transcription
S	Serine
SCNT	Somatic cell nuclear transfer
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
SNBP	Sperm nuclear basic protein
SPs	Sperm-specific proteins
TAE	Tris-acetic acid-EDTA
TBE	Tris-boric acid-EDTA
TEM	Transmission electron microscopy
TFA	Trifluoroacetic acid
TNP	Transition protein
TOF	Time of flight
v/C	Volume / C-value

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Dedication

I would like to thank my wonderful husband Michael, whose love and support kept me grounded through out my studies. Without him this thesis would not have been possible. I dedicate this thesis to our son Ethan, for all of the laughs and smiles he has given me in the last year and half.

Overview

Spermatogenesis takes place within the testes and is the process by which spermatogonia develop into mature spermatozoa, or sperm cells (Fig. 1). The differentiation of spermatogonia to mature sperm involves extreme cellular, functional, genetic and chromatin changes. First, spermatogonia are amplified in numbers by mitotic division. Some of the spermatogonia then undergo meiosis I to become first primary spermatocytes and by the end of the first meiotic division secondary spermatocytes. Spermiogenesis is the final stage of spermatogenesis, where the haploid spermatids which, were produced during a second round of meiosis (meiosis II), differentiate into mature sperm. During spermiogenesis the somatic histones are replaced by specialized sperm nuclear basic proteins (SNBPs) (Caron et al., 2005). The SNBPs can be classified into three main types; histone (H) type, protamines-like (PL) type and protamines (P) type (Ausió, 1999). This is the stage in mammals where the histones are removed and subsequently replaced by positively charged protamines. Many organisms, including some fish and amphibians, either retain somatic-like histones in the mature sperm (H-type) or contain SNBPs of the PL-type (Ausió et al., 2007).

Whether histones are replaced or retained, spermiogenesis is characterized by an extreme remodelling of the paternal chromatin to form a compacted genome (Kurtz et al., 2009). However, very little is known about the chromatin remodelling that takes place during spermiogenesis in organisms that retain histones (Kurtz et al., 2009). The first section of this thesis focuses on the chromatin composition and organization in animals that do not have histones replaced by protamines and instead retain a complement of histones (H-type) or have histone H1 related SNBPs (PL-type) in the mature sperm.

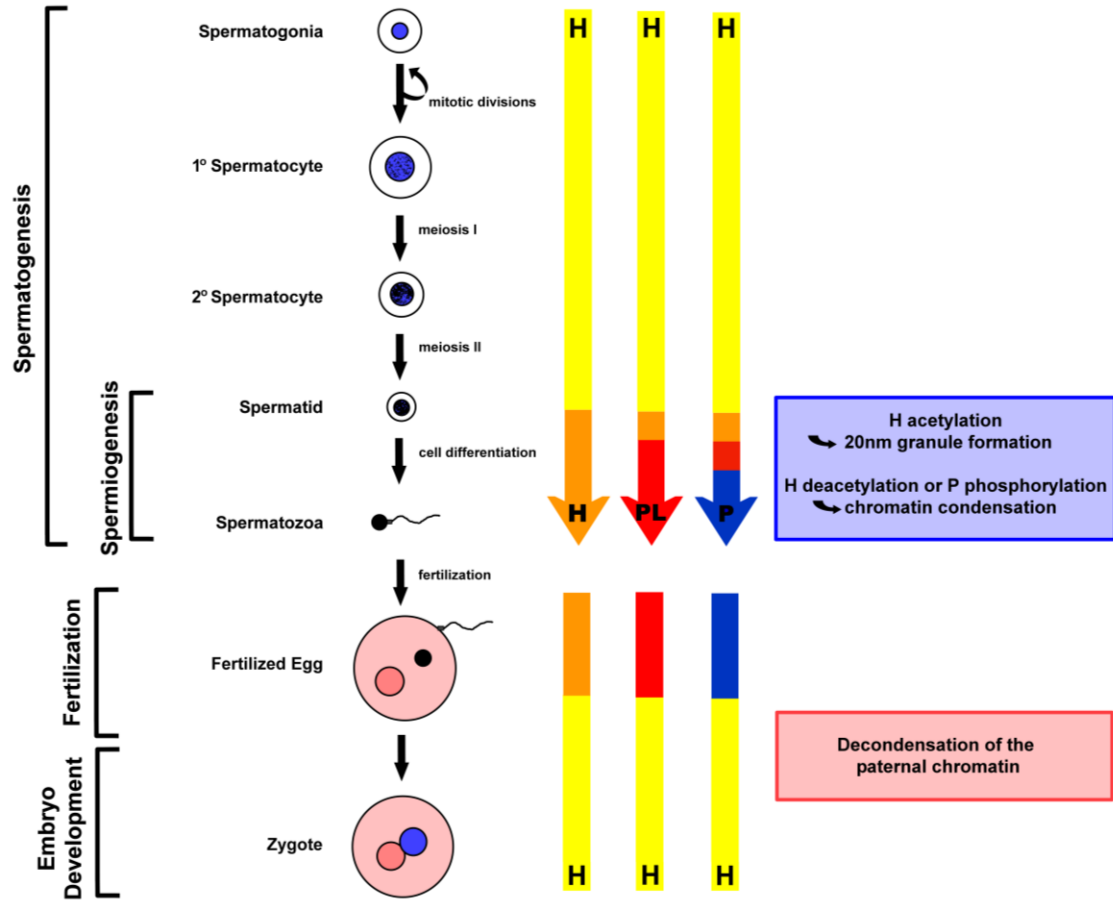


Figure 1. A schematic representation of the differentiation of spermatogonia into mature spermatozoa and the pronuclear formation after fertilization.

The arrows represent the transition from histones to the three different SNBP-types and their reversion back to histones immediately after fertilization. Somatic histones (H, yellow) are replaced by germinal histones (H, orange) in all SNBP-types. In some organisms PL protamine-like proteins (red arrow head) are incorporated into the chromatin. In P-type organism protamines (P, blue arrow head) replace histones and in mammals protamines are preceded by transition proteins (dark orange). The boxes highlight the large scale chromatin remodelling events that are conserved across organisms of all three SNBP-types that occur during spermiogenesis (blue box) and after fertilization in the egg (pink box).

Chapter 1 introduces the three types of sperm chromatin. In Chapter 2 we hypothesize that the previously identified sperm-specific proteins, SP1 and SP2, from *Xenopus laevis* are PL-I type proteins and therefore should have a winged helix domain, characteristic of linker histones, and bind to nucleosomal DNA. Chapter 3 is a preliminary study of the chromatin of *Ictalurus punctatus*. We provide evidence that *I. punctatus* has H-type SNBPs and is a good model to address our hypothesis that in cases where histones are not replaced by P- or PL-type SNBPs, epigenetic marks, histone variants and/or other factors present in the sperm are required for chromatin compaction and stabilization.

In higher chordates, the oocyte (2n), or immature egg, undergoes two rounds of meiosis to form the ovum, or unfertilized egg (1n), which is arrested at metaphase II until fertilization. The spermatozoon is responsible for delivering the paternal genome to the egg. In the fertilized egg, a second extreme remodelling event takes place to decondense the sperm chromatin (Fig. 1). In many animals protamines and protamines-like proteins completely abolish the epigenetic information of the paternal genome. This epigenetic silencing can only be reverted with the assistance of proteins in the egg, such as nucleoplasmin, which remove the protamines and decondense the chromatin following fertilization (Philpott and Leno, 1992). In the second section of this thesis the information known about nucleoplasmin and its family members is reviewed (Chapter 4). In Chapter 5 we test the hypothesis that if nucleoplasmin is important for the remodelling of chromatin, beyond its initial role in removal of P- and PL-type proteins immediately after fertilization, then it should be present in organisms of the H-type and share a similarity at the protein level between organisms with all three sperm types. Due to the relatively broad scope of this thesis the conclusions are discussed at the end of each Chapter.

Chapter 1. Introduction to sperm nuclear basic proteins and paternal chromatin remodelling

LJF's contribution to work:

This chapter contains excerpts that I originally wrote for (Frehlick et al., 2006b; Ausio et al., 2007) and then adapted for the purpose of this dissertation. I isolated sperm cells for the electron microscopy work, extracted sperm histones and separated them by AU-PAGE and prepared the figures. Electron microscopy was performed by Dr. Singla at the Electron Microscopy Laboratory, University of Victoria, Department of Biology. Juan Ausió supplied Figure 3, Part D.

Abstract

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 evolutionary process (H \rightarrow PL \rightarrow 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. From the examination of SNBP-types in fish species it has been proposed that the mode of fertilization, internal versus external, may serve as a constraint on the diversification of the SNBPs. Due to their smaller size and higher arginine content protamines and PL proteins compact sperm chromatin of the P and PL-type more efficiently than histones. However, histones are still able to significantly compact the H-type sperm chromatin compared to the chromatin of somatic cells.

Sperm Chromatin

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 and assists in the folding of the chromatin fiber (van Holde, 1988). Altering or remodelling 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 remodelling occurs within male germ cells, where changes in 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 known as sperm nuclear basic proteins (SNBPs). They can be grouped into three categories: Protamine (P-type), protamine-like (PL-type) and histone (H-type) (Ausió, 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 (Eirin-Lopez et al., 2006a).

Protamines are a compositionally and structurally heterogeneous group of proteins (Ando, 1973; Ausió, 1995; Felix, 1960; Oliva and Dixon, 1991) that are only

found in the sperm chromatin of certain organisms. These are arginine rich proteins of a relatively small size; often 50 to 70% arginine and smaller than 100 amino acids (Ausió, 1999). Due to their arginine composition, these proteins bind to DNA with high affinity and a strong electrostatic component that leads to the almost complete neutralization of the genomic DNA charge and results in a heterogeneous variety of molecular structures (Lewis et al., 2003b). They lack any secondary structure in solution but may adopt a folded conformation upon interaction with DNA.

Protamine-like proteins are an intermediate group of sperm chromosomal proteins that, like protamines, can displace somatic histones from the nucleus at the end of spermiogenesis. They exhibit an amino acid composition rich in lysine and arginine (35-50%) and have an enormous structural variability. All the proteins of this group appear to be evolutionarily related to the histone H1 family of chromosomal proteins (Ausió, 1999; Lewis et al., 2004b). Some PL proteins have a tripartite organization with a globular central core with a strong sequence similarity to the winged helix domain found in the histone family of proteins. Another, smaller type of PL-type proteins lack a winged helix domain and have share sequence similarities with the unstructured C- or N-terminal tail domains of histone H1 proteins (Eirin-Lopez et al., 2006c).

The third group of organisms retains histones in their mature sperm (H-type), which are often the same as, or indistinguishable from, somatic histones. In addition to somatic type histones, sperm-specific histone variants may also be present in this instance. 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 et al., 1997).

Evolution of vertebrate sperm nuclear basic proteins

From the examination of SNBPs from many invertebrates and vertebrates it was proposed that SNBPs have evolved from an ancestral histone to a protamine-like protein and finally to a protamine (Ausió, 1999; Saperas et al., 1994). Although a link between PL proteins and linker histone H1 had been proposed (Ausió, 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. Two recent studies have shed light on the possible mechanisms underlying this change, lending direct support for a link between H1s, PLs, and protamines (Eirin-Lopez et al., 2006c; Lewis et al., 2004b). In the first study, the SNBPs of two closely related species of tunicates, *Styela montereyensis* and *Ciona intestinalis*, revealed the presence of proteins of the PL-type in the sperm (Lewis et al., 2004b). Whereas 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 of *S. montereyensis*, establishing the evolutionary link between PL and protamine type SNBPs (Lewis et al., 2004b). A second report, focused on the evolutionary relationships between 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 (Eirin-Lopez et al., 2006c). H1 histones and PLs are descendants of an ancient group of orphon H1 replication-dependent histones, which were excluded to solitary genomic regions 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 of the SNBP lineage (Eirin-Lopez et al., 2006c). Due to the more efficient

DNA condensation properties of arginine (Ausió et al., 1984; Helene and Lancelot, 1982; Puigdomenech et al., 1976), 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 the H → PL → P process involving a primordial replication independent histone H1 (Ausió, 1999; Eirin-Lopez and Ausio, 2009; Eirin-Lopez et al., 2006a; Eirin-Lopez et al., 2006c) lends support to the old concept of a potential relationship between ontogeny and phylogeny . In mammals, somatic histones undergo post-translational modifications as well as replacement with specialized histone variants during meiotic prophase [reviewed in (Govin et al., 2004; Kimmins and Sassone-Corsi, 2005; Lewis et al., 2003a)], including the highly specialized H1 histones H1t (Seyedin and Kistler, 1980), HILS1 (Iguchi et al., 2003; Yan et al., 2003) and Hanp1/H1T2 (Martianov et al., 2005; Tanaka et al., 2005). Immediately after meiosis, histones are replaced by transition proteins (TNPs) (Meistrich et al., 1978; Meistrich et al., 2003), which are unique to mammals. Finally, during spermiogenesis, the transition proteins are replaced by protamines (Lewis et al., 2003a) in the mature spermatozoa.

Distribution sperm nuclear basic protein types within vertebrates

The different SNBP-types display a heterogeneous distribution among the vertebrates (Fig. 2). Nevertheless, as it was shown in fish, the sporadic appearance of the different types is not random and follows the phylogeny of the groups where these proteins are present (Saperas et al., 1994).

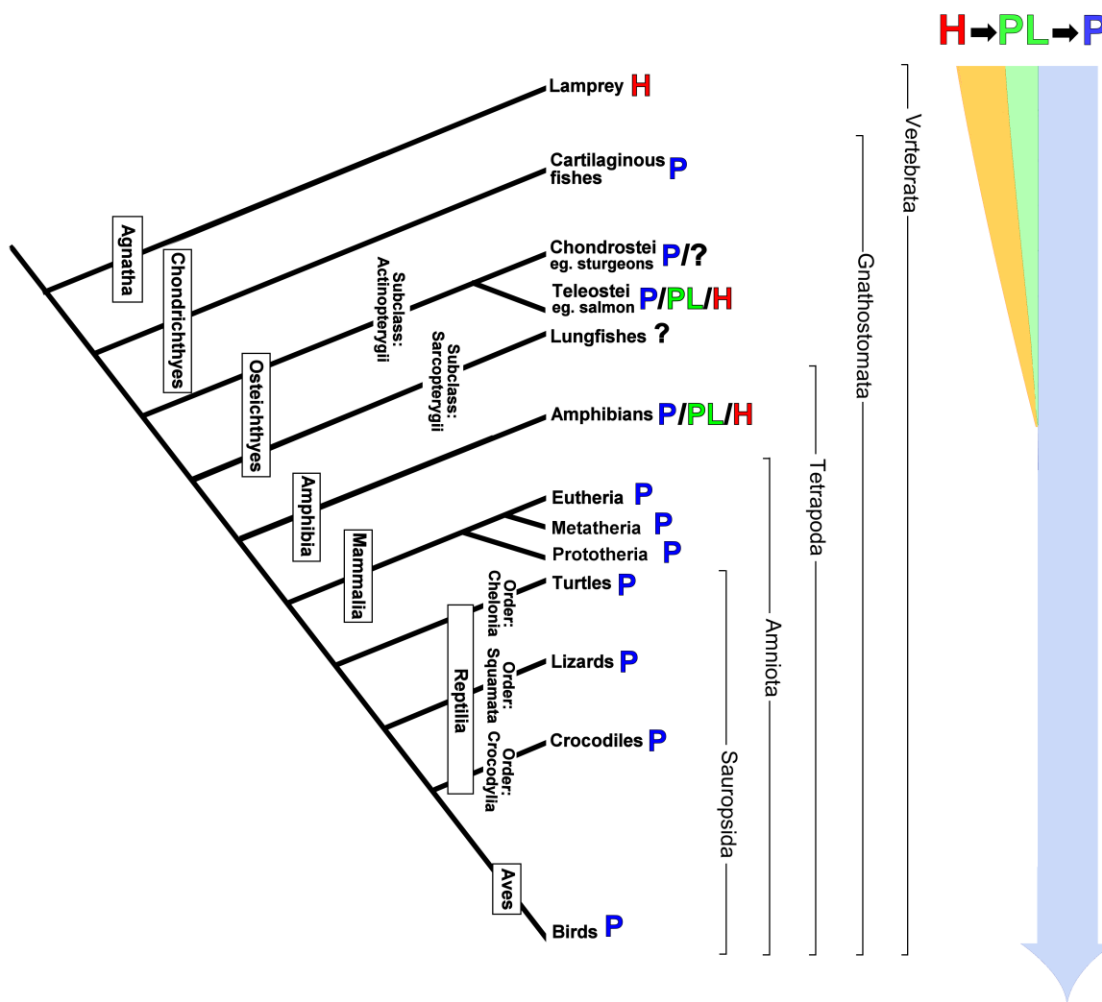


Figure 2. Cladogram of the subphylum Vertebrata showing the currently accepted relationships of monophyletic groups making up the subphylum.

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 colors) 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; Eirin-Lopez et al., 2006a; Lewis et al., 2004b).

The occurrence of protamines is seen in vertebrates as ancient as sharks and other cartilaginous fishes (Fig. 2). 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; Saperas et al., 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 sturgeons and paddle fish so far examined, 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 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 different evolutionary lines. The occurrence of this phenomenon is almost negligible during the differentiation of genera and species and minor during the differentiation of families. However, there are frequent divergences between different orders (Saperas et al., 1994).

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 fertilization (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 fertilization may select for protamines (Kasinsky, 1989; Kasinsky et al., 1985; Mann et al., 1982). Protamines would be selected for because their high arginine content lends structural and functional advantages, such as elongated and more compact

sperm, which can resist drag forces within the viscous reproductive tract. Further support for this hypothesis revealed by examining the SNBPs of two closely related species of rockfish, the internally fertilizing *Sebastes maliger* and externally fertilizing *Sebastolobus* sp. (Frehlick et al., 2006b). Both of these rockfish have protamines. However, it was found that there was a significant increase in the arginine content of the protamine in the internally fertilizing rockfish. In addition *S. maliger* has a lower histone content and a lower molecular weight protamine that lacks asparagines and glutamine residues, which suggests this more advanced internally fertilizing species has an advance protamine (Frehlick et al., 2006b).

The fact that amniotes contain only sperm of the P-type 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 in crown groups (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 (Hecht, 1989; Lewis et al., 2003b; Sautiere et al., 1988). 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 (Lewis et al., 2003b; Oliva and Dixon, 1991). 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. Interestingly cysteine is also found in the

protamines of cartilaginous fish. Cysteine also appeared in the marsupial P1 protamines of the genus *Planigales* through a process of convergent evolution (Retief et al., 1995). 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 reminiscent of the similar compositional transitions that have been observed in the equivalent invertebrate SNBP-types (Lewis et al., 2003b).

Do SNBPs affect the sperm chromatin compaction?

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 (Casas et al., 1993; Saperas et al., 2006; van Holde, 1988) that it 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).

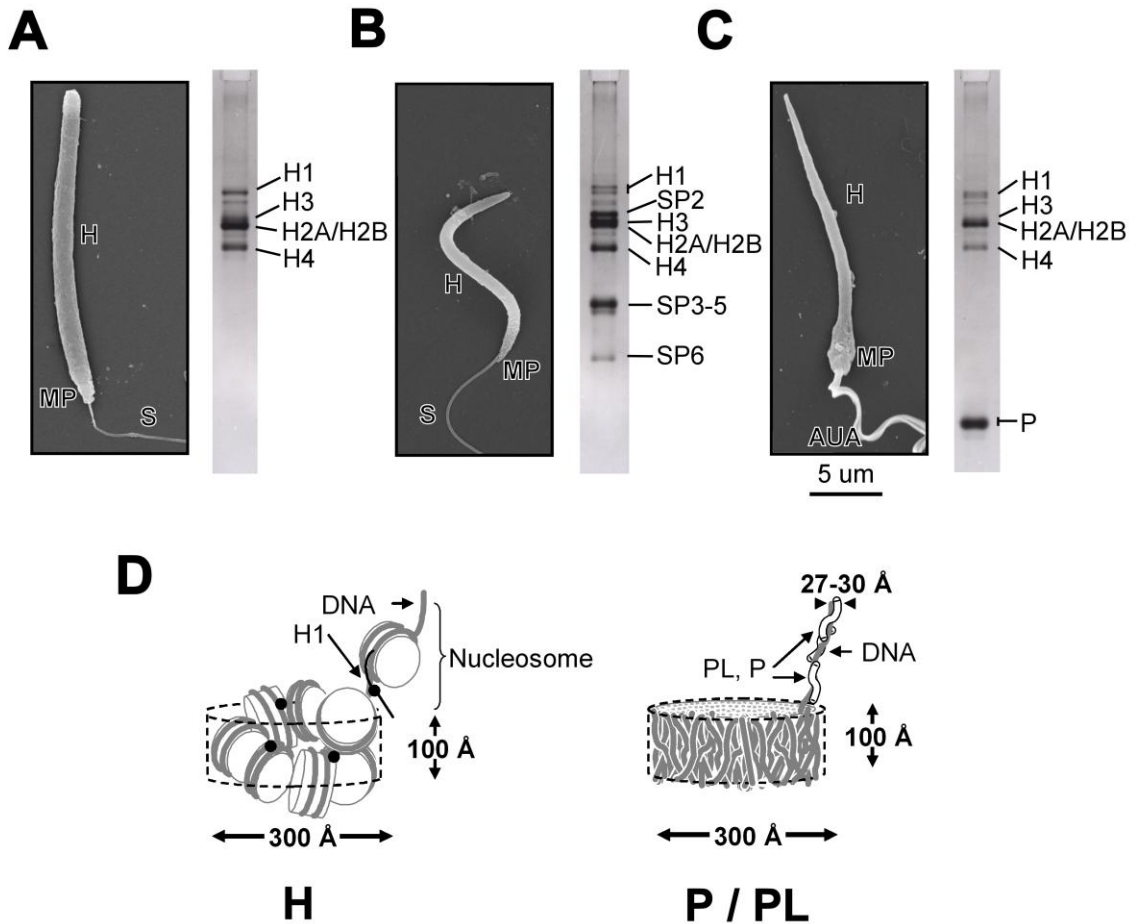


Figure 3. Scanning electron microscopy micrographs of the sperm heads and AU-PAGE analysis of the SNBPs of three different species of amphibians

Micrographs (lane 1) and AU-PAGE (lane 2) of: *R. catesbeiana* (bullfrog) (A); *X. laevis* (African clawed frog) (B); *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).

In the H-type, such as in *Rana*, the chromatin is organized in a 300 Å fiber consisting of approximately six nucleosomes per turn (see Fig. 3D, H) (Manochantr et al., 2005). In contrast, the invertebrate (Ausió and Subirana, 1982b) and vertebrate (Saperas et al., 2006) PL type chromatin consists of irregular parallel DNA bundles. In mammalian protamines, chromatin loops are organized in toroidal structures containing similar parallel nucleoprotamine bundles (Balhorn, 1999; Brewer et al., 1999; Hud et al., 1995; Ward and Zalensky, 1996) (see Fig. 3D PL/P).

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, 1982b; 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 $(100 / 3.4) \times 100$ (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). In doing so it was 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 organized chromatin fibers (Fig. 3 D, H) can interdigitate to some extent (Daban, 2003; Robinson et al., 2006) increasing the theoretically calculated value.

This demonstrates that, in general, histones are slightly less efficient in packaging sperm chromatin than P and PL proteins. However, the H-type sperm is still highly compacted compared to somatic chromatin, despite the similar histone composition. In addition to compacting the sperm chromatin P and PL proteins erase the epigenetic contribution of histones (Caron et al., 2005; Rousseaux et al., 2005). What happens to the epigenetic marks in H-type sperm chromatin is not clear and in needs to be studied.

In terms of sperm head shape, it is difficult from the three amphibian examples shown in Figure 3 to make any predictions other than the observation that whereas PL/P-types seem to lead to more conical streamlined head shapes (Fig. 3 B,C), histones 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.

Concluding remarks

The rapid evolution of sperm proteins, in particular protamines, allows 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 variable and independent of the SNBP composition. Although protamines and PL proteins compact DNA more efficiently, histones are still able to significantly compact the chromatin compared to that of somatic cells. Increased chromatin compaction by SNBPs decreases the volume and streamlines the shape of the sperm head, providing better protection against externally damaging agents and enhancing sperm mobility.

Chapter 2. Characterization of the PL-I-related SP2 protein from *Xenopus*

L.J.F.'s contribution to the work:

I prepared of the data, figures and writing. This chapter was originally published in (Frehlick et al. 2007). Alison Caestagne-Morelli contributed the reconstituted nucleosomes used in the gel mobility retardation assays.

Abstract

The complete cDNA sequence of *Xenopus laevis* sperm specific proteins SP1 and SP2 has been determined. This information when taken together with N-terminal sequencing and mass spectrometry data indicates that these two proteins share a product precursor relationship in which SP2 results from cleavage of a short N-terminal peptide of SP1. The secondary and tertiary structures of SP2 have been characterized using circular dichroism and three dimension structure prediction. These structural analyses have shown that SP1/SP2 proteins are related to proteins of the histone H1 family, particularly to vertebrate histone H1x. Hence, they can be considered *bona fide* members of the protamine-like- I (PL-I) group of sperm nuclear basic proteins (SNBPs) that have been described in other vertebrate and invertebrate groups. SP2 binds to nucleosomal DNA in a way that is very similar to that of histone H1. However its interaction with circular DNA does not exhibit an enhanced preference for the supercoiled conformation and the binding of SP2 to DNA appears to be mainly driven by ionic interactions.

Introduction

In sperm, DNA is associated with basic proteins, which often differ from somatic histone proteins. The sperm-specific nuclear basic proteins (SNBPs) are much more diverse than the nucleosomal proteins of somatic cells and can be grouped into three categories: protamine (P-type), protamine-like (PL-type) and histones (H-type) (Ausió, 1999). P and PL-type SNBPs are higher in arginine than the somatic histones that they replace, which lends to their ability to more densely compact the spermatozoa DNA (Ausió, 1999). The PL-type of proteins includes a group of SNBPs with intermediate composition between the histone and the protamine type (Ausió, 1999). It has now been shown that this group, and possibly the P-type as well, are structurally related to linker histones (histone H1) (Eirin-Lopez et al., 2006a, b). Evolutionarily PL proteins appear to have evolved from a histone H1 related PL-I protein.

Whereas most small molecular weight PL or P protamines do not have any secondary or tertiary structure, PL-I proteins all contain a central folded domain with high similarity to the winged-helix domain, which is characteristic of the members of the histone H1 family. To date, PL-I proteins have been identified in invertebrates (Jutglar et al., 1991; Zhang et al., 1999), chordates (Lewis et al., 2004a) and other vertebrate organisms (Saperas et al., 2006). However, low molecular weight PLs lacking the winged-helix domain have only been described in invertebrate organisms (Eirin-Lopez et al., 2006b).

Mature *Xenopus laevis* sperm contains six SNBPs, referred to as sperm-specific proteins SPs (SP1 to SP6) (Mann et al., 1982), which are electrophoretically distinct (Abe

and Hiyoshi, 1991). In addition, mature sperm chromatin contains somatic-type histones H3 and H4 but has dramatically reduced levels of H2A and H2B (Mann et al., 1982).

X. laevis is a well characterized model organism that has been used extensively to study the process of fertilization. A recent *in vitro* study showed that the histone chaperone nucleoplasmin was able to remove the sperm proteins of *Xenopus* and linker histones from *Xenopus* and chicken erythrocytes with similar efficiency, suggesting the possibility that these proteins may have similar structures (Ramos et al., 2005). Although some information is already available about the primary structure and gene relationship of the low molecular weight SP3-SP6 proteins, the relationship of these proteins to SNBPs of the PL-type and the sequence identity of SP1/SP2 has yet to be determined. Here we provide a detailed structural and biochemical characterization of SP2 and its interactions with circular DNA and with nucleosomes. The data show that SP2 is structurally and functionally a member of the histone H1-related PL-I family. Furthermore, we conclusively show that SP2 is a post-translational cleavage product of SP1. These proteins share a compositional similarity with SP3-SP6. Taken together, these results conclusively show that the SP proteins of *Xenopus* are members of the PL family of SNBPs and share a striking similarity to what has previously been shown in invertebrate groups such as *Mytilus* (blue mussel), which also exhibit a complex heterogeneous SNBP composition.

Materials and Methods

Extraction and purification of proteins

Samples were obtained from *X. laevis* that were reared at the University of Victoria aquatics facility. SNBPs were extracted from testes with 0.4N HCl and precipitated with acetone as described by (Wang and Ausi3, 2001). The protein extract thus obtained was resuspended in HPLC grade distilled water and fractionated by HPLC on a reverse phase 300-Å Vydac C18 column (25 X 0.46 cm) eluted at 0.4 ml/min with a 0.1% TFA-acetonitrile gradient (Ausi3, 1988).

Alternatively, for binding assays and circular dichroism experiments the sperm proteins were fractionated from sperm nuclear extracts by ionic-exchange chromatography using carboxymethyl (CM) C-25-Sephadex as described elsewhere (Ausi3 and Subirana, 1982a). The column was equilibrated in 1 M NaCl, 50 mM sodium acetate buffer, pH 6.7, and eluted with a 1-1.5 M NaCl linear gradient in the same buffer.

Gel electrophoresis

Proteins were separated by AU-PAGE (5% acetic acid-12% PAGE-2.5 M urea) according to (Ausi3, 1992). AUT-PAGE (5% acetic acid-10.5% PAGE-5.25 M urea-5 mM Triton X-100) was a modified recipe from that described in (Bonner et al., 1980). The gels were prepared by mixing the following: 7 mg thiourea, 5 ml (20:1 acrylamide-bisacrylamide), 0.48 ml of glacial acetic acid, 3 g urea, 24 µl of 45 mM NH₄OH (made fresh), 0.118 ml of 25% Triton X-100 and 1.33 ml of double distilled water. After the urea had been completely solubilized, 45 µl of 30% H₂O₂ was added and the solution was immediately poured between the glass plates as polymerization proceeds very quickly.

These gels do not need to be pre-electrophoresed and can be used immediately after polymerization. SDS-PAGE [15% acrylamide, 0.4% Bis-acrylamide] was prepared as described in (Laemmli, 1970). The gels were stained with Coomassie Brilliant Blue R [0.2% (w/v)] in 25%/10% (v/v) isopropanol/acetic acid and destained in 10%/10% isopropanol/acetic acid.

Nucleosomes were separated by 0.7% agarose in Tris-Borate-EDTA (TBE) and plasmids were separated by 1% agarose in Tris-Acetate-EDTA (TAE). Agarose gels were stained with ethidium bromide and visualize with UV light.

Trypsin digestion

SP2 was digested with trypsin (EC 3.4.21.4) (type III) (Sigma-Aldrich, St. Louis MO). Digestions were carried out in 2 M NaCl, 25 mM Tris/HCl (pH 7.5), as described elsewhere (Ausió et al., 1987) buffer at an E/S ratio of 1 : 1000 (w:w) at room temperature. Aliquots of the digestion were collected at different times, mixed with 2X gel electrophoresis sample buffer and immediately frozen and kept until used for AU-PAGE analysis.

Nucleosome gel mobility retardation assay

Histone octamers were obtained from hydroxyapatite (HAP) chromatography of chicken erythrocyte chromatin (Ausió and Moore, 1998). A 208 bp DNA fragment was obtained by RsaI (New England Biolabs, Ipswich, MA) digestion of a 208-12 oligomer consisting of 12 tandemly arranged fragments of from the 5S rRNA gene of the sea urchin *Lytechinus variegatus*.

Nucleosomes were reconstructed by histone octamer assembly onto the 208 bp DNA template by step-wise salt dialysis (Tatchell and Van Holde, 1977). The histone:DNA molar ratio was 1:1.

Aliquots of 300 ng of reconstituted nucleosome particles (in 50mM NaCl, 10mM Tris and 1mM EDTA) were incubated with increasing amounts of linker histone H1 following a protocol modified from (Sera and Wolffe, 1998). After incubation at room temperature for 30 minutes 30% sucrose was added to bring the samples to a final 5% sucrose concentration and the samples were then loaded onto a 0.7% agarose gel in 0.5× TBE.

Plasmid gel mobility retardation assay

Plasmid pBR322 was purified using the QIAprep spin miniprep kit (Qiagen, Misissauga, ON) from *E. coli* grown at 37°C overnight in LB broth, following the manufacture's protocol. For the linear plasmid, pBR322 was digested with BamHI (New England Biolab, Ipswich, MA). The reaction was cleaned up using the QIAquick PCR purification kit. DNA concentrations were determined on a Nanodrop spectrometer (Nanodrop Technologies, Wilmington, DE) using an extinction coefficient of 20 mL cm⁻¹ mg⁻¹ at 260 nm.

Increasing amounts of SP2 or chicken H5 were incubated with 0.5 µg of pBR322, in a total reaction volume of 20µl and final buffer concentration of 10 mM Tris-HCl (pH 8), 0.2 mM EDTA, 0.02% Triton X-100 and 4 mM NaCl, as described in (Ellen and van Holde, 2004). The samples were incubated at room temperature for 1 hour, then 30% sucrose was added to give a final concentration of 5% and the samples were loaded onto a 1% agarose gel in TAE.

Circular Dichroism and UV spectroscopy

Circular dichroism (CD) Spectroscopy experiments were carried out at 20 °C on a Jasco model J720 spectropolarimeter. Spectra were acquired from 200-260 nm using a bandwidth of 1 nm and data pitch of 0.1 nm at a scan speed of 2 nm/min and 1 accumulation per second. Spectra were corrected for solvent contribution and the CD signal was converted to molar ellipticity, $[\theta]$, using the formula, $[\theta] = \text{CD} (\text{MRW} / 10 \times l \times c)$, where MRW is the mean residue weight (the molecular mass divided by the number of peptide bonds), l is the path length of the cell, and c is concentration in mg/ml. The extinction coefficients in water at 230nm were determined by amino acid analysis using Norleucine as an internal standard. The values thus obtained were 2.35, 3.03, 4.53 and 8.1 $\text{cm}^2\text{mg}^{-1}$ for H5, SP2, SP3-5 and SP6 respectively. The value determined in this way for histone H5 agrees with the previously reported value of 2.34 $\text{cm}^2\text{mg}^{-1}$ (48 000 $\text{M}^{-1}\text{cm}^{-1}$) in (Carter and van Holde, 1998) using the same experimental approach. The absorbances were measured on a Cary spectrophotometer (Varian, Palo Alto, CA) and the change in absorbance of the proteins in 20 mM Sodium Phosphate pH 7.2 and in 10 mM Tris-HCl/ 0.1 M NaCl pH 7.5 were used to correct the extinction coefficients for buffer effects. On average the extinction coefficients were 15% lower in buffer than in water.

Because histones exhibit a very low β sheet structure, the percentiles of α helix were estimated from the values of the ellipticity $[\theta]$ at 220 nm according to (Verdaguer et al., 1993) using the equation,

$$\% \alpha \text{ helix} = 8.9 - (2.47 \times [\theta]_{220\text{nm}} \times 10^{-3})$$

and at 208nm (Greenfield and Fasman, 1969) using the equation,

$$\% \alpha \text{ helix} = [([\theta]_{220\text{nm}} - 4000) / (-33000 - 4000)] \times 100$$

N-terminal sequencing and Mass spectrometry

The N-terminal sequence and partial peptide sequence was obtained using SP2 purified by RP-HPLC. The N-terminal protein sequence was determined by conventional Edman degradation using an ABI Precise protein microsequencer (Applied Biosystems, Foster City, CA), as described previously (Carlos et al., 1993b). The partial peptide sequence was obtained from Glutamic endopeptidase digestion of the SP2 protein. The resulting peptides were separated by reverse phase HPLC and the most prominent peptide was sequenced by electrospray quadrupole time-of-flight (Q-TOF) mass spectrometry.

Molecular masses were determined by mass spectrometry analysis of *X. laevis* SP1 and SP2 carried out by MALDI-TOF on a Voyager Linear DE (PerSpective Biosystems Inc., Foster City, CA) using a sinapinic acid matrix following the protocol described in (Hunt et al., 1996).

cDNA sequence determination

Total RNA from testes was extracted using Trizol reagent (GibcoBRL, Burlington, ON) and mRNA from total RNA was isolated using a mRNA purification kit (Amersham Bioscience, Piscataway, NJ). The following primers were designed using protein sequence data and cDNA sequences with similarity to SP2 (such as *X. laevis* histone H1x, GeneBank accession number AAH41758):

Forward 1: CAGCCGGGCMRSTACAG

Forward 2: CAGGAACGGCTCGTCCCT

Reverse 1: GTASYKGCCCGGCTGGTT

Reverse 2: GGGACGAGCCGTTCCCTCT

Using these primers the complete cDNA sequence was amplified using the First Choice Rapid amplification of cDNA ends (RACE) Kit (Ambion, Austin, TX). For cDNA sequencing, agarose gel purified PCR products were ligated into pCR2.1-TOPO vectors (Invitrogen, Burlington, ON) following the instructions of the manufacturer and transformed into TOP10 competent cells (Invitrogen, Burlington, ON). The plasmids were purified with the QIAprep Miniprep kit (Qiagen, Mississauga, ON) and sequencing of the inserts was done by the DNA Sequencing Facility, Centre for Biomedical Research at the University of Victoria. The *X. laevis* SP2 sequence determined was aligned with similar protein sequences using the CLUSTAL_X (Thompson et al., 1997) and BIOEDIT programs (Hall, 1999) with the default parameters.

Protein structure

The secondary structures were predicted from the protein sequences using PROFsec (Rost and Sander, 1993) on the PredictProtein server (Rost et al., 2004) The three dimensional structure of *X. laevis* SP2 was modeled using the coordinates determined from the crystal structure of the globular core of the chicken erythrocyte histone H5 (Ramakrishnan et al., 1993) as a reference, using the SWISS-MODEL server (Schwede et al., 2003).

Results

The primary structure of X. laevis SP1 and SP2

It had been previously revealed that SP2 was processed from a precursor which was probably SP1, as a pulse-chase experiment showed SP2 increased while the SP1

protein band decreased concomitantly (Abe and Hiyoshi, 1991). There was also indirect evidence, based on partial N-terminal sequence that suggested that SP1 and SP2 shared a precursor – mature protein relationship (Ariyoshi et al., 1994). Here, SP1 and SP2 were purified from other basic proteins of *X. laevis* testes by either reverse phase HPLC (Fig. 4A) or cation exchange chromatography (CM Sephadex C25) (Fig. 4B). The high absorbance at 230 nm of peak 2 of the cation exchange column (Fig. 4B) was caused by the peptides of the protease inhibitor (shaded area), which eluted in the same position as SP2.

As can be seen in Figure 4A, reverse-phase HPLC allowed us to separate SP1 (lane 4) from SP2 (lane 5). The proteins thus obtained were used in N-terminal and Mass spectrometry sequencing (Fig. 5), and their masses were determined by MALDI-TOF (Table 1). The results provided N-terminal sequence for SP2 and some internal peptide sequence information. Attempts to determine the N-terminal sequence of SP1 failed, probably due to N-terminal blocking, as has been observed with other SNBPs (Saperas et al., 2006). Alanine at position 2 of the amino acid sequence had a 83% likelihood of being acetylated as predicted by the Terminator program at <http://www.isv.cnrs-gif.fr/terminator2/index.html> (Frottin et al., 2006), which supports this hypothesis. In order to obtain the complete primary structure of these proteins, and establish the precursor – product relationship between them, we cloned the cDNA (see Fig. 5 for the cDNA and translated protein sequence). The N-terminal sequence of SP2 did not start at the initial methionine. The size difference between the N-terminal sequence of SP2 and the starting methionine matched the mass difference between SP1 and SP2 as determined by MALDI-TOF (Table 1). These results, along with the fact that the N-terminus of SP1

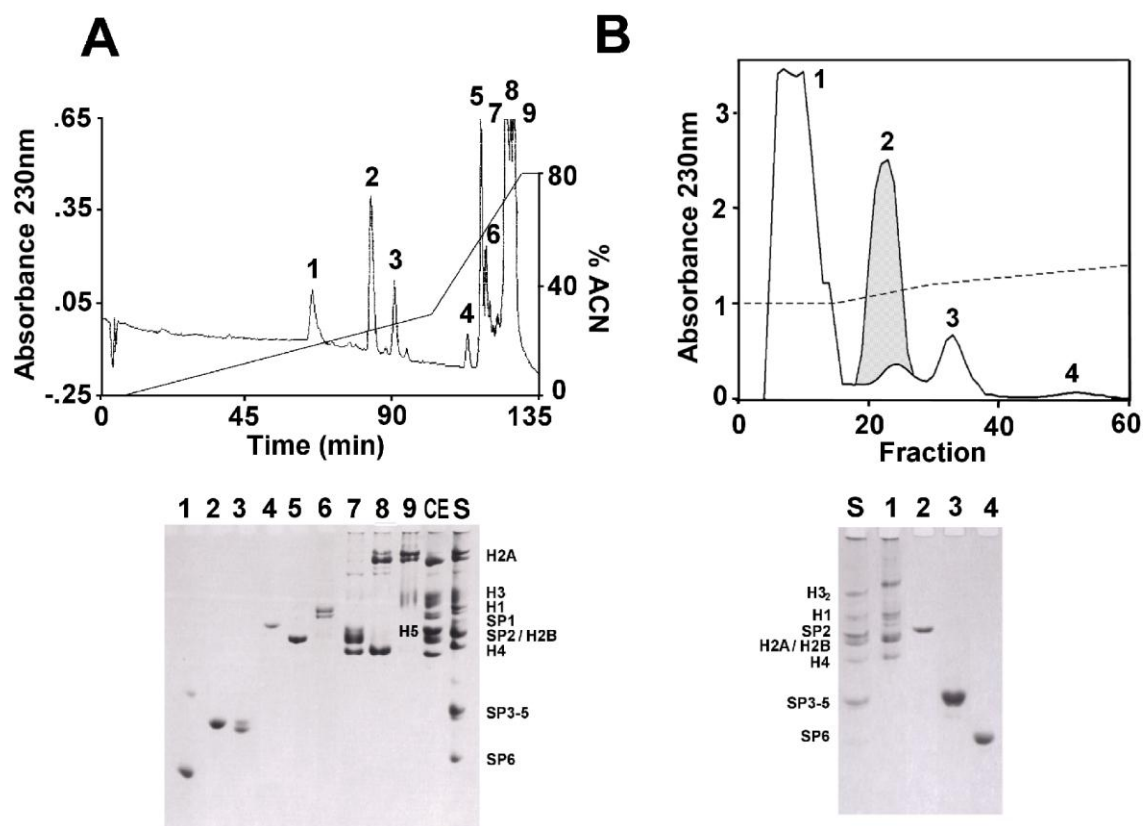


Figure 4. Fractionation and purification of *X. laevis* SNBPs.

A) Reversed phase HPLC. Proteins were eluted with an acetonitrile (ACN) gradient. Below is an AUT-PAGE analysis of the indicated peaks from A (1-9). CE, chicken erythrocyte histones; S, starting sample loaded on the HPLC. **B)** Ion exchange [Carboxymethyl (CM) C-25 Sephadex] chromatography. The solid line denotes the absorbance at 230nm and the dashed line denotes the 1M to 1.4M NaCl gradient in 50mM sodium acetate buffer (pH 6.7). The gel below shows AU-PAGE analysis of the indicated peaks from A (1-4). H3₂ indicates an H3 dimer.

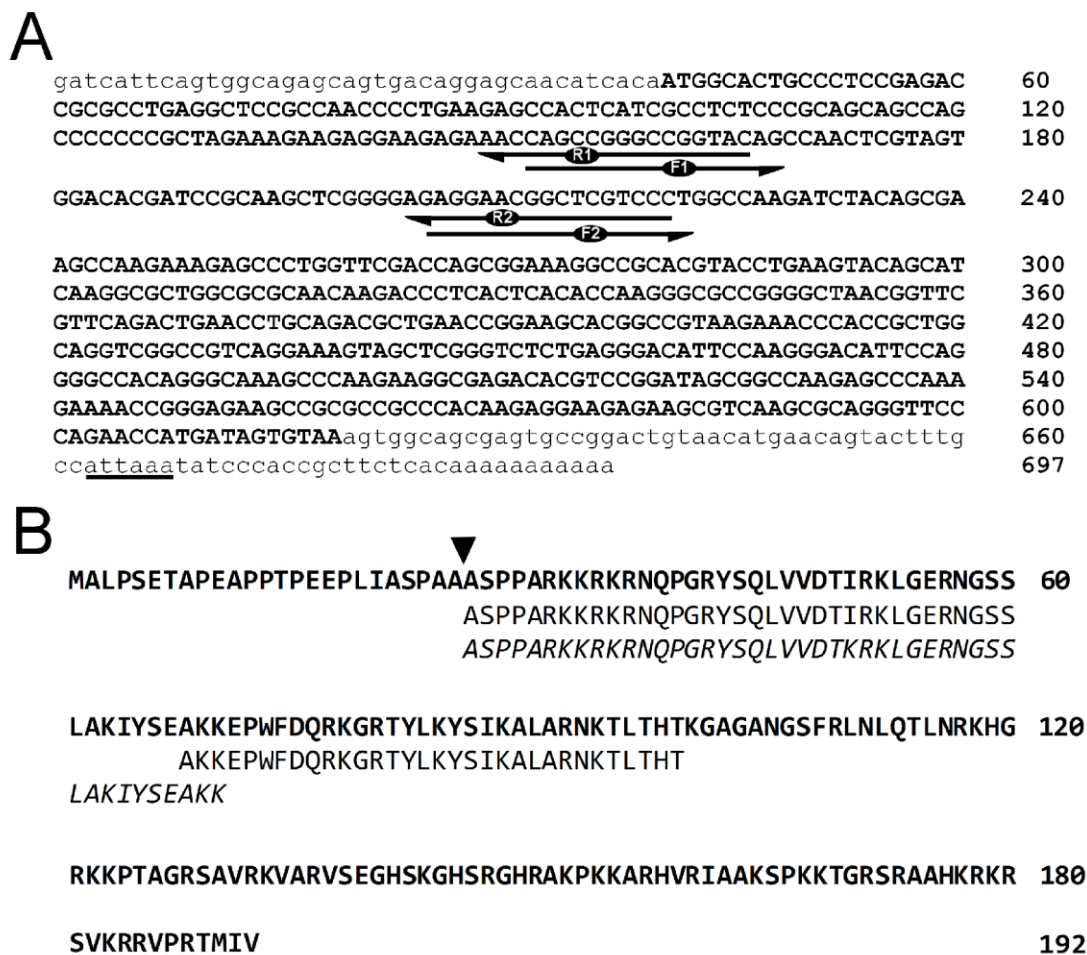


Figure 5. *X. laevis* SP1 / SP2 sequence.

The complete SP1/SP2 cDNA sequence determined from RACE PCR (Genebank accession # 920865). The 5' and 3' untranslated regions are in lower case and the coding region is in bold capitals. A putative polyadenylation sequence is underlined and the primers used for RACE are indicated by arrows. **B.** The amino acid sequence translated from the SP1 / SP2 cDNA is shown in bold. Below the full sequence is the partial peptide sequences confirmed by N-terminal sequencing and mass spectrometry and in italics the N-terminal sequence previously determined by Ariyoshi et. al. (Ariyoshi et al., 1994). The predicted cleavage site on the SP1 precursor is shown with an arrow head.

Table 1. The predicted protein size compared to the mass determined from MALDI-TOF mass spectrometry

Protein	# of amino acids	Predicted size (Da)	Size from MALDI-TOF (Da)
SP1	191	21311*	21327
SP2	167	18930	18941

*This value is based on the N-terminal Methionine being cleaved and the alanine being acetylated (addition of 42 Da)

was blocked, indicated that the cleavage site on SP1 is at the N-terminus (shown by a triangle in Fig. 5B).

The secondary and tertiary structure of SP1 / SP2.

To further characterize the structure of the protein, SP2 was analyzed by CD to determine the secondary structure composition. SP2 appears to have a structural organization similar to histone H5 (Fig. 6A), as can be seen by the shape of the CD spectra, with lows at 208 nm and 222 nm characteristic of α helices (Greenfield and Fasman, 1969; Townend et al., 1966). However, the ellipticities at 208 nm and 222 nm of the SP2 curves were not as low as H5, suggesting less α helical content. The calculated α helical content estimated for SP2 was 15.7% and 17.6% and for H5 was 18.7% and 20.3%, for Tris and phosphate buffers respectively. The α helical content of H5 determined in this way is agrees with that of the 19.5% determined from the crystallographic analysis of the winged helix domains of this protein (Ramakrishnan et al., 1993) and suggests that this is the only structural region in solution. The α helical content estimated from the predicted secondary structure analysis was 31.8% for SP2 and 20% for H5 (Fig. 6C). The high predicted α helical content of SP2, compared to that of the calculated content, is most likely due to the fact that the four helices predicted for the tail domain would not likely be folded in solution. The basic charges of the lysine and arginine residues in this region would disrupt the helical fragments and would likely need to be neutralized by binding DNA to form any helical structure (Verdaguer et al., 1993). Also, the prediction of these helices only had a reliability of 70%, relative to 100% for the helices in the globular regions. The fact that the SP2 C-terminal tail does not form helices in solution, as well as

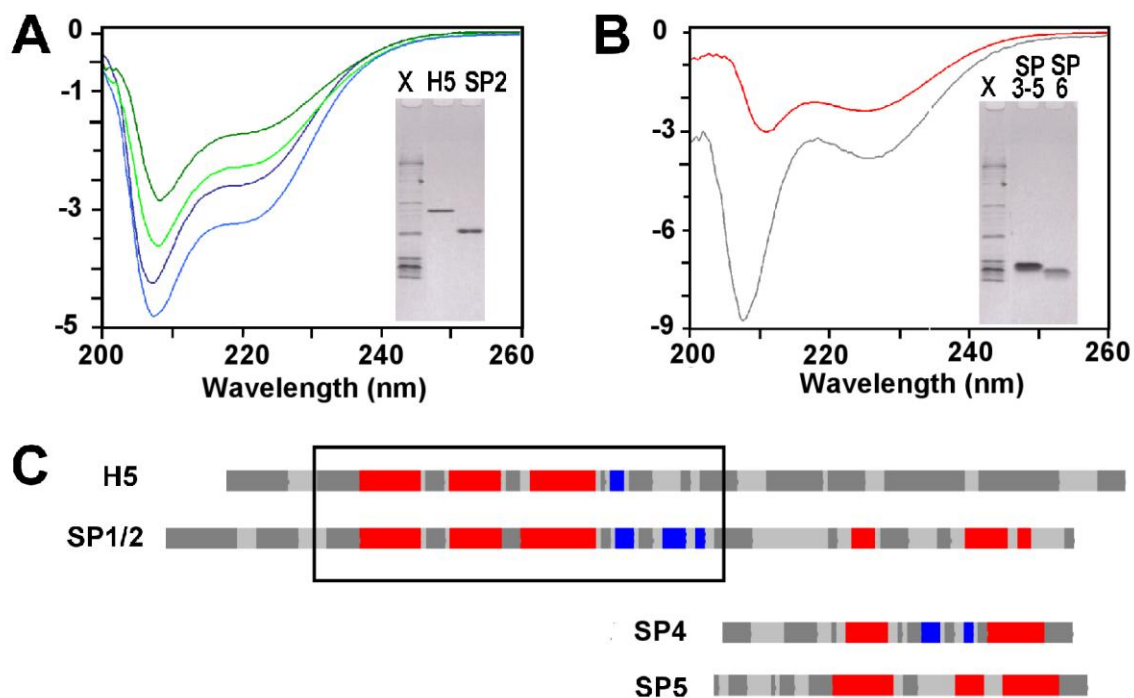


Figure 6. Protein secondary structures of chicken histone H5 and *Xenopus* SP2

A) CD spectra of *X. laevis* SP2 (green) and chicken H5 (blue) in 10mM Tris / 0.1 M NaCl buffer (pH 7.5) (dark green and blue) and 20mM phosphate buffer (pH 7.2) (light green and blue). **B)** CD spectra of *X. laevis* the SP3, SP4 and SP5 fraction (red) and SP6 (grey), both in 20mM phosphate buffer (pH 7.2). The inserts show the SDS-PAGE of the proteins used in the analysis: X1 is *X. laevis* SNBPs. **C)** The predicted secondary structures of H5, SP1/SP2, SP4 and SP5. Red, helix; Blue, extended (sheet); Dark gray, other (loop) and Light gray, no prediction is made for these residues as the reliability is too low (< 5). The globular domains of H5 and SP1/SP2 are within the black box.

the bias toward hydrophilic amino acids indicate that it is likely intrinsically disordered, similar to the C-terminal tail of mouse H1o (Hansen et al., 2006).

For comparison, the mixed SP3, SP4 and SP5 fraction and the SP6 protein were also analyzed by CD. The spectra of these proteins are shown in Figure 6B. The amount of α helix determined for these was 15 % for the SP3-5 mixture and 26 % for SP6. This, despite the shorter amino acid sequence of these proteins, is somewhat similar to that of SP2 and H5. As with SP2, the predicted secondary structure, which consists predominantly of α helix and random coil (Fig. 6C), was higher: approximately 28% for SP3-5 and 39% for SP6.

The alignment of the primary structures indicates that SP2 shares a substantial amount of sequence similarity with the core domain of linker histones and other PL-Is (Fig. 7A).

One of the structural signatures of the protein members of the H1 family of linker histones is the presence of a winged-helix domain (Ramakrishnan et al., 1993), which exhibits trypsin digestion resistance (Hartman et al., 1977). To check that this was also the case with SP2 the protein was digested with trypsin in the presence of 2M NaCl. As can be seen in Figure 7B, and similar to what has been observed with other histone H1 – related SNBPs (Ausió et al., 1987; Jutglar et al., 1991; Saperas et al., 2006), a trypsin-resistant peptide was observed. Given the sequence similarity to the globular region of H5 (Fig. 7A) and the fact that the secondary structure predictions yielded three helices in this region for SP2, which matched the size and spacing of those in H5 (Fig 6C), it is not surprising that the tertiary structure modeling gave a 3D structure that is an almost perfect match to the winged helix of H5 (Fig. 7C).

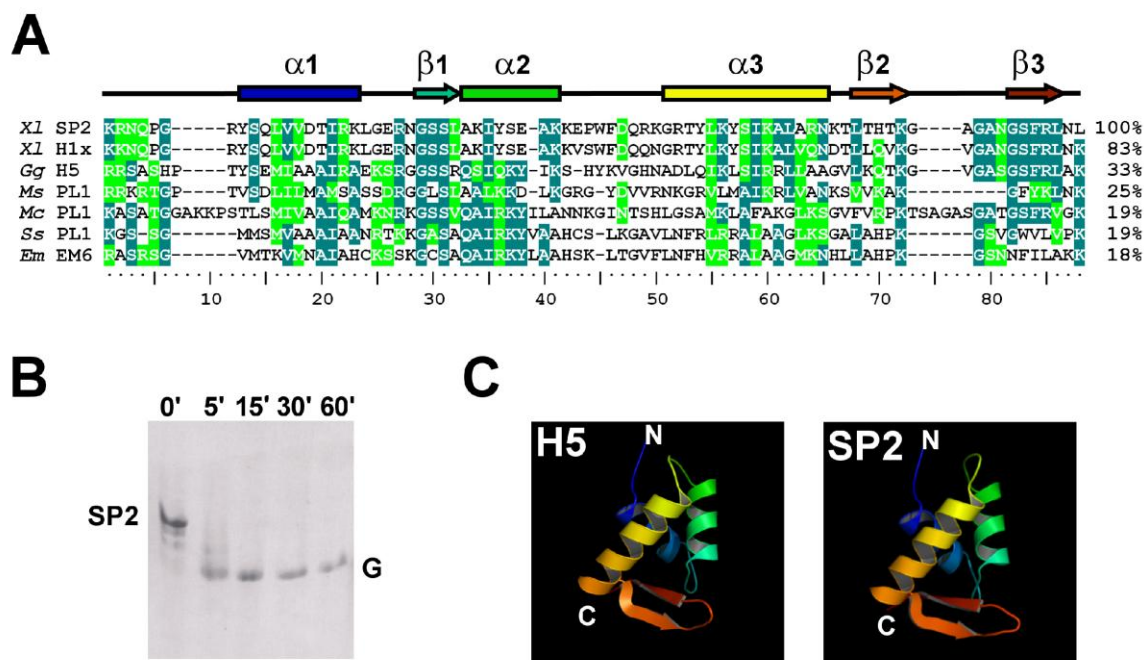


Figure 7. SP2 contains a trypsin-resistant winged-helix motif.

A) The schematic secondary structure of the globular winged-helix domain is above the corresponding amino acid sequences. β -turns and strands are indicated by arrows and α -helices are indicated by boxes. Sequence alignment of the amino acid region corresponding to the globular winged-helix domain in SP2 in comparison to other protein members of the histone H1 family. The light green shading indicates similar amino acids and teal the shading indicates identical amino acids in at least 50% of the sequences compared. The sequences and Genebank accession numbers are: *Xl* **SP2**, *X. laevis* SP2; *Xl* **H1x**, *X. laevis* histone H1x (AAH41758); *Gg* **H5**, *Gallus gallus* histone H5 (NP001038138); *Ms* **PLI**, *Mullus surmuletus* PL-I (Q08GK9); *Mc* **PLI**, *Mytilus californianus* PL-I; *Ss* **PLI**, *Spisula solidissima* PL-I (AAT45384); *Em* **EM6**, *Ensis minor* EM6 (AAA98076). The numbers at the right hand side designate percent identity. **B)** AU-PAGE analysis of the time course trypsin digestion of SP2, carried out in the presence of 2 M NaCl at room temperature. G, the resistant globular core of the protein. The digestion times (0, 5, 15, 30 and 60 min) are indicated on top of the lanes. **C)** Tertiary structure of the trypsin-resistant core of chicken erythrocyte H5 obtained from the crystallographic data determined in (Ramakrishnan et al., 1993). The H5 structure was used as a template to model the tertiary structure of the trypsin-resistant core of SP2.

Characterization of the interactions of SP2 with DNA and nucleosomes

Given of the structural similarities between SP2 and histone H5 (Fig.7), and their relation to the members of the histone H1 family, we decided to analyze and compare the binding of these two proteins to DNA and nucleosomes. The presence of a winged helix domain in linker histones imparts them with a preferential binding to DNA cruciform (four-way junction) structures (Thomas et al., 1992; Varga-Weisz et al., 1993; Varga-Weisz et al., 1994), which is reflected by their higher affinity for circular supercoiled DNA and within a more physiological relevant context, to the nucleosome.

Similar to histone H1 (Ellen and van Holde, 2004; Ivanchenko et al., 1997), histone H5 was found to preferentially bind to supercoiled DNA over linear or relaxed circular DNA (Fig. 8A, lanes 9-12). This is shown by the fact that the supercoiled plasmid (lower band) shifts before the linear or relaxed circular DNA. In addition, the protein to DNA ratios (w:w) at which the plasmids shifted were similar to that of H1 (Ellen and van Holde, 2004). A complete shift was observed with circular supercoiled DNA at a protein:DNA ratio of 0.25 – 0.5 (w:w) for both histone H1 (Ellen and van Holde, 2004) and histone H5 (Fig. 8A, lanes 5-8). In contrast, approximately 5 times the ratio of SP2:DNA compared to that of H5:DNA (1.5 w:w versus 0.3 w:w) was required to shift the plasmids. Another difference between the shifts of H5 and SP2 is that, like in the case of histone H1 (Ellen and van Holde, 2004), H5 appears to bind linear DNA non-specifically, resulting in a sudden aggregation of the DNA (Fig. 8A, lanes 1-4). This is in contrast to the supercoiled band, which shifts in a regular manner as the amount of H5 is increased. For our SP2 protein, both the supercoiled and linear DNA exhibits this regular shift, albeit the linear DNA did aggregate at a lower ratio (1-1.5 w:w) than the supercoiled DNA (1.5-2.0 w:w). Although SP2 seems to bind the supercoiled DNA first

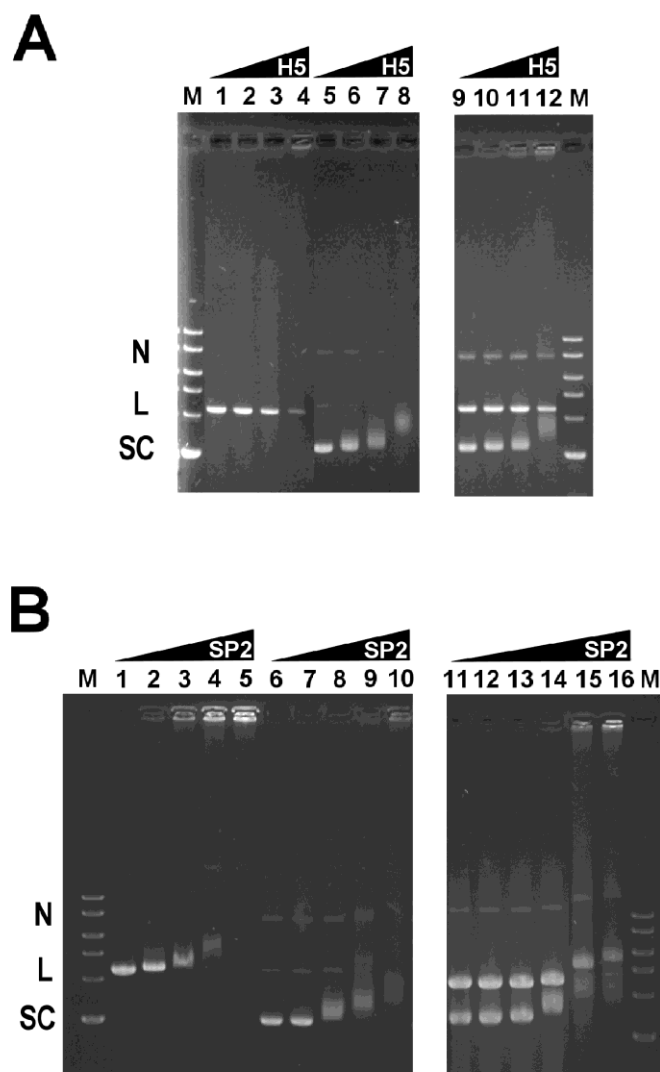


Figure 8. Gel mobility retardation assay comparing the binding of H5 and SP2 to supercoiled and linear pBR322 plasmid DNA.

A) Increasing amounts of H5 were incubated with 0.5 μ g of BamHI cut pBR322 (lanes 1-4), uncut pBR322 (lanes 5-8) or both uncut and cut plasmid (lanes 9-12). After incubation the resulting complexes were analyzed on a 1% agarose gel. The H5 to plasmid ratios (w:w) were 0, 0.075, 0.15 and 0.3 for lanes 1-4, 5-8 and 9-12 respectively. **B)** Increasing amounts of SP2 were incubated with 0.5 μ g of BamHI cut pBR322 (lanes 1-5), uncut pBR322 (lanes 6-10) or both uncut and cut plasmid (lanes 11-15). The SP2 to plasmid ratios (w:w) were 0, 0.5, 1, 1.5 and 2 from lanes 1-5, 6-10 and 11-15 respectively, lane 16 had a ratio of 2.5. A 1 kilobase marker (M) was loaded with bands of 10, 8, 6, 5 and 3 kb from the top.

(Fig. 8B, lane 14), at higher ratios both the linear and supercoiled DNA experience a comparable shift (Fig. 8B, lanes 15 and 16).

The different ability of SP2 to bind to supercoiled DNA compared to somatic linker histones (H1 and H5) contrasts with the similar ability of these two proteins to bind to nucleosomal DNA (Fig. 9). Furthermore, discrete nucleosomal-histone H1 complexes are formed at very similar input ratios (Fig. 9).

X. laevis SP proteins are genuine members of the sperm nuclear basic proteins of the PL-type.

The relationship of SP1/SP2 with members of the histone H1 family clearly identifies these proteins as belonging to the protamine-like (PL-I) type of SNBPs (Fig. 10) (Ausió, 1999; Eirin-Lopez et al., 2006b). PL-proteins and H1 histones are closely evolutionarily related and are descendants of a common ancient orphon group of H1-replication-dependent histones (Eirin-Lopez et al., 2004). PL proteins have been described in invertebrate (Ausió, 1986; Ausió et al., 1987; Bandiera et al., 1995; Carlos et al., 1993b) and chordate groups (Lewis et al., 2004b; Saperas et al., 2006; Watson and Davies, 1998; Watson et al., 1999). Interestingly, in both instances a precursor-product relationship involving protein post-translational cleavage of the N-terminal (Bandiera et al., 1995) or C-terminal domain (Carlos et al., 1993a) of these proteins has been described. For example, N-terminal cleavage of the *X. laevis* SP1 precursor yields the SP2 protein (as shown in this paper) and in mussel, cleavage at a C-terminal cut sight in the PL-I precursor yields both PL-II and PL-IV (Fig. 10). However, the cleavage site (SPAA*ASP) at the N-terminal region of SP1 has no sequence similarity to the proteolytic processing sites of invertebrates (NKSNN*AK) whether they occur at the

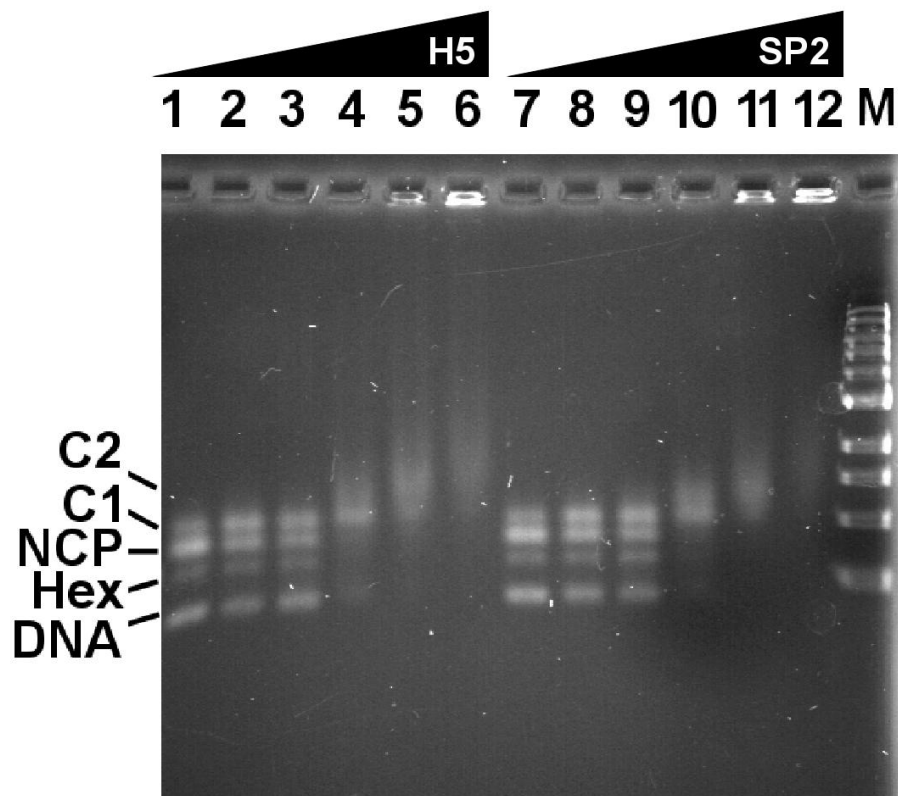


Figure 9. Gel mobility retardation assay comparing the binding of H5 and SP2 to nucleosomes.

Increasing amounts of H5 were incubated with 300 μ g of nucleosomes and analyzed on a 0.8 % agarose gel. The H5 to nucleosome ratios (mol:mol) were 0, 1, 2, 4, 6 and 8 for lanes 1-6.

Increasing amounts of SP2 were incubated with 300 μ g of nucleosomes. The SP2 to plasmid ratios (mol:mol) were 0, 1, 2, 4, 6, and 8 for lanes 7-12. A 1 kilobase marker (M) was loaded with bands of 10, 8, 6, 5 and 3 kb from the top. C1, chromatosome with one linker protein; C2 chromatosome with two linker proteins; NCP, nucleosome core particle; Hex, hexamer and DNA, free DNA.

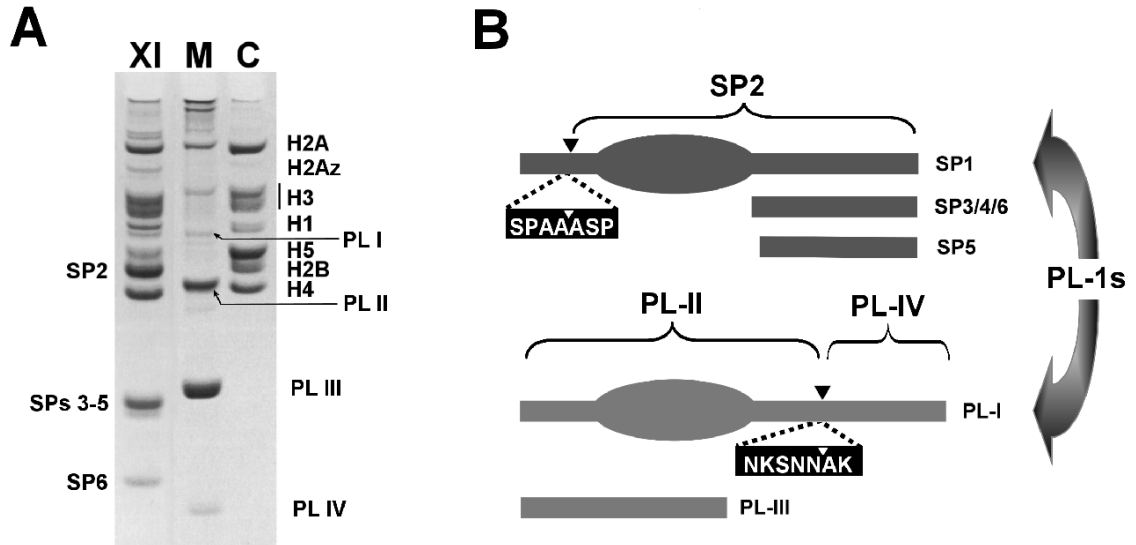


Figure 10. The SP proteins from *X. laevis* are structurally related to the SNBPs of the PL-type.

A) Acid-Urea-Triton (AUT)-PAGE characterization of SNBPs from *X. laevis* (XI), and *Mytilus californianus* (M) and a histone marker from chicken erythrocytes (CE). PL, protamine-like. **B)** Schematic representation of sperm protein structure in *X. laevis* (SP1 to SP6) and *Mytilus* (PL-I, II and III), showing the globular (trypsin-resistant) portions (oval) and tails (rectangular). See text for details.

N- or C-terminal regions of these proteins (Fig. 10B) (Agelopoulou et al., 2004; Carlos et al., 1993a).

Discussion

Xenopus SP2 is the cleavage product from an SP1 precursor.

Nucleotide sequences are available for SP4 and SP5 (Ariyoshi et al., 1994; Hiyoshi et al., 1991) and partial N-terminal peptide sequences are available for SP1-6 (Ariyoshi et al., 1994). Cloning of SP4 and 5 and N-terminal sequence information indicates that the six SPs are derived from three different mRNA. SPs 3, 4 and 6 are derived from one mRNA and SP5 from a separate mRNA (Ariyoshi et al., 1994). These mRNAs are polyadenylated and likely replication-independent and expressed in primary spermatocytes. From the available N-terminal protein sequence data SPs 1 and 2 are suspected to be encoded by a common mRNA species (Ariyoshi et al., 1994). However, no nucleotide sequence is currently available to support this notion.

The results provided show that a precursor-product relationship exist between SP1 and SP2 where SP2 is the result of post-translational cleavage of SP1 with removal of the first 25 N-terminal amino acids. A precursor product relationship between *X. laevis* SP1 and SP2 had been earlier demonstrated by Katagiri and co-workers using [14C] arginine - [14C] lysine incorporation during spermiogenesis (Abe and Hiyoshi, 1991). However, based on N-terminal sequencing of the two proteins it was concluded that the protein processing must have taken place at the C-terminal end of SP1 (Ariyoshi et al., 1994). The disagreement regarding the site of cleavage is most likely due to the method (AUT gel purification and extraction of the bands with 0.4 N SO₄H₂) used by (Ariyoshi et al.,

1994). As we have shown, the N-terminal end of SP1 appears to be blocked and contamination and/or partial cleavage by the method of extraction used could have led to the misidentification of the SP-1 N-terminus in (Ariyoshi et al., 1994).

Sperm specific SP1/SP2 proteins are closely related to vertebrate histone H1x.

The protein sequence identities shown in Figure 7A indicate that, of all the histone H1 proteins, SP1/SP2 exhibits the highest similarity with H1x. Histone H1x has been recently described to be present in mammalian cells and to be closely related to the replacement subtype H1o (Happel et al., 2005). The genes for these two linker histones are orphans and like SP1/SP2 are transcribed into polyadenylated mRNA (Happel et al., 2005). Interestingly human H1x is present in chromatin regions that are resilient to micrococcal nuclease digestion suggesting that they contribute to enhance chromatin compaction (Happel et al., 2005) and accumulates in the nucleolus during G1 phase at inactive ribosomal genes (Stoldt et al., 2007).

Although SP1/SP2 shows a high similarity with H1x proteins within the core domain, there is greater variability in the N- and C- tail regions. Following cleavage of the SP1 precursor, the resulting SP2 protein has only a short highly basic C-terminal tail and together the C- and N-terminal tail domains of SP2 are higher in arginine than a typical H1. This high arginine content is clearly seen when comparing the amino acid composition of *X. laevis* SP2 to *X. laevis* H1x. SP2 has 15.6% arginine whereas H1x only has 4.1% arginine. This increase in arginine is at the expense of lysine, as SP2 has only 15.6% lysine and H1x has a 21.2 % lysine content. The high arginine content is characteristic of PL and P proteins (Ausió, 1999).

The chromatin compacting role of the histone SP1/SP2-related histone H1s is in agreement with the role of SP proteins in the condensation of *X. laevis* chromatin during spermiogenesis. The molecular mechanisms by which SP2 contributes to the highly condensed chromatin organization of the mature sperm nucleus are not clear. Our results indicate that SP2 can bind to nucleosomes in a similar way to histone H5 and other related histone H1 proteins. Such binding does not appear to be impaired by the partial depletion of H2A-H2B dimers (see Fig. 9 Hex), which is present to a higher extent in native *X. laevis* sperm chromatin (Mann et al., 1982). However, in contrast to histone H1 (Ellen and van Holde, 2004) or to histone H5, for SP2 no preferential binding with supercoiled DNA is observed (Fig. 8). This suggests that, as in the case of other vertebrate PL-I proteins that bind where nucleosomes are nonexistent (Saperas et al., 2006) and in the case of protamines (Lewis et al., 2003b), the interaction of SP2 with DNA is electrostatically driven.

SP2 interaction with DNA is electrostatically driven but is able to form chromatosomes

The molecular weights of H5 and SP2 are 20.6 kDa (189 amino acids) and 18.0 kDa (170 amino acids) and their basic amino acid compositions are 31.5% and 35%. From this it is possible to calculate that in both instances charge saturation (mol $-ve$ / mol $+ve$) in terms of their binding to DNA is achieved at 1:1 w:w ratios. However, whereas H1 and H5 can shift circular supercoiled DNA at a ratio of 0.3-0.5 w:w below the charge neutralization, in SP2 a shift is only observed above charge saturation, approximately 5-fold higher w:w. This indicates that SP2 binds non-specifically to circular supercoiled DNA and this binding appears to be electrostatically driven. However, the ability of SP2 to produce chromatosome structures (Fig. 9) shows that this electrostatic component does

not impede the protein from assembling into nucleosome substrates to form chromatosomes containing 1-2 molecules of SP2 (Fig. 9, C1 and C2).

Evolutionary implications

In addition to the biochemical information on amphibian PL-I related proteins, this work has some evolutionary relevance. Despite the clear relationship between SP1/SP2 and PL-I proteins, the origin of *X. laevis* SP3-SP6 and their relation to other proteins of the PL-type is not as clear. In some *Xenopus* species, such as *X. tropicalis*, these smaller sperm-specific proteins SP3-6 are absent. This absence also occurs in some molluscs. For instance, in *Mytilus*, SNBPs contain a small PL-III in addition to PL-I (PL-II/PL-IV) (Fig. 10) however, the former is absent in other species such as the surf clam *Spisula solidissima* (Lewis et al., 2004a). Whereas *Mytilus* PL-III can be distinctively related to the N-terminal region of some PL-I proteins (Eirin-Lopez et al., 2006c), a BLAST search for proteins similar to the full length precursor SP4 or SP6 produced only matches to themselves or each other. Interestingly, in the case of *X. laevis*, there is a trend toward an increase in arginine at the expense of lysine in going from the PL-I like SP2 to SP4 (precursor to SP3, SP4 and SP6) and SP5, which are more similar to the protamine group of SNBPs (Ariyoshi et al., 1994; Hiyoshi et al., 1991). This trend is reminiscent of the K to R transition that has been observed in tunicates (Lewis et al., 2004b). There is also a substantial decrease in size between SP1/SP2 and SP3-SP6, which is another characteristic feature of the protamine group of SNBPs (Ausió, 1999). These trends lend support to the hypothesis that the smaller forms of PLs or SPs represent an early gene transition from H1-related PL-I proteins to the protamines.

The coexistence of PL proteins containing (PL-I, SP2) and lacking (PL-III, SP3-SP6) the winged-helix motif appears to have occurred repeatedly in different phylogenetic groups (Ausió, 1999). The lack of a structural relationship between the shorter PLs in these different groups provides support to the notion that this is most likely the result of a process of convergent evolution that is constrained by the structural requirement needed to achieve sperm chromatin compaction. This would also apply to the seemingly unrelated post-translational cleavage of these proteins in different organisms (Fig. 10).

Concluding remarks

The process that leads to the N-terminal cleavage observed by us is currently unknown. However, it is reminiscent of the N-terminal processing undergone by some vertebrate and invertebrate protamines where a short terminal portion of the protein is removed during spermiogenesis [see (Lewis et al., 2003b) for a review]. In molluscs (Caceres et al., 1999) and cephalopods (Martinez-Soler et al., 2007) the N-terminal processing results in chromatin condensation. It is not known if the processing of SP2 may also result in chromatin condensation in *Xenopus*. Thus, it would be interesting to determine if the unprocessed protein, SP1, binds to DNA and forms chromatosomes in a similar way as SP2 and to compare the binding of SP1 and SP2 on a chromatin template.

The findings that SP2 has a trypsin resistant core domain and forms chromatosomes *in vitro* are in good agreement with the previous *in vitro* report that *Xenopus* nucleoplasmin removed SP2 with similar efficiency to the removal of H1/H5 (Ramos et al., 2005), suggesting that SP2 could be arranged like an H1 on chromatin. The

data in this current chapter go further to show that indeed SP2 has a similar structure to H1/H5.

The possibility that the interaction of SP2 with DNA is electrostatically based, but is able to form chromatosomes, leads us to propose that SP2 may bind to the H2A/H2B deficient nucleosomes (Mann et al., 1982) in the *Xenopus* sperm chromatin through its winged-helix motif, while using its N- and C-terminal tails to neutralize the charge in the linker domains. *In vivo* this would be achieved in conjunction with SP3-SP6 to create a highly compacted sperm chromatin organization (Lewis and Ausió, 2002). More information, including understanding how the different SPs work together, is needed to determine if this is indeed the case.

Chapter 3. Characterization of channel catfish (*Ictalurus punctatus*) histone-type sperm chromatin

L.J.F.'s contribution to the work:

I prepared the data, figures and writing. Channel catfish samples I prepared were imaged by the UVic, Department of Biology EM Laboratory. The MALDI-TOF analysis of HPLC fractions was done by the UVic Genome BC proteomics centre.

Abstract

During spermatogenesis, one of the most dramatic examples of chromatin remodelling takes place. In many organisms this is concurrent with major changes in chromatin composition where histones are replaced by small and highly charged proteins, called protamines. However, many fish species do not undergo this composition change and instead retain histones in their sperm (H-type). Despite the lack of protamine displacement, a remodelling of chromatin and a reduction in nuclear volume still occurs. The factors that lead to the condensation of chromatin in organisms with H-type sperm are unknown. Therefore, we have begun a study of *Ictalurus punctatus* (channel catfish) chromatin which has sperm of the H-type with sperm histones that are compositionally indistinguishable from those in somatic cells. Electron microscopy indicated that the sperm chromatin is still maximally compacted. Micrococcal nuclease digestion indicated that the testes chromatin has an organized, highly repetitive nucleosome structure. These characteristics make *I. punctatus* an ideal candidate for the further study of the structural determinants of chromatin compaction in organisms with H-type sperm. Western analysis results are presented for several histone variants and post-translational modifications.

Introduction

Chromatin is important for the regulation of the cellular events transcription, DNA replication, and the repair and maintenance of DNA integrity. Somatic chromatin can be classified into two groups depending on its condensation state. The first group is euchromatin, which is in a decondensed or extended conformation and typically contains genes that are transcriptionally active. Characteristics and post-translational modifications (PTMs) of euchromatin include histone hyperacetylation, methylation at lysine 4, 36 and 79 of histone H3, and poor staining of DNA (Berger, 2007). The second group is heterochromatin, which is highly compacted, transcriptionally silenced, and stains more intensely. Constitutive heterochromatin is irreversibly condensed and its distribution varies between different cell lineages. These stable heterochromatinized regions are found at centromeres, telomeres, repetitive sequences and non-coding sequences (Grewal and Jia, 2007; Trojer and Reinberg, 2007). In these regions, histones are hypoacetylated, histone H3 is trimethylated at lysine 9, histone H4 is trimethylated at lysine 20 and DNA is methylated. Heterochromatin protein 1 alpha and beta (HP1 α/β) are also found in these regions. A second heterochromatin type, facultative heterochromatin, has a distribution that is more varied between both cell lineages and chromosomal domains. Examples of this type are X-chromosome inactivation, autosomal imprinting of genomic loci, long range silencing and local gene silencing (Grewal and Jia, 2007; Trojer and Reinberg, 2007). Like its distribution, the epigenetic marks of facultative heterochromatin can vary. Typically, histones are hypoacetylated, histone H3 is methylated at lysine 9 and 27, Histone H4 is methylated at lysine 20, H2A is mono-ubiquitinated at lysine 119 and DNA is methylated in these regions. Other factors

associated with many of the different regions of facultative heterochromatin include macroH2A and Polycomb group (PcG) proteins (Grewal and Jia, 2007; Trojer and Reinberg, 2007).

Unlike somatic cells, where chromatin is found in equilibrium between the different condensation states, sperm cells contain an extreme example of chromatin compaction in which the genome is completely silenced and organized into heterochromatin. As discussed in Chapter One, the replacement of histones by small arginine rich proteins called protamines is credited for the compaction of mammalian sperm chromatin. However, some other vertebrates, such as several species of fish and amphibians, retain histones in their mature sperm and still have highly compacted sperm chromatin. The epigenetic marks, histone variants and other trans-acting factors that may be involved in the tight compaction of H-type sperm chromatin and maintenance of its heterochromatic state are unknown as most research into chromatin compaction during spermiogenesis has focused on SNBPs of the P-type. It is possible that H-type sperm chromatin may be similar in composition and structure to the constitutive or facultative heterochromatin found in somatic cells. Alternatively, it could be unique, with sperm specific factors involved in creating and maintaining its condensed state.

Following meiosis in mammalian spermatogenesis, histones become modified by post-translational marks and canonical histones are replaced by sperm-specific histone variants. These histone changes are important for their replacement by transition proteins and protamines and ultimately in chromatin compaction (Govin et al., 2004; Kimmins and Sassone-Corsi, 2005; Lewis et al., 2003a). For example, in mammals, histone H4 becomes acetylated during the early elongation stage in correlation with partial chromatin compaction. Also at this stage, histone H2A becomes monoubiquitinated. Acetylated H4

may aid in the displacement of canonical histones for their replacement with transition proteins. Alternatively, acetylated H4 may have a more direct role in compaction by acting as a signal for the binding of the testes-specific bromodomain protein (Brdt), which has been shown to compact chromatin (Pivot-Pajot et al., 2003). Many histone and chromosomal proteins become phosphorylated during spermiogenesis and at least one of these phosphorylation sites, H4 serine 1, has been directly linked to chromatin compaction (Krishnamoorthy et al., 2006). These mammalian histone PTMs are but a few of the possible marks that could be involved in the regulation of H-type sperm heterochromatinization in other vertebrates.

To date, the only study on histone PTMs in organisms of the H-type focused solely on histone acetylation and compared fish of the H-, PL- and P-types (Kurtz et al., 2009). It was found, in all of the three sperm types, that the onset of spermiogenesis is characterized by the remodelling of chromatin into 20 nm granules that are uniformly distributed throughout the nucleus and at this time, histones H3 and H4 are acetylated. During the final stage of spermiogenesis in the H-type fish, histones H3 and H4 become deacetylated at the same time that the 20nm granules condense (Kurtz et al., 2009).

The nucleated blood cells of vertebrates are an example of a terminally differentiated cell type that also contains abundant heterochromatin. Myeloid and Erythroid Nuclear Termination stage-specific protein (MENT) is an avian erythrocyte protein that binds to nucleosomes and causes crosslinking and hence condensation of chromatin (23). MENT is a non-histone protein that can cause compaction. It works synergistically with the specialized linker histone variant, histone H5.

Histone H1 binds to the linker region of DNA between nucleosomes and stabilizes the folding of chromatin into more compacted, higher order structures. In

addition, H1 histones may participate in the regulation of transcription. Thus far, eleven different histone H1 homologues have been identified in humans. These include replication dependent subtypes (H1.1 to H1.5) and replication independent subtypes (H1o, H1x and H1t) expressed in somatic cells as well as in sperm and oocyte specific variants [reviewed in (Ausio, 2006; Eirin-Lopez et al., 2005)]. These wide ranges of homologues are differentially expressed, suggesting specific functions. As histone H1s have a role in compaction and sperm-specific variants exist not only in humans but in a broad range of other species (Ausio, 1999) it is possible that H1s could have a role in compaction of H-type sperm chromatin.

Ictalurus punctatus (channel catfish) is an aquaculturally important freshwater bony fish belonging to the family Ictaluridae. In this work we determine that *I. punctatus* testes contain H-type sperm as it does not contain protamines or sperm-specific histone variants. Despite this, the sperm chromatin is still maximally compacted as assessed by electron microscopy. For comparison, liver and blood tissues were used as mitotically active and post-mitotic tissue examples. Nuclease digestion showed that the testes chromatin has an organized, highly repetitive structure indicative of chromatin in a uniform condensed state. These characteristics make *I. punctatus* an ideal candidate for the study of H-type sperm chromatin compaction. We also begin to address the question of how H-type chromatin is able to compact chromatin in the absence of protamines by examining several histone variants and PTMs.

Materials and Methods

Living Organisms

Samples were obtained from *I. punctatus* (channel catfish, Family Ictaluridae), *Carassius auratus* (gold fish, Family Cyprinidae) and *Danio rerio* (zebrafish, Family Cyprinidae) that were housed at the University of Victoria aquatics facility.

Investigations were conducted in accordance with the National Research Council (NRC) publication *Guide for Care and Use of Laboratory Animals* (copyright 1996, National Academy of Science) under the approval of the University of Victoria's Animal Care Committee. Cryopreserved sperm samples from *Ictalurus furcatus* (blue catfish, Family Ictaluridae) and *Xyrauchen texanus* (razorback sucker, Family Catostomidae) were generously supplied by Terrence Tiersch (Louisiana State University Agricultural Center).

Extraction and purification of proteins

Sperm cannot be stripped from channel catfish (Guest et al., 1976). Therefore testes tissues were removed by dissection. Mature catfish, approximately 4-6 years of age, were sampled during their spawning season (late June). The tubules of ripe catfish testes contain only two cell types, large numbers of mature sperm and some scattered type A spermatogonia (Schulz et al., 2009) and estimates of 1^{10} sperm per gram of testes have been reported (Bart and Dunham, 1996). Testes maturity and sperm presence were monitored by light microscopy, but not quantified. SNBP extracts were obtained following a method modified from (Wang and Ausio, 2001). Testes were briefly homogenized with a Polytron homogenizer in 0.1 M KCl, 25mM Tris (pH 7.5), 1 mM $MgCl_2$ buffer containing 1 tablet of Complete protease inhibitor per 100 mL of buffer

(Roche Diagnostics, Laval, QC). The homogenate was strained through two layers of presoaked cheese cloth to remove tissue particulate. After homogenization, the samples were centrifuged at 2000g for 10 min at 4 °C. The pellet was resuspended in 0.1 M KCl, 25mM Tris (pH 7.5), 1 mM MgCl₂, 0.2% Triton X-100 buffer containing 1 tablet of Complete protease inhibitor per 100 mL of buffer (Roche Diagnostics, Laval, QC) and centrifuged as above. This step was repeated and the resulting pellet was then resuspended in approximately 6 mLs 0.4 N HCl per gram of starting tissue, homogenized with a dounce homogenizer and centrifuged at 10,000g for 10 minutes at 4°C. The HCl supernatants were precipitated with 7 volumes of cold acetone at -20 °C overnight and then centrifuged as described above. The pellets were resuspended in room temperature acetone and centrifuged as described above. The acetone extracted SNBP pellets were dried under a vacuum and then resuspended in sterile distilled water for analysis. SBNPs extracts were fractionated by reverse phase HPLC as described in Chapter 2 of this thesis. Proteins were analyzed by AU-PAGE, AUT-PAGE or by SDS-PAGE as described in Chapter 2.

Chromatin digestions

Testes tissue and blood were homogenized in ice cold homogenization buffer [0.1M KCl, 1mM MgCl₂, 25 mM Tris pH 7.5 and 1/5000 Complete protease inhibitor]. The homogenate was centrifuged at 2000 g for 5 min at 4°C. The pellets were resuspended in the same buffer containing additionally 0.2% Triton X-100. After incubation for 5 min on ice, the homogenate was centrifuged as before and then this step was repeated. The pellets were then resuspended in the starting buffer without Triton X-100 and centrifuged again. The nuclear pellets thus obtained were washed in

homogenization buffer without Triton X-100 and then resuspended in digestion buffer [KCl, CaCl₂ and Tris pH 7.5].

For the liver samples, the above protocol was modified to include 25 mM EDTA in the homogenization buffers to inhibit metal dependant endogenous nucleases. In the final step, the pellet was washed with the digestion buffer (no Triton X-100) to remove the EDTA before the final resuspension in the digestion buffer.

The nuclei suspensions were diluted in the digestion buffer to an absorbance of 20 at 260 nm. Micrococcal nuclease (Worthington) digestions were carried out at 37°C at 25 units of enzyme per mg DNA. Digestion aliquots were withdrawn at selected times, EDTA was added to a final concentration of 10 mM and the samples were frozen in liquid nitrogen.

DNA was analyzed by 6% Native PAGE (Yager and van_Holde, 1984) or 1% agarose in Tris-Acetate-EDTA (TAE). Native PAGE and agarose gels were stained with ethidium bromide and visualize with UV light.

Repeat length determination

The fragment lengths of the micrococcal nuclease digested DNA products were determined from the agarose gels. The average repeat lengths at each time point were calculated from the slope of the line obtained by plotting the fragment length versus band number at each given digestion time (Johnson et al., 1976). The repeat length for each tissue was determined by plotting the average repeat lengths against time and then extrapolating back to time zero.

Determination of the Histone:DNA ratios and the H1:nucleosomes ratio

Nuclei from catfish liver, blood and testes were prepared as described above. The concentration of DNA for each of the nuclei was determined from the absorbance at 260 nm in 0.5% SDS as described in (Ausió et al., 1989) using a Cary-1 UV-Visible Spectrophotometer (Varian Inc.). The nuclei were diluted to 0.82 mg/ml DNA, mixed with SDS-PAGE and loaded in triplicate onto an SDS gel.

Scanning densitometry was used to quantify the relative amounts of histones H1 and H3 in the gel described above. H3 was used as a representative of the core histones as it ran separately, H2A and H2B ran as one electrophoretic band, and had straighter bands that stained more consistently than H4. The ChemiImager 4000 scanner was used with the AlphaEase™ Version 3.3d software (Alpha Innotech Corp.). A background measurement was taken within each lane and subtracted from the values for H1 and H3 in dots per inch (DPI) to give corrected values. By plotting HPLC purified H1 and H3 DPI values of various concentrations it was determined that H3 stained approximately 1.4 times more intensely than H1 with coomassie blue and this was corrected for as well. Alternatively the same value of 1.4 can also be obtained by dividing by the amino acid (aa) number, using 190 aa for H1 and 136 aa for H3. Also taking in to account that there are two H3 molecules per octamer, the following equation was used to calculate the H1 to nucleosome ratios:

$$\frac{\text{Background corrected DPI for H1}}{\text{Background corrected DPI for H3} / 1.4 / 2 \text{ H3 molecules per nucleosome}}$$

Catfish testes histone samples of known concentration were loaded on the same SDS gel described above to create a standard curve. The amount of total histone protein in micrograms loaded in the SDS gel was determined using this standard curve and the

H3 DPI values. The extrapolated total histone protein values were divided by the known micrograms of DNA loaded in the lanes to give the measured Histone:DNA (w:w) values.

Western Blotting

Approximately 2 µg of acid extracted histones were loaded onto SDS–polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Mississauga, ON) at 100 volts for 2.5 hours at 4 °C in 25 mM Tris-HCl (pH 7.5) and 192 mM glycine. The blotted membranes were blocked in PBS, 0.1% Tween, 3% skimmed milk for 2 hours at room temperature. The primary antibodies were diluted in blocking buffer and the blots shaken in the primary antibodies' dilutions as described below. The blots were subsequently washed with PBS and 0.1% Tween. The membrane was then incubated with secondary antibody, washed with PBS and 0.1% Tween followed by washes with PBS and rinsed with distilled water. Chemiluminescent detection of the protein bands was carried out according to the manufacturer's instructions (NEN, Boston, MA).

The primary antibodies and dilutions used were: macroH2A [1:3 500, a rabbit polyclonal antibody (Costanzi and Pehrson, 1998)]; histone H4 [1:10000, an in house raised rabbit polyclonal], pan-acetyl H4 [1:2 000, Upstate], H3 dimethyl lysine 9 [1:2000, Upstate]; H3 tri-methyl lysine 27 [1:750, Abcam]; H2A.X [1:5 000, Abcam]; H1 phosphoserine 1 [1:10 000,(Barber et al., 2004)]; H2A.Z [1:1 000, Abcam] and MENT [1: 5000 a rabbit antibody (Grigoryev and Woodcock, 1998)]. The secondary goat anti-rabbit and goat anti-mouse horseradish peroxidase conjugate antibodies (Abcam) were diluted 1:10 000 and 1:3 000 respectively.

Mass spectrometry

Molecular masses were determined by mass spectrometry analysis of *I. punctatus* histone H1s using MALDI-TOF on a Voyager Linear DE (PerSpective Biosystems Inc., Foster City, CA) with a sinapinic acid matrix following the protocol described in (Hunt et al., 1996).

Alternatively, sample masses were determined with an Applied Biosystems 4800 MALDI-TOF mass spectrometer using the Mid-Mass Linear Positive mode. A 3mg/mL hydroxycinnamic acid matrix was used and the samples were resuspended in 70% ACN + 0.1% TFA and 10 pmoles samples were spotted.

Results and Discussion

Electrophoretic characterization of nuclear proteins in I. punctatus and other fish species

The basic proteins of several fish containing H-type SNBPs were extracted with 0.4N HCl and analyzed using SDS and AU- PAGE. Both species of catfish (*I. punctatus* and *I. furcatus*) have SNBP extracts consisting exclusively of histones, as do all the other fish examined (Fig. 11). The razorback sucker (*X. texanus*) is an endangered fish found in the Colorado River basin. Knowledge of the sperm types (H-, PL- or P-type) may have implications for development of cryopreservation techniques (Ausio et al., 2009), which is particularly important for endangered and farmed species. Goldfish (*C. auratus*) was the first organism shown to have no specialized SNBPs (Munoz-Guerra et al., 1982). In the H1 region of the AU-PAGE, the goldfish histone extract has a broad band which

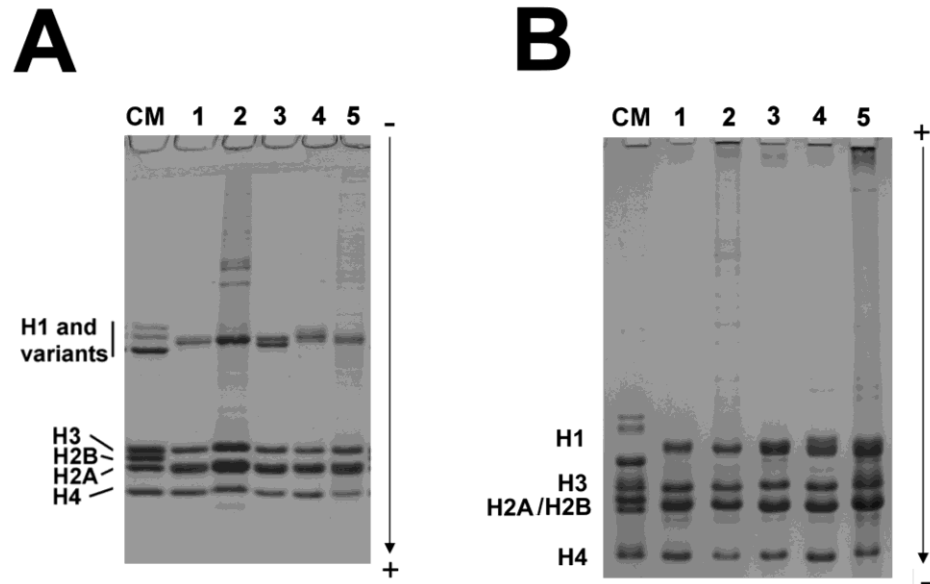


Figure 11. Characterization of H-type fish SNPBs

(A) SDS-PAGE and (B) AU-PAGE. Lane 1: *Ictalurus punctatus* (channel catfish); lane 2: *Ictalurus furcatus* (blue catfish); lane 3: *Xyrauchen texanus* (razor back sucker); lane 4: *Carassius auratus* (goldfish); lane 5: *Danio rerio* (zebrafish). CM; Chicken erythrocyte histones used as a histone marker.

may be a doublet (Fig. 11). In comparison, both species of catfish have a single narrow band in this region indicating that they may have fewer, or less variant, linker histones. Zebrafish (*D. rerio*) is a widely used organism for studying vertebrate development and transgenics and the fact that it has H-type sperm has been overlooked. Knowledge of the SNBP type is important for the interpretation of epigenetic data such as the methylation state of the DNA in zebrafish sperm. Upon running the AU gel for a shorter period of time, no lower molecular weight bands, which would correspond to protamines, were seen in any of the fish species (data not shown).

Channel catfish was chosen as the model for additional characterization as has H-type SNBPs and sufficiently large amounts of sample could be collected. Liver and blood were used as mitotically active and post-mitotic tissue controls. The histones from liver, blood and testes were characterized by SDS-PAGE, AU-PAGE and AUT-PAGE (Fig. 12). No sperm-specific linker or core histone variants that could account for the increased compaction of chromatin were apparent. The main difference between the three samples was an H1 variant in the liver.

I. punctatus chromatin organization and nuclear compaction

The transmission electron microscopy (TEM) image of the hepatocyte nucleus showed electron-dense heterochromatin regions mainly at the periphery of the nuclear envelope and the nucleolus (Fig. 13). Regions of heterochromatin were seen throughout the erythrocyte nucleus. In the sperm, all the chromatin appeared to be heterochromatinized. The sperm chromatin had a tightly compacted structure similar to that previously observed in the sperm chromatin of organisms containing protamines. Overall, there was a decreasing nuclear size and increasing extent of chromatin

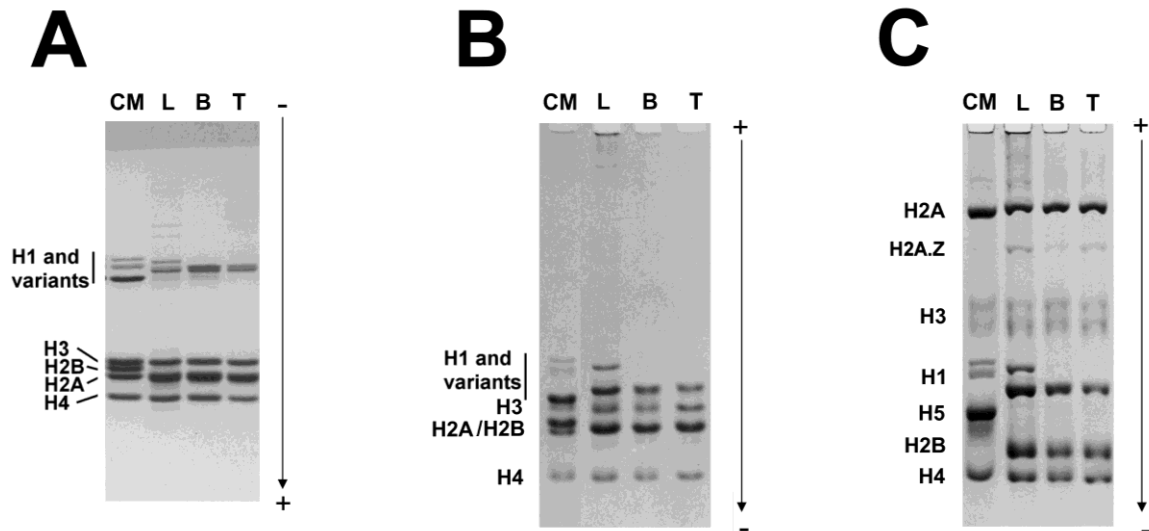


Figure 12. Electrophoretic characterization of *I. punctatus* histones from different tissues (A) SDS-PAGE, (B) AU-PAGE and (C) AUT-PAGE. The lanes are labelled as: CM, Chicken erythrocyte histones used as a histone marker; L, liver; B, blood; T, testes.

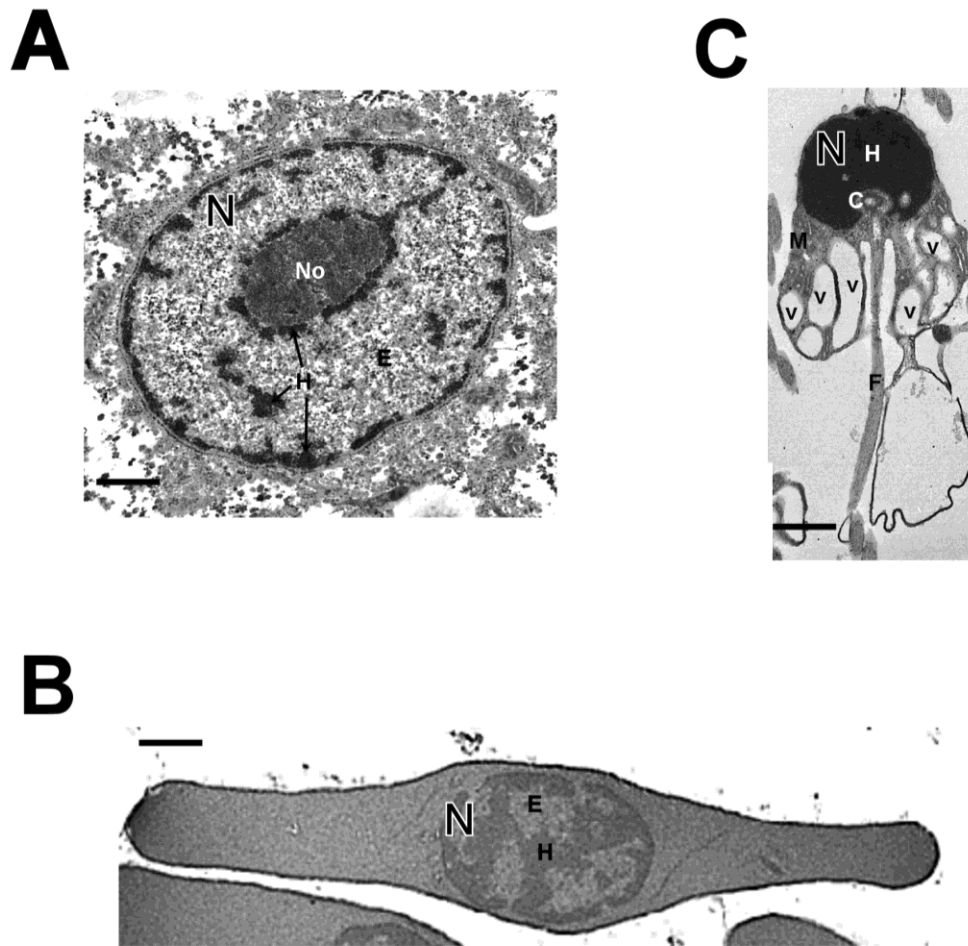


Figure 13. Electron microscopy images of sections through the hepatocyte nucleus, erythrocyte and sperm.

C, centriole; E, euchromatin; F, flagella; H, heterochromatin; M, mitochondria; N, nucleus; No, nucleolus; V, midpiece vacuole. The bar is 0.5 microns.

compaction from liver to blood to sperm (Fig. 13).

Micrococcal nuclease (MNase) digestions were carried out in order to assay the accessibility of DNA to nucleases and to characterize the nucleosomal organization of the different chromatin. MNase digestions for the different tissue types are shown in Figure 14. The liver had an extremely high level of endogenous nucleases and the procedure had to be modified to include EDTA in the nuclei isolation buffer so that the DNA did not become digested before it could be analyzed. Therefore, the protocols varied slightly and the digestion rates could not be directly compared between the three samples. A portion of the full length liver DNA remained undigested after 24 minutes, which was likely an artifact of the EDTA. The rate of digestion in the blood, which was prepared in parallel with the testes sample and is therefore comparable, appeared to be faster than that of the testes. This was likely due to the greater degree of chromatin compaction in the testes (i.e. more heterochromatin) which resulted in a greater protection of the DNA. However, the possibility that these differences in rates could have been due to endogenous nucleases in the blood could not be ruled out.

The repeat lengths were determined from the agarose gels, in Figure 14B, to be 192 base pairs (bp) for the liver chromatin, 199 bp for the blood chromatin and 209 bp for the testes chromatin (Table 2). The value of 209 bp for the catfish testes was similar to the value found for *C. auratus* sperm of 205 bp (Munoz-Guerra et al., 1982). The longer repeat length found in the testes chromatin compared to that of the chromatin of the liver sample may cause the increased chromatin compaction in the sperm, or could be an effect of the compaction.

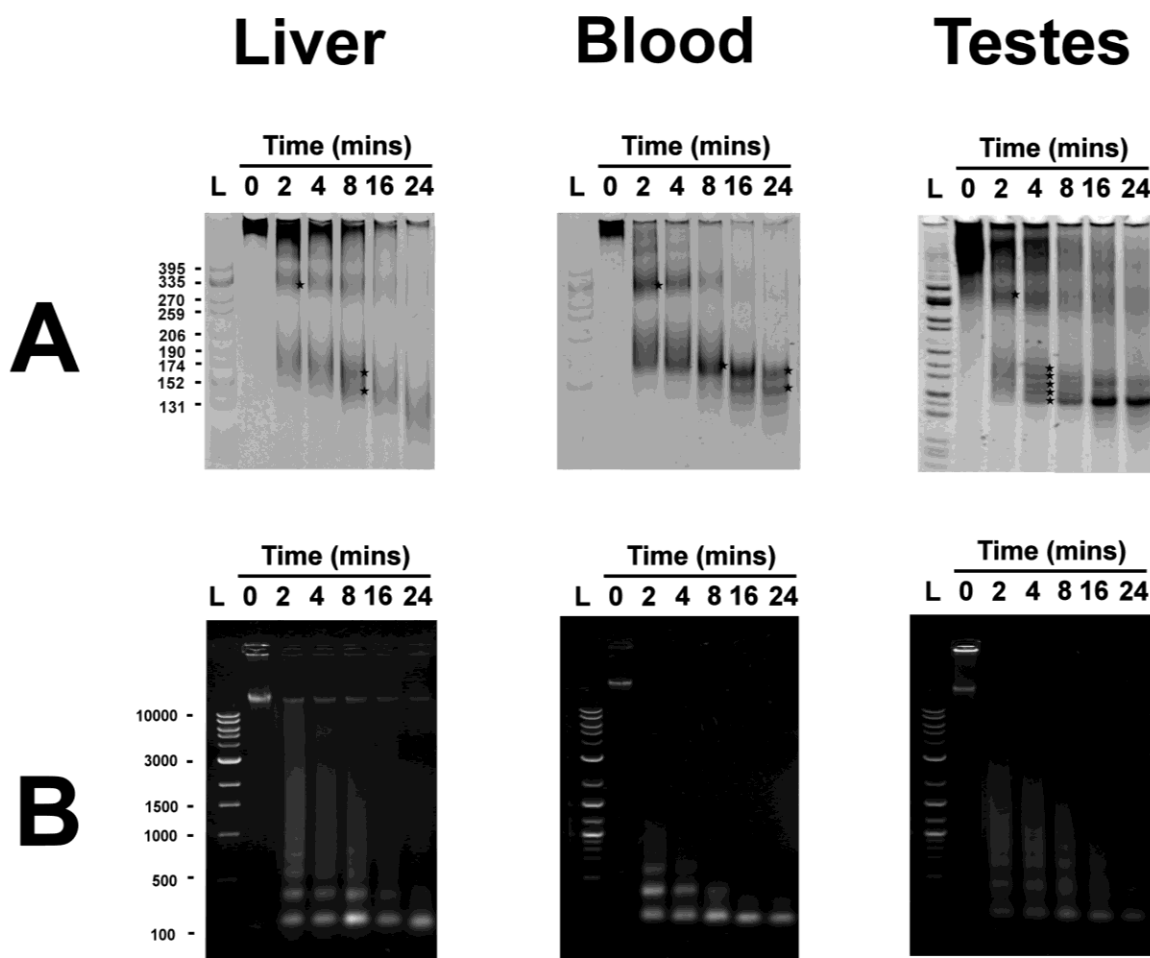


Figure 14. Micrococcal nuclease digestions of *I. punctatus* nuclei.

The DNA fragments obtained from 0 time and 2, 4, 8, 16 and 24 minute digestions with 25U of MNase per mg of DNA at 37°C were separated by (A) native PAGE and (B) agarose gel electrophoresis. The lane labelled L in each gel contains a DNA ladder. pBR322 plasmid DNA digested with the restriction enzyme CfoI was used as the ladder for the native gels. Combined 1 Kb and 100 bp DNA ladders (NEB) were used for the agarose gels.

Table 2. *I. punctatus* chromatin characteristics and stoichiometric values

Tissue	Repeat length	H1 per nucleosome	Histone:DNA (w/w)
Liver	192	0.74	0.75
Blood	199	0.99	0.86
Testes	209	0.94	1.16

The liver had a dinucleosome band, following a 2 minute digestion with MNase at 339 bp, and a mononucleosome smear centered around 178 bp (Fig. 14b). After 8 minutes of digestion, a smear was seen from approximately 132 bp to 196 bp. The smearing seen in the liver sample indicated that the spacing between the nucleosomes in liver chromatin was varied. This was expected as liver chromatin contains a mixture of both active and inactive chromatin regions and therefore the nucleosomes are not evenly spaced. For instance, promoter regions of active genes are often devoid of nucleosomes (Yuan et al., 2005). The lower base pair smear after a 24 minute digestion of liver chromatin was due to over digestion of the nucleosome. The blood had dinucleosome and mononucleosome bands of 353 bp and 182 bp. After a 24 minute digestion there were two main bands at 170 bp and 150 bp. The testes sample in the 2 minute lane had a dinucleosome band of 354 bp. In the testes sample, distinct bands at 185 bp, 175 bp, 165 bp, 155 bp and 145 bp, all exactly 10 base pairs apart, were seen in the mononucleosome region of the gel. This suggested a regular chromatin organization as compared to liver and blood. The nucleosome core particle protects 145 bp of DNA from digestion, and the longer DNA fragments include portions of the linker DNA. MNase digestion of different chromatin samples, including sea urchin sperm, also yield DNA products that increase by 10 bp increments, starting from the 145 bp nucleosome core particle (Bavykin et al., 1990). The discrete bands at 10 bp intervals in the testes chromatin digestion is indicative of a regularity in the chromatin structure and may be due to linker DNA in the sperm being organized into a super helix, possibly in a continuous manner with the DNA of the core particle (Bavykin et al., 1990).

The addition of histone H1 to nucleosomes is thought to be important for formation of the 30nm fiber and higher order chromatin structures (Felsenfeld and McGhee, 1986). The sperm of some fish species have increased amounts of H1 and it is thought that this may, in part, account for sperm chromatin compaction in these species (Saperas et al., 1993). The values obtained for the ratios of H1 molecules per nucleosome for catfish liver and blood of 0.74 and 0.99 (Table 2) were similar to those previously determined for chicken liver and erythrocytes of 0.8 and 1.3 (Bates and Thomas, 1981). The values for chicken blood were likely higher than those for catfish blood due to high levels of H5 in the chicken blood. The value determined for the catfish sperm of 0.94 is similar to that found for catfish blood and higher than that of the liver. These values indicate that all of the sperm chromatin was organized into compacted higher order structures that required approximately one histone H1 per octamer. In contrast, the liver, which may have regions of transcriptionally competent chromatin organized into 11 nm fibers devoid of histone H1, had a lower H1 to nucleosomes ratio.

The histone to DNA ratios, determined by scanning densitometry analysis of a SDS polyacrylamide gel, were 0.75, 0.86 and 1.16 for the liver, blood and testes samples respectively. Liver chromatin is more transcriptionally active than blood and likely had the lowest histones to DNA ratio because sections of chromatin may be devoid of histones. In contrast, sperm cells are generally considered transcriptionally silent. It may be that all of the DNA is equally assembled into nucleosomes and therefore, over the entire genome, there are more nucleosomes even though the sperm had a longer repeat length. In support of this, the differences in compaction between euchromatin and

heterochromatin are lost during spermiogenesis and the chromatin takes on a uniform distribution of 20nm granules, each containing 4 to 6 nucleosomes (Kurtz et al., 2009).

Histone H1 variants

Specialized sperm histone H1s containing high amounts of lysine have been attributed to causing compaction in some H-type species, such as *Rana* (Itoh et al., 1997). Therefore, we were interested in determining if any unique sperm histones were present in catfish testes. Histones from liver, blood and testes were separated by HPLC (Fig. 15). From this analysis, no unique histone H1 subtypes were apparent in the testes. Histone H1s were also extracted from liver, blood and testes nuclei using 5% PCA and then separated on a polyaspartic acid cation-exchange (PolyCAT) HPLC column (data not shown). However, this did not reveal any additional H1 subtypes other than those seen in Figure 15, and the peaks were less defined (data not shown).

For further analysis of H1 subtypes, the fractions were sent for mass spectrometry analysis. Four main variant and/or post-translationally modified H1 histones were identified. The main difference in the testes was the lack of the 23 kDa variant, which was prevalent in the liver. The masses of the proteins in the HPLC fractionated SNPB extracts were 20,797 Da, 20,702 Da and 20,280 Da. Unfortunately, the masses alone were not sufficient to identify the variants present.

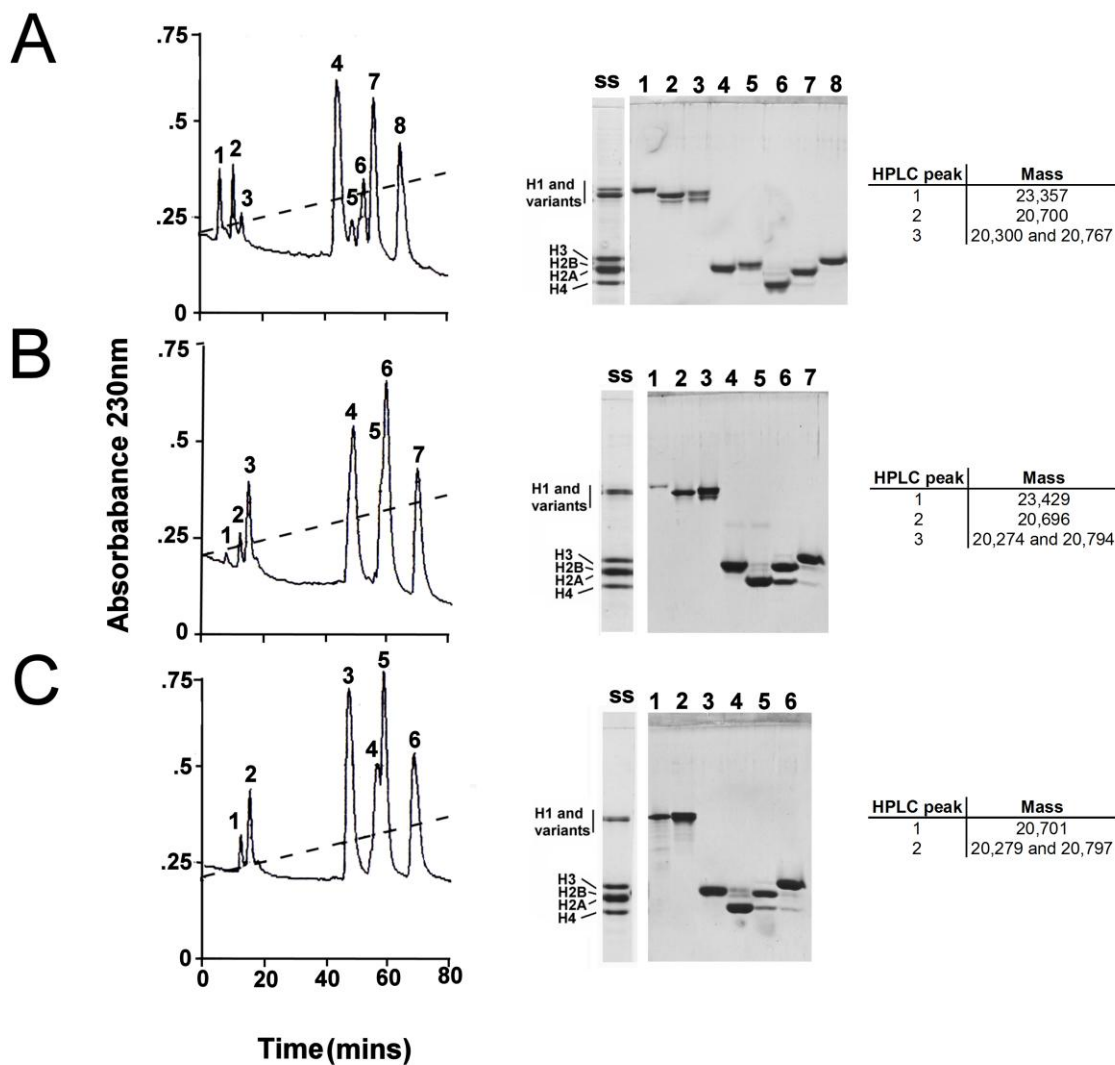


Figure 15. Fractionation and purification of *I. punctatus* liver. (A), blood (B), and testes (C) histones.

Proteins were separated by reverse phase HPLC and eluted with a 30% to 50% acetonitrile (ACN) gradient (denoted by the dashed line). In the middle are SDS-PAGE analyses of the indicated peaks from the HPLC profiles. On the right are the most prominent masses determined by MALDI-TOF for the HPLC fractions in the histone H1 region. SS; starting sample loaded on the HPLC.

Histone variant and histone post-translational modification distribution in liver, blood and testes

Western blot analysis (Fig. 16) was used to determine the tissue distribution of specific histone PTMs and histone variants to assess whether histone PTMs or variants with an increased expression in the testes could contribute to the compaction of the sperm chromatin. In this analysis histone H4 was used as a loading control.

Histone H4 acetylation

During spermiogenesis in organisms of the P-type, histone H4 becomes hyperacetylated before the displacement of histones by protamines (Lewis et al., 2003a). As hyperacetylation neutralizes the charge of the lysine residues, it gives the chromatin a more open conformation by decreasing the electrostatic interaction between the negatively charged DNA and histones (Zheng and Hayes, 2003). This open conformation allows access to the nucleosomes and weakens their interaction with DNA. Pan-acetylated histone H4 (H4Ac) was detected in the blood but not in the liver or the testes. The acetylation marks in blood are not dynamic, but are instead in a permanently acetylated state, with approximately 30% of the histones acetylated (Brotherton et al., 1981). In a recent collaboration with our lab, Kurtz et al. (Kurtz et al., 2009) showed that during condensation of chromatin in the H-type sperm of *Sparus aurata* (gilthead seabream), histone H3 and H4 were acetylated in immature sperm and deacetylated in the mature sperm. Thus, the lack of detectable acetylation in the Catfish testes (Fig. 16b) is a good indication of its maturity (Kurtz et al., 2009).

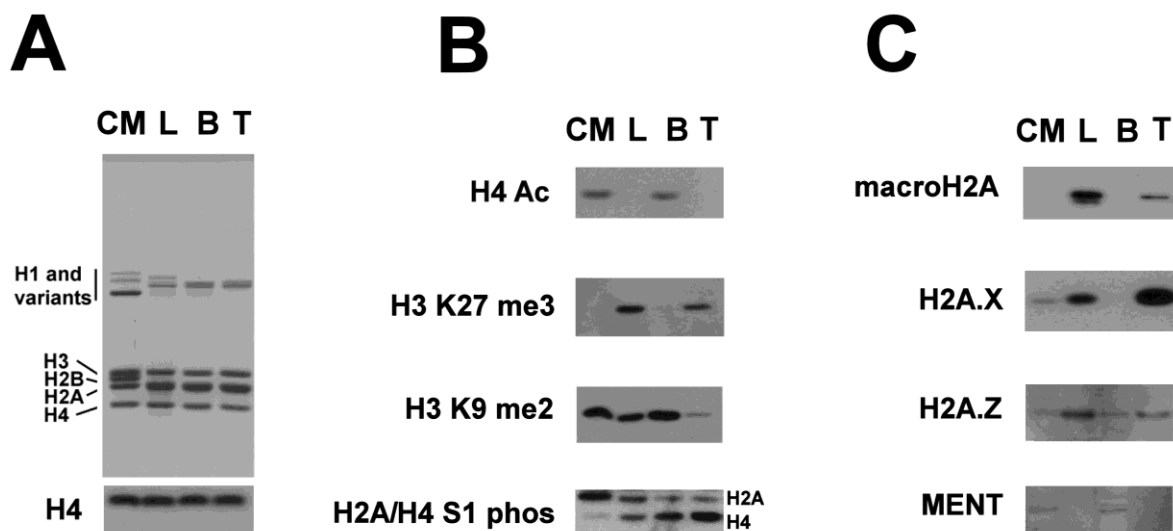


Figure 16. Western Blot analysis of the distribution of histone PTMs and histone variants in *I. punctatus* tissues.

(A) Coomassie stained SDS-PAGE gel of histones used for western analysis. A histone H4 antibody was used as a loading control. (B) Western blotting analysis of histones using various antibodies to histone PTMs. (C) Western blotting analysis of histone H2A variants and the non-histone protein MENT. The samples loaded in each lane were: CM, chicken erythrocyte histones used as a histone marker; L, liver; B, blood; T, testes.

Histone H3 methylation

Histone H3 is tri-methylated at lysine 27 (H3K27me3) in most facultative heterochromatin and is correlated with transcriptional repression (Peters et al., 2003). The polycomb repressor complex 2 (PRC2) is responsible for the trimethylation at lysine 27 of H3 and PRC1 recognizes the H3K27me3 mark (reviewed by Schwartz and Pirrotta, 2008). In human sperm, H3K27me3 is present at the promoters of transcription factors and signalling proteins that are important for the later stages of embryo development but are repressed in early embryos (Hammoud et al., 2009). This mark is not present in blood (Fig. 16) which is consistent with a previous study (Gilbert et al., 2003). However, this mark is present in both liver and testes (Fig. 16). As the abundance of H3K27me3 appears to be slightly less prevalent in testes than in the liver sample, it is likely not solely responsible for the maintenance of the global chromatin condensation in the sperm. It is possible that, like in human sperm, this mark is located at promoters that are repressed in the early embryos.

Lysine 9 of histone H3 (H3K9) can be methylated and is a well characterized epigenetic marker of heterochromatin. It is recognized by heterochromatin protein 1 (HP1) in constitutive heterochromatin. An antibody that recognizes H3K9 dimethyl showed a low level of binding to this mark in testes. An elevated level of staining was detected in chicken and fish blood, which is consistent with a previous report on chicken erythrocytes (Gilbert et al., 2003). Gilbert and colleagues found that HP1 proteins were absent from chicken and zebrafish erythrocytes, suggesting a HP1 independent condensation in these cells. The low level of H3K9 dimethylation suggests that the condensation in sperm may also be HP1 independent, but unlike erythrocyte chromatin is H3K9 dimethyl independent.

MENT

In chicken erythrocytes, two chromatin proteins, histone H5 and the non-histone protein MENT, act synergistically and are able to form protein bridges that connect separate chromatin fibers, resulting in a more compact higher-order chromatin structure (Grigoryev et al., 1999; McGowan et al., 2006). The linker histone variant H5 was not present in the catfish sperm, as indicated by its absence in the electrophoresis shown in Figures 11 and 12. MENT, which was present in catfish blood in the same stoichiometry as in the chicken blood, was absent in the sperm (Fig. 16). This suggested that the mechanism acting to condense chromatin in testes did not require MENT or H5. However, the absence of these proteins does not rule out unknown proteins acting in a similar manner.

Histone H2A/H4 phosphorylation

Serine 1 of histone H4 (H4S1) is an evolutionarily conserved residue that is phosphorylated during mitosis (Barber et al., 2004), yeast sporulation and spermiogenesis compaction (Krishnamoorthy et al., 2006). During all three of these cellular events, chromatin undergoes changes resulting in its compaction. In yeast, H4S1 remained phosphorylated in spores after sporulation and the mutation of H4S1 resulted in an increased nuclear volume, indicating that H4S1 phosphorylation may have a role in chromatin compaction (Krishnamoorthy et al., 2006). In the fly and mouse, H4S1 is phosphorylated in spermatocytes and round spermatids and remains phosphorylated even after meiotic divisions. A reduction in H4S1 phosphorylation is only seen once the histones are replaced with transition proteins in the elongating spermatids. In catfish, where the histones are not replaced, phosphorylation levels of H4S1 are increased in testes relative to the levels in liver and blood (Fig. 16). In mammalian spermatogenesis,

H4S1 phosphorylation may help compact chromatin prior to histone replacement by transition proteins (Krishnamoorthy et al., 2006). In mature yeast spores (Krishnamoorthy et al., 2006) and mature H-type sperm, it may help to stabilize and maintain chromatin compaction. How this PTM functions is unknown, but several possibilities have been hypothesized by (Wendt and Shilatifard, 2006). One possibility is that its function could involve the direct interaction between the tail of H4 and DNA (Suto et al., 2000; Zheng and Hayes, 2003) as N-terminal tails of histone H4 and H3 are important for folding and compaction of chromatin fibers (Moore and Ausio, 1997). Alternatively, trans-factors may recognize and bind to phosphorylated H4S1 and then compact the chromatin fiber.

Phosphorylated serine 1 of H2A (H2AS1phos) was also recognized by the above antibody. The abundance of this mark was inversely proportional to H4S1phosphorylation. Phosphorylation of H2AS1 has been shown to down regulate transcription *in vitro* (Zhang et al., 2004). In the catfish tissues examined, H2AS1 was most phosphorylated in the liver and less in the blood and testes. Although the reason for this is unclear, it may be that the blood and testes do not need to have H2AS1 phosphorylated for regulation of transcription because they have transcription down regulated by another means.

Histone H2A variants

Histone H2A has a number of variants including H2A.X, H2A.Z and macroH2A. In mature human sperm, where 15% of the chromatin remains associated with histones (Tanphaichitr et al., 1978), H2A.X is the major H2A variant present and low amounts of H2A.Z have also been detected (Gatewood et al., 1990). H2A.X is most often associated

with its role in double stranded break repair. Phosphorylated H2A.X has also been shown to accumulate in the XY body at the pachytene stage (Fernandez-Capetillo et al., 2003) and to colocalize with heterochromatin in the inactive X chromosome (Chadwick and Lane, 2005). H2A.X is activated by phosphorylation. An antibody to the mammalian phosphorylated H2A.X (γ H2A.X) was unable to recognize this epitope in catfish. Therefore the role of H2A.X in the catfish testes and whether or not it is phosphorylated needs further investigation.

H2A.Z is another H2A variant whose precise function remains uncertain.

Although it is found at the promoter regions of genes, it is unclear if it has a repressive or active function and may act as a signal for chromatin poised for transcription [reviewed in (Dryhurst et al., 2004; Thambirajah et al., 2009)]. During mouse spermatogenesis the X and Y chromosomes become enriched in H2A.Z after meiotic sex chromosome inactivation (MSCI) and this enrichment persists in the condensed sex chromosomes in round spermatids (Greaves et al., 2006). H2A.Z was present in all of the catfish tissues examined; however it was most abundant in the liver and testes (Figures 12 C and 16). H2A.Z in a mono-ubiquitinated form is found in the inactive X chromosome and this monoubiquitination mark seems to be specific for H2A.Z in transcriptionally inactive chromatin domains (Thambirajah et al., 2009). However, there were no upper bands in the western blot that would correspond to ubiquitinated H2A.Z (data not shown). This suggests that H2A.Z is not always mono-ubiquitinated in heterochromatin, at least not in the case of H-type sperm.

The histone variant macroH2A is found associated with the facultative heterochromatin in inactive X-chromosomes (Costanzi and Pehrson, 1998), the XY-bodies of spermatocytes (Hoyer Fender et al., 2000; Richler et al., 2000) and centrosomes

in somatic cells (Chadwick and Willard, 2002). MacroH2A has a large C-terminal non-histone domain. This H2A variant increases inter-nucleosomal contacts (Changolkar and Pehrson, 2002) and inhibits transcription, histone acetylation and the activity of chromatin remodelling complexes (Doyen et al., 2006); which may be how it promotes heterochromatinization. In the catfish, two electrophoretic bands were seen in the liver, possibly corresponding to mH2A1.1 and mH2A1.2, no bands were seen in the blood and one faint band was seen in the testes. The high levels of macroH2A in the liver were consistent with previous results (Abbott et al., 2004; Pehrson et al., 1997). Given the low levels in the testes, it is unlikely that this variant contributes significantly to the maintenance of heterochromatin in the sperm. In mice, macroH2A has been detected in the pericentric heterochromatin of round spermatids (Greaves et al., 2006). In catfish, perhaps macroH2A in the sperm is a remnant from the earlier spermatid stage. However, it is possible that low levels in the mature catfish sperm may have been due to contamination in the sample used for this particular western blot by other testes cells where macroH2A is prevalent (Abbott et al., 2004).

Conclusions

This work showed that the testes of catfish contains histones similar to those found in somatic cells. Despite this, the sperm nuclei are highly compacted and fully heterochromatinized, making catfish an ideal candidate to study chromatin compaction by H-type SNBPs. Catfish testes had a longer repeat length, an increased ratio of H1 per nucleosome and an increased ratio of histone to DNA, compared to catfish liver. It is not

possible to say if these characteristics cause chromatin compaction or are a consequence of the compaction. Either way, they are likely not the sole factors involved in compacting the sperm chromatin.

An initial analysis of the histone PTMS and core histone variants yielded some interesting results. The testes chromatin does not contain MENT or H5, the two proteins that work synergistically to condense the chromatin of blood cells (Grigoryev et al., 1999), and therefore does not condense by the same mechanism. One of the most interesting modifications that we analyzed was H4S1 phosphorylation. The increase in this PTM has been shown to affect nuclear size and may help stabilize chromatin compaction (Krishnamoorthy et al., 2006). Although macroH2A and dimethylated H3K9, which are common to many types of facultative heterochromatin such as the inactive X chromosome, are absent, histone H3K27me₃, another common facultative heterochromatin mark, is prevalent. The H-type sperm chromatin is likely a unique form of facultative heterochromatin. Although it is fully condensed for the life span of the sperm cell, following fertilization it is rapidly decondensed by maternal factors such as nucleoplasmin (see Chapter 4 and 5).

To further analyze the histone PTMs and the chromatin composition of the catfish testes, it would be useful to isolate sperm and fractionate it by levels of maturity. For example, in catfish testes histones are not acetylated, but we do not know if histones undergo acetylation during sperm maturation as they do in some other fish (Kurtz et al., 2009) and at what stage of spermatogenesis this occurs. Alternatively, instead of fractionation of sperm, immunodetection using cross sections of testes where spermatocytes in different stages of maturation are present could be used.

Although western blot analysis is quick and relatively straightforward, it is limited to finding only the proteins already known to function as potential condensing factors. Thus, a mass spectrometry approach will be used as it has the potential to analyze a greater number of proteins and possibly identify novel proteins. Proteins of interest will be further characterized by Western blotting or immunodetection. These results will be compared to the thousands of proteins that have been identified in humans and several model species which have had already had their spermatozoon proteomes examined (Oliva et al., 2009). All of the vertebrates studied so far have had sperm of the P-type. Therefore, catfish will be the first organism with H-type sperm to have its spermatozoon proteome analyzed. This data will be an important because comparisons with P-type sperm may give insight into which PTMs and variants are important for the replacement of histones by protamines and which are important for other chromatin transitions.

Catfish sperm gives us a unique model in which to study chromatin condensation. H-type sperm is the only example of a cell type that has almost all of its chromatin in a compacted state, but does not contain special SNBPs like those found in P and PL-type spermatozoa. Almost no information is available on the factors involved in H-type sperm condensation (Kurtz et al., 2009). Determination of the epigenetic marks, histone variants and other factors present in catfish sperm chromatin is important for the understanding of how this chromatin is compacted. Elucidating the steps and mechanisms by which H-type sperm is condensed may also have implications for the understanding of the condensation of chromatin that takes place in P- and PL-type sperm before the replacement of histones and formation of heterochromatin in other cell types.

Chapter 4. Introduction to the nucleophosmin / nucleoplasmin family of nuclear chaperones

L.J.F.'s contribution to the work:

I prepared the data, figures and writing which were originally published in (Frehlick et al. 2007). José María Eirín-López helped with the figures and contributed the section “Evolution and functional diversification”. Juan Ausio prepared Figure 20.

Abstract

Basic proteins and nucleic acids are assembled into complexes in a reaction that must be facilitated by nuclear chaperones in order to prevent protein aggregation and formation of non-specific nucleoprotein complexes. The nucleophosmin/nucleoplasmin (NPM) family of chaperones [NPM1 (nucleophosmin), NPM2 (nucleoplasmin) and NPM3] have diverse functions in the cell and are ubiquitously represented throughout the animal kingdom.

The importance of this family in cellular processes such as chromatin remodelling, genome stability, ribosome biogenesis, DNA duplication and transcriptional regulation has led to the rapid growth of information available on their structure and function. This review covers different aspects related to the structure, evolution and function of the NPM family. The evolutionary mechanisms leading to the functional diversification of the family members is reviewed. Emphasis is placed on their role as chaperones, particularly as it pertains to their ability to aid in the reprogramming of chromatin, and the importance of NPM2 as an essential component of the amphibian chromatin remodelling machinery during fertilization and early embryonic development.

Introduction

Oppositely charged molecules within the cell, such as nucleic acids and proteins, must have their charges shielded to prevent aggregation that can occur when opposing charges on molecules interact in an uncontrolled manner. This is the case for nucleic acids and their interactions with histones in which incorrect binding may result in aggregation instead of the assembly of nucleosome core particles (NCPs). NCPs are the complex of DNA and histones in which approximately 146 base pairs of DNA are wrapped around an octamer of core histones (two H2A/H2B heterodimers and an H3/H4 tetramer). The addition of histone H1, which binds to the linker DNA between the NCPs, protects an additional 20 base pairs of DNA creating the chromatosome (Simpson, 1978; van Holde, 1988). *In vitro*, nuclear chaperones are needed for the correct assembly and spacing of nucleosomes and the attainment of higher order chromatin which is structurally identical to that found *in vivo* (Shintomi et al., 2005). The nucleophosmin/nucleoplasmin (NPM) family of nuclear chaperones has members found throughout the animal kingdom. Members can be subdivided into four groups based on their protein sequences: NPM1, NPM2, NPM3 and invertebrate NPM proteins (Eirin-Lopez et al., 2006a; Shackelford et al., 2001)(see Table 3).

Nucleoplasmin (NPM2) was first isolated from eggs and oocytes of the African clawed frog, *Xenopus laevis* (Laskey et al., 1978). It is the the most abundant nuclear protein in *Xenopus* eggs (Mills et al., 1980). Nucleoplasmin binds to histones and mediates the assembly of nucleosomes from DNA and histone proteins (Earnshaw et al., 1980; Laskey et al., 1978). It also binds sperm nuclear basic proteins (SNBPs) (Philpott and Leno, 1992; Prieto et al., 2002; Rice et al., 1995) in order to facilitate the

Table 3. NPM family members

Member	Also known as *	Location	Properties and functions
Nucleophosmin/ nucleoplasmin member 1 (NPM1)	Nucleophosmin, also called B23 (Kang et al., 1974; Prestayko et al., 1974) or numatrin (Feuerstein and Mond, 1987) in mammals and NO38 in amphibians (Schmidt-Zachmann et al., 1987)	Mainly nucleolar; wide tissue distribution (Michalik et al., 1981; Spector et al., 1984)	roles in several cellular processes, including: ribosome biogenesis (Herrera et al., 1995; Savkur and Olson, 1998; Spector et al., 1984; Yung et al., 1985), nucleocytoplasmic transport (Borer et al., 1989; Szebeni et al., 1995; Szebeni et al., 1997; Valdez et al., 1994), centrosome duplication (Okuda et al., 2000; Tokuyama et al., 2001), embryonic development and genome stability (Grisendi et al., 2005), DNA duplication (Okuwaki et al., 2001a; Takemura et al., 1999; Umekawa et al., 2001), transcriptional regulation (Swaminathan et al., 2005; Weng and Yung, 2005) histone chaperoning (Okuwaki et al., 2001b; Swaminathan et al., 2005), binding and folding of denatured proteins (Szebeni et al., 2003; Szebeni and Olson, 1999) and nucleic acid binding (Dumbar et al., 1989; Palaniswamy et al., 2006; Wang et al., 1994)
Nucleophosmin/ nucleoplasmin member 2 (NPM2)	Nucleoplasmin in amphibians (Earnshaw et al., 1980)	Nuclear; only found in eggs and oocytes (Laskey et al., 1978; Mills et al., 1980)	binds histones and promotes chromatin assembly (Dingwall et al., 1987; Laskey et al., 1978) paternal chromatin decondensation (Ohsumi and Katagiri, 1991; Philpott et al., 1991)
Nucleophosmin/ nucleoplasmin member 3 (NPM3)	NO29 in amphibians (Zirwes et al., 1997)	Mainly nucleolar (Huang et al., 2005); wide tissue distribution (Shackleford et al., 2001)	ribosomal RNA biogenesis (Huang et al., 2005) and potentially paternal chromatin decondensation in mammals (McLay and Clarke, 2003)

* The nomenclature for this family has been complicated by different names given to the proteins by different groups. For this review the abbreviations NPM1, NPM2 and NPM3 will be used.

decondensation and remodelling of paternal chromatin following fertilization.

Nucleophosmin (NPM1) was first identified as a phosphoprotein that is highly expressed in the nucleolus (Kang et al., 1974; Prestayko et al., 1974). NPM1 was initially thought to be important for the assembly of ribosomes and has since been found to have roles in many important

cellular processes (see Table 3). NPM1 is the most studied of the NPM family members, largely due to the fact that it is often up-regulated in tumours and is often genetically altered in cancers (recently reviewed in Grisendi et al., 2006). In this review, I will focus specifically on the role of NPM1 as a histone chaperone. The least amount of data is available for NPM3 (MacArthur and Shackelford, 1997; Zirwes et al., 1997), which is the most recently discovered family member. NPM3 has been implicated in the regulation of NPM1 in ribosomal RNA genesis (Huang et al., 2005) and, in addition, its expression in mammalian oocytes has been correlated with paternal chromatin decondensation (McLay and Clarke, 2003), similar to NPM2 in lower vertebrates.

Invertebrates also have NPM-like family members, the best characterized of which is the *Drosophila* nucleoplasmin-like protein (dNPL) (Ito et al., 1996). Although dNPL is able to bind histones and decondense sperm chromatin, this nucleoplasmin homologue is different from the archetypical nucleoplasmin in *Xenopus* in that it is dependent on ATP to assemble nucleosomes (Dimitrov and Wolffe, 1996; Ramos et al., 2005).

In this review, the information on NPM gene and protein structures and evolution is presented and discussed with respect to the chaperone role fulfilled by the members of this family, particularly as it pertains to their contribution to chromatin remodelling and reprogramming.

A family of evolutionarily related proteins

Gene and protein structure

NPM1 members contain twelve exons in human and rat, and eleven exons in mouse (Fig. 17). The human NPM1 gene can be transcribed as three variants among which isoform 1 is the predominant type and results in the longest transcript. Isoforms 2 lacks an in-frame exon VIII and isoform 3 utilize an alternate 3' exon X. Rat NPM1 mRNA is also alternatively spliced, which result in two different proteins termed B23.1 and B23.2 (Chang and Olson, 1989). NPM2 members have eight exons whereas most of the NPM3 members have five exons ((Fig. 17). *Drosophila* dNLP, taken here as a representative of the invertebrate NPM-like lineage, has a coding region composed of only three exons. The relative number of exons is generally proportional to the overall protein length of the corresponding NPM type and, although the intron sizes differ, the sizes of the exons are relatively conserved within each type.

The evolutionarily related members of the NPM family share similarities not only in their amino acid sequences, but also in their domain organization and tertiary structure (Fig. 18). An N-terminal protease resistant core, which is the most conserved domain of these proteins and in most cases includes a short A1 acid tract, is responsible for oligomerization and chaperone activity. The C-terminal tail domain contains up to two additional acids tracts (A2 and A3) and the sizes and functional motifs differ between the family members (Prado et al., 2004). In NPM2, the first of the C-terminal acid tracts (A2) is the longest whereas in NPM1 this first tract is relatively short and followed by an additional region that is not conserved in the NPM2 or 3 sequences (Fig. 18). For NPM1, the A3 region is the longest of the three acid tracts, similar in length to that of A2 in

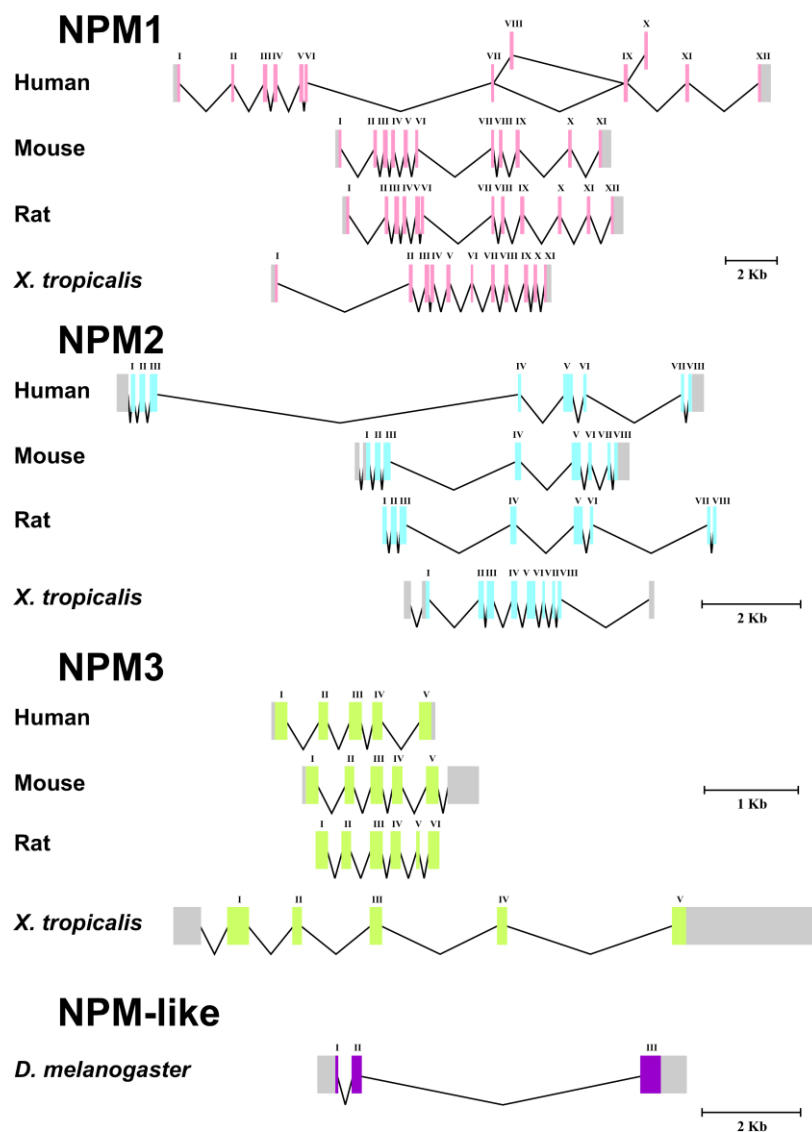


Figure 17. The intron and exon organization of NPM genes.

Exons coding for translated regions of NPM1, NPM2 and NPM3 NPM-like proteins are schematically represented by pink, blue, green and purple boxes respectively. Exons coding for untranslated regions are depicted by grey boxes. Introns are indicated by black lines. The *Xenopus tropicalis* NPM1, 2 and 3 sequences were found on the JGI *Xenopus tropicalis* v4.1 database and had assigned names of fgenes1_kg.C_scaffold_24000023, fgenes1_kg.C_scaffold_34000011 and fgenes1_kg.C_scaffold_238000002 respectively. In the case of mammals, the accession numbers for the sequences used are as follows: NPM1 (human, NC_000005.8; mouse, NC_000077.3; rat, NC_005109), NPM2 (human, NC_000008.9; mouse, NT_039606; rat, NW_047454), NPM3 (human, NC_000010; mouse, NC_000085; rat, NW_047567); NPM-like (*Drosophila*, NT_033777.2).

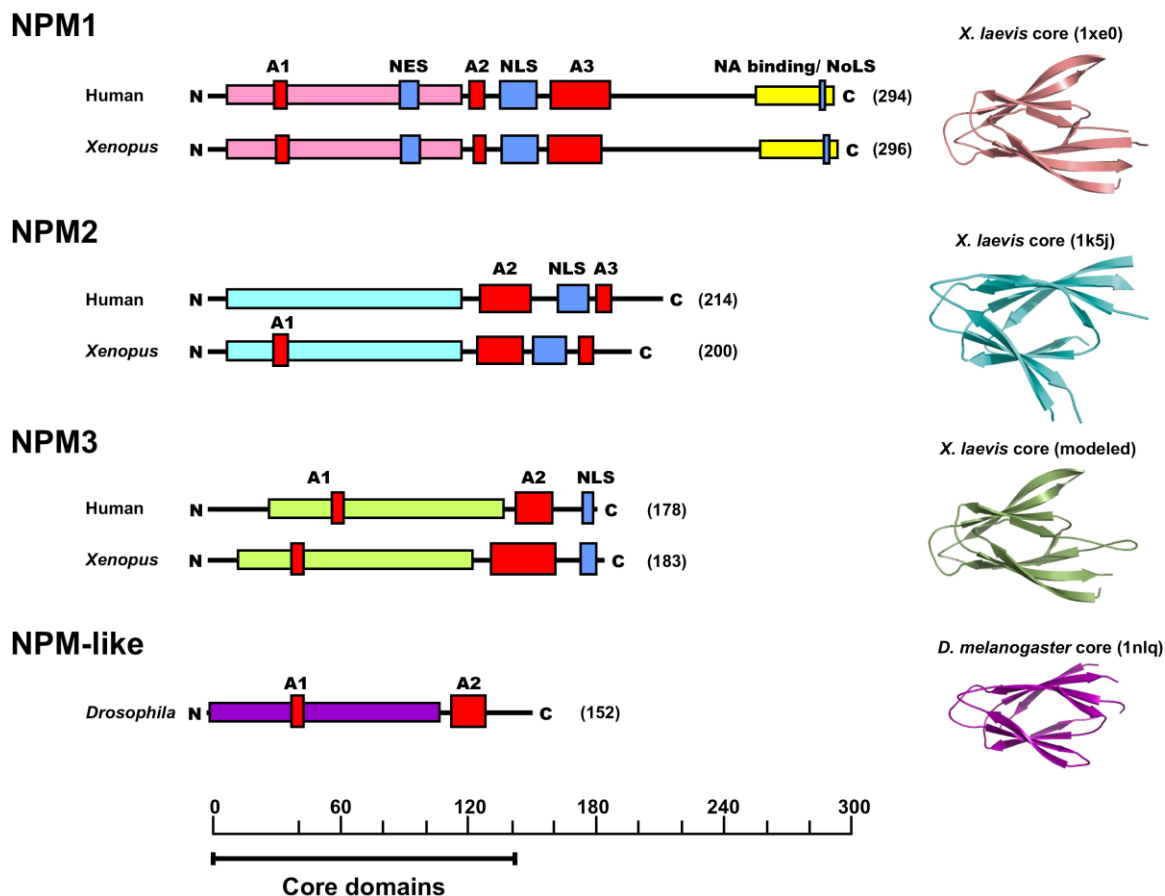


Figure 18. Selected NPM protein structures.

Depictions of the protein domains on the left and the corresponding protein tertiary structures of the monomer core domains on the right. Acid tracts are shown in red. Localization signals are shown in blue (nuclear localization signal, NLS; nuclear export signal NES; and nucleolar localization signal NoLS). The core domains are shown in pink, blue, green and purple for NPM1, NPM2, NPM3 and NPM-like proteins respectively. NPM1 members also have a nucleic acid (NA) binding domain at the C-terminus which is shown in yellow. The NCBI accession numbers of the sequences used to draw the protein domain structures were as follows: Human NPM1, NP_002511; *X. laevis* NPM1, X05496; Human NPM2, NP_877724; *X. laevis* NPM2, X04766; Human NPM3, EAW49745; *X. laevis* NPM3, X99293.1; *Drosophila* NPM-like, AAF56988. The coordinates for the three-dimensional structures of the *X. laevis* NPM1 core (PDB ID: 1xe0), the *X. laevis* NPM2 core (PDB ID: 1k5j) and the *D. Melanogaster* NPM-like core (PDB ID: 1xe0) were from the NCBI protein database. The *X. laevis* NPM3 core monomer was modeled with the SWISS-MODEL server (Schwede et al. 2003) using the coordinates of the *X. laevis* NPM1 core crystal structure (PDB ID: 1xe0) as a reference.

NPM2. All three have classic bipartite nuclear localization signals (NLS) with the consensus sequence $KRN_{10}KKK$, where N accounts for any amino acid (Dingwall et al., 1987). However, whereas NPM3 terminates immediately following this NLS, NPM2 extends into the last acid tract, which is absent in NPM3. Additional motifs, which are exclusively found in NPM1, include the putative nuclear export signal (NES) and the extension at the C-terminal end containing the putative nucleolar localization signal (NoLS) and the domain for nucleic acid binding and RNA cleavage (Hingorani et al., 2000).

It is worth noting that the human, mouse and rat NPM2 proteins do not contain an A1 acid tract in the N-terminal core region. It has been found that mutation of only four of the acidic amino acid residues of the A1 tract to glutamine residues drastically reduces the sperm decondensing activity of *Xenopus* nucleoplasmin and the ability of this mutant chaperone to swell sperm nuclei (Salvany et al., 2004). This is very interesting considering that mouse NPM2 was found not to be essential for protamine removal and sperm decondensation in knock out experiments (discussed below). In contrast, human NPM3 protein, which does have an A1 tract in the N-terminal region, was shown to be required for decondensation of sperm chromatin (McLay and Clarke, 2003).

The tertiary structures of the *Xenopus* NPM2 (nucleoplasmin) (Dutta et al., 2001) and NPM1 (NO38) (Namboodiri et al., 2004) N-terminal core domains have been determined by X-ray crystallography, as has the *Drosophila* nucleoplasmin-like core domain (Namboodiri et al., 2003). All three have monomer subunits with eight-stranded β -barrel topologies (Fig. 18).

Evolution and functional diversification

The intron and exon organization of NPM genes described above (Fig. 17) is not enough to infer an evolutionary relationship among the NPM genes. However, it does support the functional evolution and diversification of the NPM family described by (Eirin-Lopez et al., 2006a). The phylogenetic analyses of the NPM family members among different metazoan taxa reveal a clustering pattern by type, instead of by species (Fig. 19) (Eirin-Lopez et al., 2006a). The observed topologies revealed a process of functional evolution operating in NPMs which ultimately must have imposed strong selective constraints on the family members, resulting in the appearance of the different NPM (1-3 and NPM-like) types (Eirin-Lopez et al., 2006a).

The NPM1 and the invertebrate NPM-like lineages are characterized by a monophyletic origin in the protein topologies, whereas NPM2 and NPM3 have a polyphyletic origin due to what appears to be independent differentiation in mammals with respect to the rest of the vertebrate groups (Eirin-Lopez et al., 2006a). The NPM-like lineage from invertebrates shows a closer phylogenetic relationship with the NPM1 lineage, although within invertebrates the characteristic acidic tracts are not as conserved as within vertebrates in terms of their spatial arrangement and consensus sequences. The diversification and distribution of the potential phosphorylation sites in NPM proteins across these different groups of vertebrates is also consistent with the polyphyletic origin described for NPM2 and NPM3, and further supports their differentiation early in NPM evolution (Eirin-Lopez et al., 2006a).

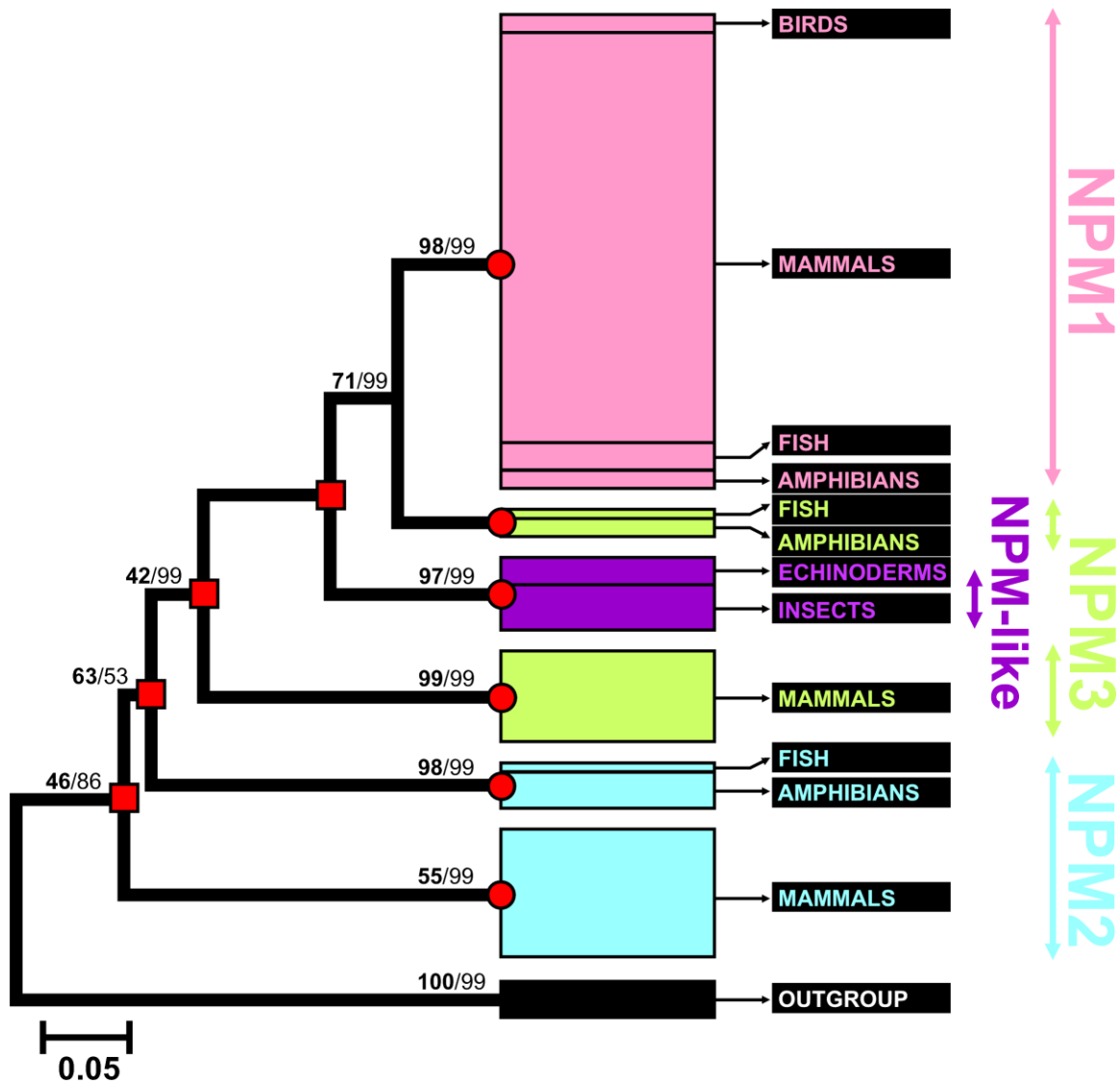


Figure 19. Schematic representation of the phylogenetic relationships among NPM proteins based on results obtained by (Eirin-Lopez et al., 2006a)

The tree (Eirin-Lopez et al., 2006a) was reconstructed based on evolutionary protein *p*-distances using the pairwise deletion option and the neighbour joining tree building method. Numbers for interior nodes indicate bootstrap (boldface) and interior branch-test (normal) confidence values based on 1000 replications. The differentiation events leading to the appearance of the different NPM lineages are represented by red squares at the corresponding nodes in the tree, while diversification events are represented by red circles. The heights of the boxes in the right margin of the tree are proportional to the number of sequences analyzed for each taxon. The reader is referred to (Eirin-Lopez et al., 2006a) for a full description of the methods used in reconstructing the tree, which is not part of this dissertation.

In terms of gene evolution, NPM coding sequences diverge extensively through silent substitutions, which in all cases are significantly greater than the observed nonsilent variation (Eirin-Lopez et al., 2006a). This reveals a deviation from neutrality that suggests the presence of selection acting on specific residues (Eirin-Lopez et al., 2006a). Indeed, the triplets encoding the glutamic and aspartic acid residues in the acidic tracts show bias in terms of nucleotide frequencies at the second codon positions, further supporting a departure from the neutral expectations and pointing towards a selection process favouring specific amino acids (Eirin-Lopez et al., 2006a). It appears that the high frequencies presented by acidic residues at C-terminal regions (which are critical for the correct interactions of NPMs with other proteins) are maintained by selection, altering the nucleotide composition of these domains (Eirin-Lopez et al., 2006a). Such a selection process for highly biased amino acid frequencies has been previously described in other genes such as the *tola* gene from proteobacteria (maintaining high levels of alanine and lysine (Rooney, 2003)) and the mammalian protamine 1 gene (high levels of arginine (Rooney et al., 2000)).

The evolution of NPM proteins shares some similarities with that of histones, for which NPM1 and NPM2 operate as chaperones. Both groups of proteins are under a strong purifying selection process at the protein primary structure level and diverge extensively through silent substitutions at the nucleotide level (Eirin-Lopez et al., 2006a; Eirin-Lopez et al., 2004). An intriguing possibility would involve the parallel evolution of these two groups of interacting proteins, which could have ultimately resulted in the functional diversification of the different NPM lineages. However, more functional and structural data are still needed before this hypothesis can be tested.

Overview of chaperone functionality

The archetypical Xenopus nucleoplasmin: Sperm chromatin remodelling

Accumulated biochemical studies have given hints to the possible mechanisms of nucleoplasmin binding to sperm-specific proteins (SPs) and histones (Prado et al., 2004) (Fig. 20). A model has been put forward for *X. laevis* in which histones bind to the nucleoplasmin pentamer through stereospecific interactions (Prado et al., 2004). When hyperphosphorylated nucleoplasmin, with H2A-H2B dimers bound, comes into contact with the highly compacted sperm chromatin, the SPs subsequently dissociate and bind to the pentamer through electrostatic interactions. This SP-nucleoplasmin interaction may cause a conformational change in the nucleoplasmin pentamer resulting in the dissociation of the bound H2A-H2B dimers. Such a dissociation occurs within the vicinity of the paternal DNA with H3-H4 tetramers, thus allowing nucleosomes to form (Prado et al., 2004).

In the egg, chaperones other than nucleoplasmin are also present and aid in chromatin remodelling. In *Xenopus* oocytes, H2A/H2B and H3/H4 are found complexed with nucleoplasmin and N1/N2 (Kleinschmidt et al., 1985; Kleinschmidt and Franke, 1982). Both chaperones are able to separately reconstitute nucleosomes *in vitro*, but are more efficient when used together (Kleinschmidt et al., 1990). In addition to the better known role of NAP1 as an H2A/H2B chaperone, *Xenopus* NAP1 (xNAP1) was recently found to immunoprecipitate with the *Xenopus* early embryonic linker histone B4 (Shintomi et al., 2005). In the absence of xNAP1, nucleosomes were

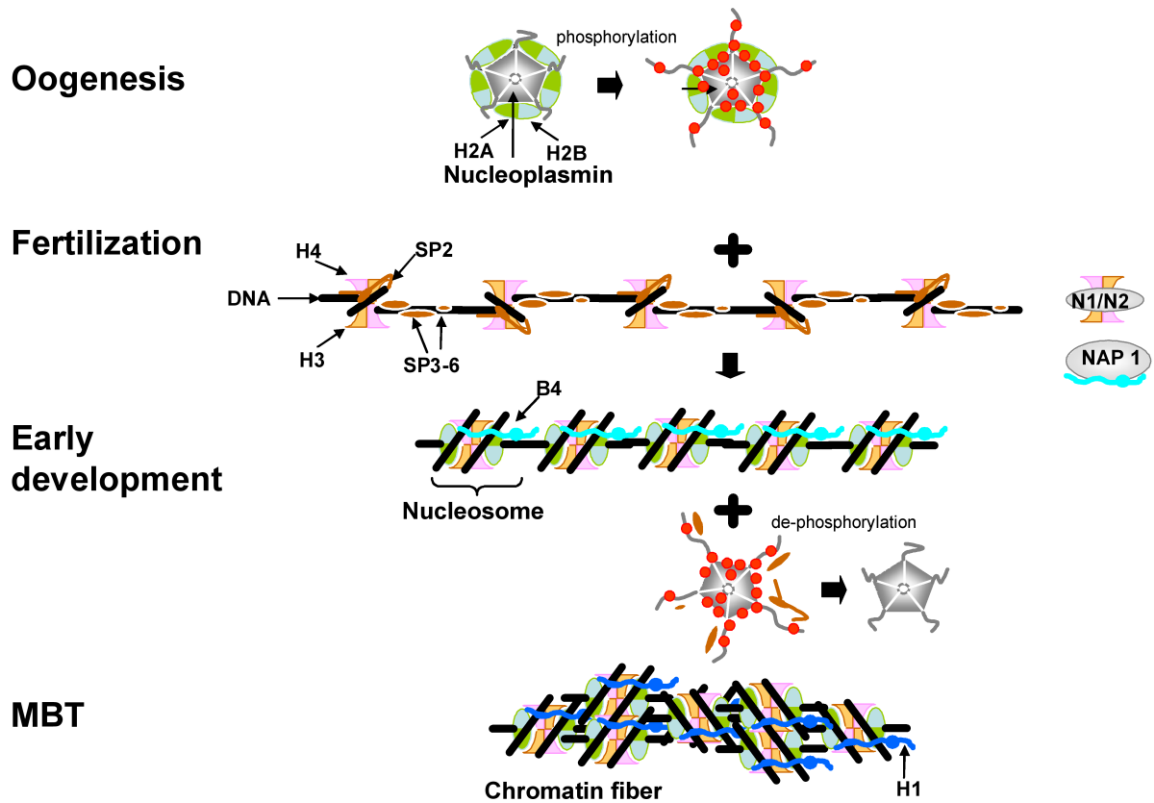


Figure 20. A schematic representation of *Xenopus* nucleoplasmin functions.

In the unfertilized egg, nucleoplasmin (grey pentamer) stores H2A/H2B dimers. During oocyte maturation nucleoplasmin becomes phosphorylated, represented by red dots. The paternal chromatin in *Xenopus laevis* has sperm-specific proteins SP1-6 and H3/H4 tetramers.

Nucleoplasmin removes the SPs from the paternal chromatin and supplies H2A/H2B dimers. The other chaperones, N1/N2 and NAP1, supply H3/H4 octamers and oocyte-specific linker histone B4. Nucleoplasmin remains phosphorylated until the mid-blastula transition (MBT).

assembled on sperm chromatin. However, there was an excessive deposition of B4 and micrococcal nuclease digestion showed abnormal chromatosome formation. These observations support the notion that xNAP1 plays a chaperone role for the proper deposition of B4 in *Xenopus* eggs during early development (Fig. 20).

A larger picture in which these histone chaperones (nucleoplasmin, N1/N2 and NAP1) act together to coordinate chromatin remodelling following fertilization is beginning to be unveiled. Nucleoplasmin seems to be the most important of the three for the initial decondensation of the sperm chromatin. Due to the fact that histones H3 and H4 are already present in the paternal chromatin of *Xenopus*, N1/N2 would have less of a role to play in this initial step. Following the exchange of H2A/H2B dimers for SPs by nucleoplasmin, NAP1 adds B4 and the chromatin in the paternal pronucleus then resembles that of the maternal pronucleus. Following replication of the paternal DNA, N1/N2 would be needed to deposit H3/H4 tetramers before nucleoplasmin could add H2A/H2B dimers (Fig. 20). This model suggests a role for nucleoplasmin that continues into early development. In support of this, phosphorylated nucleoplasmin is present until the mid blastula transition (MBT) (Litvin and King, 1988). In addition, nucleoplasmin was also found to be present in the oocytes of *Rana catesbeiana*, which contains only histones in the sperm and does not necessarily require nucleoplasmin for the initial step following decondensation, except in its capacity as a general decondensation factor (Frehlick et al., 2006a).

Why is there a need for specialized chaperone proteins in eggs and why does NAP performance of a different task in eggs compared to somatic cells? Before the MBT in *Xenopus* rapid and synchronous rounds of replication occur approximately every half hour without increase in the size of the embryo. *Xenopus* eggs have enough stored

histone proteins to assemble nucleosomes in 20,000 cells (Woodland, 1980). For this storage, and possibly to keep up with the replication machinery, the cell has embryo specific histone chaperones. In addition, embryos also contain specialized histones such as the linker histone B4 which has a longer, less basic C-terminal tail than somatic H1 and may bind less tightly to chromatin, thus allowing for better access by chromatin remodelling factors (Saeki et al., 2005). This would be important during early embryonic development when rapid rounds of replication are taking place. Nucleoplasmin becomes dephosphorylated at the MBT, and at this point, histone B4 is replaced by the conventional set of somatic linker histones.

Chromatin remodelling in somatic cells by Xenopus nucleoplasmin and implications for cell cloning

Somatic cell nuclear transfer (SCNT) involves removing or destroying the nucleus of an unfertilized egg, replacing it with the nucleus of a somatic cell and stimulating this new fused cell to begin dividing and differentiating (see Wilmut et al., 2002 for a review). This technology has allowed for the cloning of animals (such as Dolly the sheep (Wilmut et al., 1997)), as well as for the production of embryonic stem cells. However, a limitation of SCNT is the high mortality rate of cloned embryos and therefore, increasing the success rate of SCNT is a major goal in current cloning research (Wilmut et al., 2002). The low efficiency rate is most likely due to an incorrect reprogramming (including that of epigenetic marks such as DNA methylation and histone post-translational modifications) of the donor chromatin, which could ultimately lead to aberrant gene expression during development (Tian, 2004). During gametogenesis, germ cell chromatin naturally undergoes an epigenetic reprogramming process that must be

reversed in the donor nucleus during cloning by SCNT. Whether this reversal is caused by many specific or a few general reprogramming factors is not yet known.

Terminally differentiated erythrocytes and sperm cells treated with either *Xenopus* egg extracts or nucleoplasmin alone, undergo nuclear swelling and become transcriptionally active (Dimitrov and Wolffe, 1996) and are able to undergo DNA replication (Gillespie and Blow, 2000; Lu et al., 1999). Nucleoplasmin is able to remove linker histones from somatic chromatin, yielding a more open and extended chromatin structure (Ramos et al., 2005). Recently, nucleoplasmin was found to decondense heterochromatin in these cells in an ATP-dependent manner and without the removal of histones (Tamada et al., 2006). In contrast, ATP is not required for the removal of SNBPs or linker histones during decondensation of sperm or erythrocyte chromatin (Dimitrov and Wolffe, 1996; Ramos et al., 2005). The undetectable histone displacement and the ATP-dependency indicate a different mechanism of action. Furthermore, there was an active removal of the centromeric heterochromatin proteins HP1 β and TIF β , although their displacement alone was not sufficient to cause chromatin decondensation. Although mouse embryonal F9 cells displayed chromatin decondensation, some other cell lines did not.

In addition, several epigenetic modifications were reported to occur during this process. Histone H3 was phosphorylated at serine 10 and 28 and at threonine 11 and 38 when nuclei were incubated with recombinant nucleoplasmin in the presence of ATP. Additionally, there was an active acetylation of histone H3 at lysine 14 in 50% of the cases. These epigenetic modifications occurred in cell lines regardless of nuclear swelling, suggesting that these modifications were not due to chromatin decondensation. This observation also indicates that the modifications were not the mechanism of action

for decondensation. The ability of nucleoplasmin to promote chromatin decondensation, histone modifications and to displace heterochromatin proteins was dependant on its A2 polyglutamic acid tract in the C-terminal tail. In fact, polyglutamic acid was found to actively promote such processes by itself, although considerably higher amounts of polyglutamic acid were needed when compared with nucleoplasmin which is in agreement with other studies (Betthausen et al., 2006; Philpott et al., 1991; Tamada et al., 2006).

The pretreatment of F9 (but not B16 or NIH 3T3) nuclei with nucleoplasmin, or polyglutamic acid, increased the transcription of mouse oocyte specific genes (H1foo, Msy2, c-Mos and mNpm2) when these nuclei were subsequently injected into *Xenopus* oocytes (Tamada et al., 2006). Therefore, the use of nucleoplasmin or polyglutamic acid may provide considerable advantages in improving cloning efficiency. In fact, injection of specific concentrations of nucleoplasmin or polyglutamic acid into bovine oocytes during SCNT resulted in an increase in the rate of pregnancy initiation and blastocyst development (Betthausen et al., 2006). However, although microarray analysis of these treated embryos showed an upregulation of over 200 genes relative to untreated embryos, there was no increase in the number of live births suggesting that the nucleoplasmin mediated reprogramming of gene expression may not be complete (Betthausen et al., 2006).

Overall, there is now evidence that nucleoplasmin can promote the epigenetic reprogramming of somatic chromatin during SCNT (Betthausen et al., 2006), and this process may act through a different and yet to be determined mechanism than that followed by nucleoplasmin during sperm chromatin decondensation (Tamada et al., 2006). It will be interesting to see if nucleoplasmin proves to be useful for improving

SCNT efficiency in other species and if other nuclear chaperones will have similar results for improving cloning procedures.

Regulation of chromatin condensation during apoptosis: a not so typical function of Xenopus nucleoplasmin

Early in vertebrate development, oocytes are stockpiled and are later depleted over time by apoptotic cell death if they are not fertilized. Gamma-irradiated embryos undergo apoptosis before the mid-blastula transition when the cell cycle is short with no growth phase, but afterwards they are resistant to apoptosis (Anderson et al., 1997). Morphological changes that take place during apoptosis include condensation of chromatin, shrinking of the nucleus, fragmentation of DNA and finally, formation of apoptotic bodies. During fertilization in *Xenopus*, nucleoplasmin colocalizes with the sperm chromatin undergoing compositional changes, decondensation and nuclear assembly to form the male pronucleus. This can be examined *in vitro* using demembrated sperm in *Xenopus* crude egg extracts. Using this *in vitro* system Lu et al. (2005) found that following nucleosome assembly, and the addition of cytochrome c to induce apoptosis, the DNA of the male pronucleus underwent condensation. It was found that nucleoplasmin is excluded from the condensed apoptotic chromatin and is undetectable in the nuclei (Lu et al., 2005). In addition, in *Xenopus* XTC cells induced to undergo apoptosis, nucleoplasmin was excluded from condensed chromatin but remained dispersed within the nucleus (Lu et al., 2005).

The specific dephosphorylation of tyrosine 124 in the *Xenopus* nucleoplasmin protein was directly linked to apoptotic chromatin condensation, suggesting a novel role for nucleoplasmin as a regulator of apoptotic chromatin condensation in *Xenopus* (Lu et

al., 2005). Nucleoplasmin may act as a general decondensation factor, and dephosphorylation of tyrosine 124 inactivates nucleoplasmin so that apoptotic DNA condensation can occur. This tyrosine residue is conserved in amphibians and even encoded by the same codon (Eirin-Lopez et al., 2006a). The involvement of NPM2 in mammalian apoptosis, in a similar fashion as that presented by amphibians, still needs to be addressed given that tyrosine at position 124 is not conserved in mammals.

NPM1 expression is decreased in human cells undergoing apoptosis and is considered an anti-apoptotic protein (Li et al., 2004). In humans, NPM1 is up-regulated in response to hypoxic stress in normal cells and cancer cells, stopping them from undergoing apoptosis through inhibition of p53 activation, a known binding partner of NPM1 (Li et al., 2004). Although both NPM1 and NPM2 have involvements in apoptosis, the processes appear to be quite different.

Nucleoplasmin homologues in mammalian oocytes

Similar to nucleoplasmin in *Xenopus* (Burglin et al., 1987; Dingwall et al., 1987), the mammalian NPM2 ortholog is expressed only in the oocyte (Burns et al., 2003). Initially, mammalian NPM2 protein is in the nucleoplasm of the oocyte. Following fertilization and the break down of the germinal vesicle, NPM2 disperses into the cytoplasm until formation of the pronuclei, where it is until the eight-cell stage. Unlike NPM1 and NPM3, NPM2 is excluded from the nucleolus.

Npm2 (-/-) mice are viable, although the females showed reduced fertility (Burns et al., 2003). Oocytes from *Npm2*-null females did not have normal heterochromatin formation surrounding the nucleolus and exhibited decreased nucleolar clearing of acetylated H3 and dispersed nucleolar bodies. Protamine removal and chromatin

decondensation proceeded normally following fertilization, leading to the formation of the paternal pronucleus. This is surprising as, in *Xenopus*, nucleoplasmin is essential for remodelling of the male pronucleus. The transition from the one to two-cell stage exhibited an abnormal exit from the first round of meiosis in *Npm2* (-/-) mice. *In vitro*, fertilized *Npm2*-null eggs showed a reduced progression to the two-cell stage and *in vivo* embryos had delays in early development. Therefore, NPM2 is needed for nucleolar heterochromatin organization, chromatin compaction and deacetylation of H3 in mice (Burns et al., 2003).

It is possible that protamine removal is compensated for in *Npm2*-null mice by other NPM family members in the absence of NPM2. However, it is interesting that NPM2 in mammals does not have an A1 acid tract, which in *Xenopus* is important for sperm chromatin decondensation (Salvany et al., 2004). In addition, it has been shown that human NPM3 may be required for decondensation of sperm chromatin (McLay and Clarke, 2003). Oocytes injected with *Npm3* antisense oligonucleotides before fertilization to decrease the level of NPM3 presented a significantly reduced ability to replace protamines with histones in the male pronucleus (McLay and Clarke, 2003). However, it may be possible that injection of antisense oligonucleotides may have decreased the expression of other NPM family members which have similar sequences. Therefore, more studies are needed to determine which protein is in fact required for protamine removal in mammals.

In addition to its many other roles, human NPM1 acts as a histone chaperone

Although NPM1 has been implicated in many functions in the cell, it has (like nucleoplasmin) also been shown to bind histones. Like most histone chaperones, NPM1

can bind to H3/H4 tetramers and H2A/H2B dimers, although binding to H3/H4 is preferred (Namboodiri et al., 2004; Okuwaki et al., 2001b; Swaminathan et al., 2005).

The H3/H4 tetramers interact with the N-terminal core domain, however, the first C-terminal acid tract (A2) may be needed for H2A/H2B dimer binding (Namboodiri et al., 2004; Swaminathan et al., 2005).

Through the ability to bind histones, NPM1 is able to assemble nucleosomes (Okuwaki et al., 2001b). Deletion of the last 94 C-terminal amino acids resulted in a 40% reduction in this ability, deletion of the C-terminus and the second acid tract resulted in a 70% reduction and the N-terminus region alone had no ability to form nucleosomes (Swaminathan et al., 2005). Thus, in contrast to the histone binding domain, the nucleosome assembly function is localized to the C-terminus and last acid tract.

Human NPM1 is able to decondense demembranated *Xenopus* sperm and increases the transcription of acetylated chromatin (Swaminathan et al., 2005). Several lysine residues in the N-terminus of NPM1 itself are specifically acetylated by p300, making NPM1 the first known histone chaperone amenable to acetylation. There is sufficient acetylated NPM1 in HeLa cells to be detected by Western analysis without treatment with HDAC inhibitors. Acetylated NPM1 is able to increase transcription activity by 5-fold over unacetylated NPM1 in an in vitro assay and acetylation of NPM1 increased its binding affinity to acetylated histones. In addition, overexpression of NPM1 increased the transcription of a p53-responsive reporter gene whereas expression of antisense *NPM1* decreased transcription (Swaminathan et al., 2005). However, as in the case of amphibian nucleoplasmin, the detailed molecular mechanisms involved in the facilitation of transcription are not completely understood.

Thus, NPM1 likely functions as a histone chaperone in the nucleolus, where highly active transcription creates a need for a histone chaperone to bind any released histones and aid in the assembly of nucleosomes. NPM1 has also been shown to interact with FRGY2a/YB1 and act as a mediator in nucleolar disassembly (Gonda et al., 2006), emphasizing NPM1's importance for nucleolar structure.

Potential ways in which NPM family members may control their multiple functions

Interaction with other family members

How does NPM1 function in ribosome biogenesis, histone chaperoning and other activities? It could have to do with post-translational modifications, variants or binding to other proteins, possibly even other members of the NPM family. In support of this, NPM1 localized to centrosomes has different immunogenic characteristics (i.e., preferentially recognized by a different antibody) than nucleolar NPM1 (Shinmura et al., 2005).

Heterogeneous complexes of different NPM family members may allow for regulation of function. This has been implicated for NPM1 and NPM3. Results of a yeast two-hybrid screen indicated that human NPM3 was a major binding partner of human NPM1 (Huang et al., 2005). This was consistent with the first report of the *Xenopus* homologue of NPM3, NO29, which was immunoprecipitated from cellular extracts with NPM1/NO38 (Zirwes et al., 1997). The interacting regions of the human homologues were narrowed down to amino acids 35 to 90 of NPM1 and 30 to 90 of NPM3, which are located within the core domains. It is yet to be determined whether this

association occurs in the form of heterogeneous pentamers or if a set of NPM1 and NPM3 homopentamers form decameric complexes.

NPM3 is mainly located in the nucleoli and maintaining this localization requires active rRNA transcription (Huang et al., 2005), but unlike NPM1, NPM3 does not seem to associate with rRNA. The overexpression of NPM3 resulted in a decrease in rRNA production and processing and this was dependent on binding to NPM1 (Huang et al., 2005). Thus, it seems binding of NPM3 to NPM1 may create a complex in which NPM1 can no longer perform its ribosomal biogenesis activities, possibly because it can no longer bind to RNA (Huang et al., 2005). Similar to NPM3, NPM1 variants, which lack the nucleic acid binding domain, inhibits ribosome biogenesis (Itahana et al., 2003) (Okuwaki et al., 2002). Thus, variants of NPM1 as well as other NPM family members can act as binding partners to modulate its cellular activities. So far no studies have been done to assess the effect of the NPM1-NPM3 association on histone chaperone function.

Post-translational modifications of NPM proteins: Phosphorylation, acetylation, glutamylation, polyribosylation

Post-translational modifications, especially phosphorylation, are common mechanisms for controlling the function of proteins. NPM1 is phosphorylated by several kinases at multiple sites. One example is phosphorylation of Threonine199 by CDK2/cyclin E, which is involved in the regulation of centrosome duplication (Okuda et al., 2000; Tokuyama et al., 2001) and targets NPM1 to nuclear speckles, enhances RNA-binding and represses pre-mRNA splicing (Tarapore et al., 2006). Thus, phosphorylation seems to regulate NPM1 activity, a process that takes place in a cell cycle-dependent manner. Among other sites, Thr199 as well as Thr219, Thr234, and Thr237 were

identified as potential phosphorylation targets for cyclin B/cdc2 during mitosis (Okuwaki et al., 2002). In addition, NPM1 is acetylated by histone acetyltransferase p300 resulting in an increase in transcription of a p53-responsive synthetic reporter gene (Swaminathan et al., 2005).

NPM1 was found to co-precipitate with components of a neuronal gene repressor complex (Ju et al., 2004). The components of this repressor complex, including NPM1, are modified by polyribosylation which allows for their dissociation from the complex and gene promoter. Poly(ADP)ribosylation may not be a general modulator of NPM1 activity, but may instead be specific to this example, as all of the other proteins in the repressor complex are also modified.

Phosphorylation is required for the biological activity of nucleoplasmin, with the protein having a higher degree of phosphorylation in eggs than oocytes (Leno et al., 1996). The exchange of H2A/H2B heterodimers for SPs by nucleoplasmin mediated by the phosphorylation of up to 14-20 phosphates per nucleoplasmin monomer (Cotten et al., 1986; Prado et al., 2004). Phosphorylation of nucleoplasmin during maturation into the egg releases the highly negatively charged unstructured C-termini of the nucleoplasmin pentamer. This release most likely provides the complex with a more open structure amenable for sperm chromatin remodelling. It is important to note that while the histone interactions involve stereospecific constraints (both in their interaction with nucleoplasmin and with the negatively supercoiled DNA of the nucleosome), binding of protamines is mediated by electrostatic interactions. Indeed, the nucleoplasmin region responsible for the binding of protamines seems to be able to bind generically to polycationic chromosomal proteins (protamines, SPs, histone H1) which are part of the 'accessible' linker DNA connecting nucleosomal structures. As mentioned above,

phosphorylation of nucleoplasmin at tyrosine 124 further acts as a switch in chromatin condensation during apoptosis.

NAP1 and nucleoplasmin are both characterized by exhibiting acid tracts in their C-terminal domains. Within this region, NAP1 has two glutamylation motifs which may allow for a reversible increase in the charge of its C-terminal tail by the addition of poly-glutamate side chains (Regnard et al., 2000). Nucleoplasmin has also been observed to be amenable to glutamylation (Regnard et al., 2003). The addition of acid side chains could alter the binding specificity of these chaperones to different proteins.

Concluding remarks

From an evolutionary perspective, the members of the NPM family follow a functional diversification process and are subject to purifying selection. NPM1 and NPM3 are more closely related and appear to have differentiated later during evolution than NPM2 (Eirin-Lopez et al., 2006a). NPM2 represents the most specialized NPM lineage and acts as a chaperone in oocytes and eggs (Eirin-Lopez et al., 2006a). The understanding of the role played by NPM2 in H2A and H2B storage (Dilworth et al., 1987), as well as in sperm chromatin remodelling through the removal of SNBPs (Leno et al., 1996; Philpott and Leno, 1992), has more recently been expanded to hypothetically include assisting in nucleosome assembly during early development until the MBT (Frehlick et al., 2006a; Philpott et al., 2000). NPM 2 also acts as a general decondensation factor and inhibits chromatin condensation during apoptosis in *Xenopus* (Lu et al., 2005). On top of numerous functions fulfilled by NPM1 in the cell lies its role as a histone chaperone in the nucleolus. The different roles played by this protein are

regulated by binding partners, including NPM3. Post-translational modifications are also critical in the regulation of NPM family members and allow them to perform different activities in the cell. A better understanding of the locations and temporal occurrence of PTMs is still needed, especially in the case of nucleoplasmin, which is known to become heavily phosphorylated (Leno et al., 1996), but the amino acids modified have not been determined.

Very little is known about the function played by nuclear chaperones in remodelling mammalian sperm chromatin following fertilization. It is not clear which chaperone is responsible for the removal of protamines in this taxonomic group, indicating that more research is needed in this field. Given that NPM2 is one of only a few proteins that are known to be required for the transition from the one to two cell-stage in mammalian development (Burns et al., 2003), further research into NPM2 could be important for treatment and diagnosis of infertility in humans. Similarly, characterization of the activity of NPM3 following fertilization, which may be responsible for sperm chromatin decondensation in mammals (McLay and Clarke, 2003), is important and should be a focus of future research in this field.

Chapter 5. Characterization of amphibian nucleoplasmins

L.J.F.'s contribution to the work:

This chapter was adapted from (Frehlick et al., 2006) for the purpose of this dissertation. I prepared the data, figures and writing. Erin Jeffery in Donald Hunt's lab at the University of Virginia and the UVic Genome BC Proteomic Center performed the mass spectrometry analysis. Juan Ausio prepared Figure 28.

Abstract

Nucleoplasmin is a nuclear chaperone protein that has been shown to participate in the remodelling of sperm chromatin immediately after fertilization by displacing highly specialized sperm nuclear basic proteins (SNBPs), such as protamine (P-type) and protamine-like (PL-type) proteins, from the sperm chromatin and by the transfer of histone H2A-H2B. The presence of SNBPs of the histone type (H-type) in some organisms raises uncertainty about the need for a nucleoplasmin-mediated removal process in such cases. It also raises a question regarding the appearance and further differentiation of the sperm chromatin remodelling function of nucleoplasmin and its relationship with SNBP diversity. The amphibians represent a unique opportunity to address this issue as they contain genera with SNBPs representative of each of the three main types: *Rana* (H-type); *Xenopus* (PL-type) and *Bufo* (P-type).

In this work, the presence of nucleoplasmin in oocyte extracts from these three organisms has been assessed using Western Blotting. We have used mass spectrometry and cloning techniques to characterize the full-length cDNA sequences of *Rana catesbeiana* and *Bufo marinus* nucleoplasmin. Northern dot blot analysis shows that nucleoplasmin is mainly transcribed in the eggs of *Rana*. Phylogenetic analysis of nucleoplasmin family members from various metazoans suggests that amphibian nucleoplasmins group closely with mammalian NPM2 proteins.

We have shown that these organisms, in striking contrast to their SNBPs, all contain nucleoplasmins with very similar primary structures. This result has important implications as it suggests that nucleoplasmin's role in chromatin assembly during early zygote development could have been complemented by the acquisition of a new function

of non-specifically removing SNBPs in sperm chromatin remodelling. This acquired function would have been strongly determined by the constraints imposed by the appearance and differentiation of SNBPs in the sperm.

Introduction

Nucleoplasmin was first isolated from African clawed frog (*Xenopus laevis*) egg extracts and it was described as an acidic chaperone protein capable of assembling nucleosomes (Earnshaw et al., 1980; Laskey et al., 1978). In the egg, nucleoplasmin is found natively bound to histones H2A and H2B, facilitating the storage of these proteins (Dingwall and Laskey, 1990; Kleinschmidt et al., 1985; Philpott et al., 2000). *In vivo*, nucleoplasmin has been shown to mediate the *X. laevis* sperm decondensation in the egg cytoplasm that takes place immediately after fertilization (Philpott et al., 1991). This process involves the binding of the *X. laevis* sperm nuclear basic proteins (SNBPs) and transfer of H2A-H2B to the sperm chromatin (Philpott et al., 1991).

The SNBPs of *X. laevis* consist of a structurally heterogeneous mixture of protamine-like proteins named SP1-SP6 that coexist with a complement of only histones H3 and H4 (Mann et al., 1982). This composition varies significantly between different species of *Xenopus*, which in some instances only contain SP1-2 (such as in *X. tropicalis*) and often contain a stoichiometric complement of nucleosomal H2A-H2B and H3-H4 (Mann et al., 1982).

The SP1-SP6 proteins from *X. laevis* have an amino acid composition intermediate between histones and protamines and hence belong to the Protamine-like (PL) type (Ausió, 1999). In contrast, the toad *Bufo* contains protamines in its sperm that are very rich in arginine and can be considered representatives of the vertebrate protamine (P) type SNBP (Takamune et al., 1991). In the frog *Rana*, the mature sperm contains histones that are almost indistinguishable from the somatic counterparts as is characteristic of the histone (H) type (Itoh et al., 1997; Kasinsky et al., 1985).

If nucleoplasmin is involved in sperm chromatin remodelling during fertilization (Philpott and Leno, 1992), the existence of three main types of structurally and compositionally different SNBP raises the question of whether there are three different nucleoplasmins specific for each of these types or whether, in contrast to SNBPs, nucleoplasmin is a highly conserved molecule. Amphibians provide a unique opportunity to address this question as the group contains representative organisms of each of the main SNBP-types.

In this paper we have characterized the structure of the *Xenopus* nucleoplasmin gene as well as the complete sequences of both the *Bufo marinus* and *Rana catesbeiana* nucleoplasmin cDNAs. The analysis of these sequences shows that nucleoplasmin is indeed a highly conserved protein that is closely related to mammalian NPM2, and suggests that its functional role goes beyond chromatin assembly immediately after fertilization, into the early stages of development. We propose that nucleoplasmin may have acquired the function of SNBP removal after fertilization in many vertebrates because of the appearance and vertical evolution of SNBPs.

Materials and Methods

Living Organisms

X. laevis were reared at the University of Victoria, *B. marinus* were purchased from Wards Natural Science Ltd. (St. Catherines, Ontario) and *R. catesbeiana* were purchased from Island Bullfrogs (Nanaimo, B.C.). Investigations were conducted in accordance with the National Research Council (NRC) publication *Guide for Care and*

Use of Laboratory Animals (copyright 1996, National Academy of Science) under the approval of the University of Victoria's Animal Care Committee.

Electrophoretic Analyses of SNBPs

SNBPs were extracted from testes with 0.4N HCl and precipitated with acetone as described by (Wang and Ausió, 2001). Proteins were separated by AU-PAGE (5% acetic acid-12% PAGE-2.5 M urea) or AUT-PAGE (5% acetic acid-10.5% PAGE-5.25 M urea-5 mM Triton X-100) as described in Chapter 2.

Polyclonal antibodies

Rabbit polyclonal antibodies were raised against recombinant nucleoplasmin (Burglin et al., 1987) which had been expressed and purified as described elsewhere (Prieto et al., 2002).

Western blot analysis with nucleoplasmin antibody

For preparation of high speed extracts enriched in nucleoplasmin, ovaries were dissected from mature females after three injections of human chorionic gonadotropin (3000 units per frog) (Sigma, Oakville, ON) given equally over 5 days. The high speed extract purification procedure followed the protocol of (Sealy et al., 1989) with 1/100 v/v complete protease inhibitor (Roche Diagnostics, Laval, Qc) added to the buffers. In addition, nucleoplasmin was further purified by HPLC. For this, the high speed extract was filtered with a Nanosep MF microconcentrator (Pall Filtron Corporation, Northborough, MA) and fractionated by HPLC on a reverse phase 300-A° Vydac C18

column (25 x 3 x 0.46 cm) (Vydac, Hesperia, CA) and eluted at 1 ml/min with a 0.1% TFA-acetonitrile gradient (Moore et al., 1997).

For Western analysis, nucleoplasmin proteins or high speed extracts were separated by 10% SDS-PAGE (Laemmli, 1970), electro-transferred to polyvinylidene difluoride membrane (Bio-Rad, Mississauga, ON) and processed as described in (Abbott et al., 2004). The anti-nucleoplasmin serum was diluted 1:5000 and a secondary goat anti-rabbit horseradish peroxidase conjugate (Sigma, Oakville, ON) was diluted 1:3000.

Mass Spectrometry partial peptide sequences

SDS-PAGE separated *R. catesbeiana* and *B. marinus* protein bands, identified by Western Blot analysis as nucleoplasmin, were excised and the tryptic digested gel plugs analyzed on a Q-Star nanospray MS/MS analysis at the UVic Genome BC Proteomics center. Alternatively, the gel plugs were digested with Glu-C endoproteinase (*EC 3.4.21.19*) using 50ng/μL Glu-C enzyme overnight at room temperature following the in-gel digestion and extraction protocol described by (Shevchenko et al., 1996) and analyzed using electrospray ionization to spray the analyte into a LTQ-FTMS instrument (ThermoElectron, San Jose, CA).

cDNA sequence obtained from RT-PCR and RACE

For RT-PCR total RNA was extracted from oocytes using Trizol reagent (GibcoBRL, Burlington, ON) and cDNA was synthesized using Superscript II RNase H⁻ reverse transcriptase (Invitrogen, Burlington, ON). Degenerate primers for PCR were created based on the determined amino acid sequences for *B. marinus* and *R. catesbeiana* and the *X. laevis* nucleoplasmin cDNA sequence. Polyadenylated mRNA was purified

using the MicroPoly(A) Purist small scale mRNA purification kit (Ambion, Austin, TX) and used for 3' and 5' rapid amplification of cDNA ends (RACE) using the FirstChoice RLM-RACE Kit (Ambion, Austin TX) following the manufacturer's directions. PCR was performed using a PCR Sprint thermal cycler (Hybaid, Teddington, UK) with cDNA as the template. The following primers were designed for RACE from the internal sequence:

Rana 3' RACE inner 5'-GCAAAGGATGAGTTCCACATAGTA-3'

Rana 3' RACE outer 5'-CATCAACTGGCGTTAAGGAC-3'

Rana 5' RACE inner 5'-ACTGCTTGAGGGACTATTTCTACTA-3'

Rana 5' RACE outer 5'-CGGTTTTGCGTCACTCCCTTC-3'

Bufo 3' RACE inner 5'-GATGAAGACAAGAGCGAGCA-3'

Bufo 3' RACE outer 5'-AGCTGGCCCTGAGAACTGTA-3'

Bufo 5' RACE inner 5'-CATCGCTCCCTTCTACCTGA-3'

Bufo 5' RACE outer 5'-CTGGCTATAGGAACAGGTTG-5'

The cDNA was sequenced as described in Chapter 2. In addition to the cDNA sequences of *R. catesbeiana* and *B. marinus*, the partial genomic DNA sequence of the *X. laevis* gene was determined to confirm that *X. laevis* had a similar intron / exon structure to *X. tropicalis*. PCR and sequencing was done as described for the cDNA using *X. laevis* genomic DNA as a template and the following PCR primers:

Xenopus forward inner 5'-GTGAGCATCAGTTGGCGTTGC-3'

Xenopus forward outer 5'-CTGGGGACAAGGCAAAGGA-3'

Xenopus reverse inner 5'-ACCGGAAAGTAASTGGAGGAG-3'

Xenopus reverse outer 5'-GAGGTTCACTTCTTAGCAGCCG-3'

Phylogeny of nucleophosmin/nucleoplasmin family members from various metazoans

Amino acid sequences were aligned using the program CLUSTAL W (Thompson et al., 1994) and the phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987). Amino acid sequence distances were estimated as the uncorrected differences (*p*-distance) resulting in smaller variance. The reliability of the topology was tested by the bootstrap method (1000 replications) and values are shown in the corresponding internal nodes of the topology. Two other histone-binding chaperone proteins (NASP and N1/N2), which are closely related to each other but unrelated to the nucleophosmin/nucleoplasmin family were used to root the tree. All the molecular evolutionary analyses were conducted using the computer program MEGA version 3.1 (Kumar et al., 2004).

Northern dot blot hybridizations comparing Npm2 and Npm1 mRNA levels in different R. catesbeiana tissues

Total RNA was extracted from oocytes using Trizol reagent (GibcoBRL, Burlington, ON) and the purity and concentration were determined by UV absorbance and agarose gel electrophoresis of denatured samples. Total RNA samples (7.5 µg for *Npm1* and *Npm2* or 1.5 µg for 18S ribosomal) were dissolved in 10mM NaOH, 1mM EDTA and transferred to Zeta-Probe GT Blotting membrane (BioRad, Mississauga, ON) using a Bio-Dot Microfiltration Apparatus (BioRad, Mississauga, ON) following the manufactures directions, then crosslinked to the membrane with UV (120 000 µJ). The cDNA probes used were produced by PCR using the following primers:

18S forward 5'-TGCATGGCCGTTCTTAGTTGGTGG-3',

18S reverse 5' CACCTACGGAAACCTTGTTACGAC-3',

NPM2 forward 5'-TCCAGAATCTCCTCCAAAACC-3',

NPM2 reverse 5'-AGGGCTTCCTTCCTCTTCCT-3'

NPM1 forward 5'-CTCAAAGTYRAATCACAATGG-3'

NPM1 reverse 5'-TGTCTCCATTKCCARAGATCTT-3'

NPM 1 and 2 primers were designed based on nucleotide regions unique to each sequence. The 18SCOMF and 18SCOMR primers were from (Zhang and Lin, 2002). The 18S and *Npm2* probes were from *R. catesbeiana* and the *Npm1* probe was from *X. laevis*. PCR products run on agarose gels gave single bands of the expected sizes which were excised, purified using the QIAquick Gel extraction kit (Qiagen, Mississauga, ON) and sequenced as described in Chapter 2 to confirm their identity. The purified PCR products were labelled with the Random Primer DNA Labeling System (Invitrogen, Burlington, ON) following the Standard Labeling protocol to produce probes. Hybridization of $\sim 10^6$ cpm 32 P-labeled cDNA probes in PerfectHybtm Plus buffer (Sigma, Oakville, ON) were incubated overnight with the blots. Membranes were then washed to high stringency, exposed and analyzed.

Results

Ubiquitous presence of nucleoplasmin in the eggs of amphibians

As it can be seen in Figure 21 A-C, *R. catesbeiana* sperm contains only core histones that, except for the histone H1 counterpart, exhibited identical electrophoretic mobility to those of somatic histones (Fig. 21 A-C, lane 3). Indeed, the sperm of this amphibian has been shown to consist of core histones that have amino acid compositions

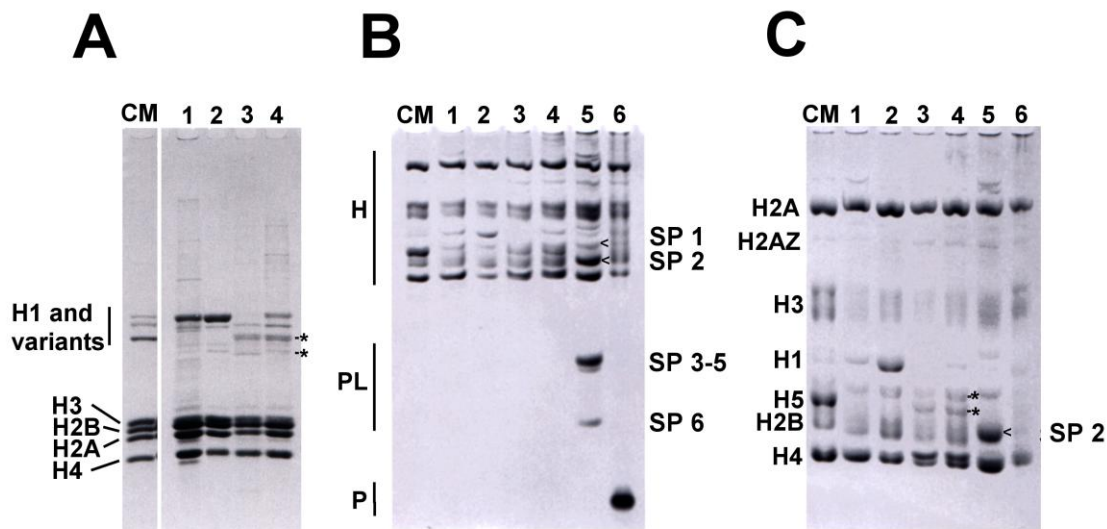


Figure 21. Electrophoretic characterization of *R. catesbeiana*, *X. laevis* and *B. marinus* SNPBs.

Characterization of histones and SNBPs extracted from different tissues by: **A)** SDS-PAGE; **B)** AUT-PAGE run for a short time duration to separate histones (H), protamine-like proteins (PL) and protamines (P); and **C)** AUT-PAGE run for a longer duration to separate histones. Lane 1: *R. catesbeiana* liver; lane 2: *R. catesbeiana* blood; lane 3: *R. catesbeiana* sperm; lane 4: *R. catesbeiana* testes; lane 5: *X. laevis* testes; lane 6: *B. marinus* testes. CM; Chicken erythrocyte histones used as a histone marker. The SNBPs of *X. laevis* in lane 5, called sperm-specific proteins (SP1-6), are labelled and arrows (<) are used when needed to clearly indicate which bands the labels refer to. The asterisks point to the sperm-specific histone H1 complement in *R. catesbeiana*.

identical to those of somatic tissues and a set of specific linker histones with similar composition to histone H1 (Itoh et al., 1997).

In contrast to *R. catesbeiana*, the SNBPs of *X. laevis* in testis showed the presence of a complex mixture of SNBPs of the PL-type (SP1-SP6) (Mann et al., 1982) (Fig. 21B, lane 5), which in the mature sperm coexist with a H3-H4 complement. Alternatively, *B. marinus* contained a typical vertebrate protamine (Kasinsky et al., 1985; Takamune et al., 1991) (Fig. 21B, lane 6). Thus, the protein composition of the sperm chromatin of these organisms is very different.

With the exception of *X. laevis* nucleoplasmin, which has been extensively characterized and has been for many years used as a generic prototype for nucleoplasmin, only a small amount of information is available on *Bufo* nucleoplasmin (Ohsumi and Katagiri, 1991) and very little is known about its existence in *Rana* (Lohka and Masui, 1983). Therefore, we took advantage of an antibody developed in our lab against recombinant *X. laevis* nucleoplasmin to identify the nucleoplasmin-like proteins in oocyte extracts of these two organisms. The candidate bands from gels were then used to obtain some partial protein sequence information in order to further confirm the bands identity and to produce primers that would allow us to obtain the complete cDNA sequences of these proteins.

One of the distinctive features of nucleoplasmin is its ability to retain its native pentameric conformation ($M_r = 110,000$) in the presence of the SDS used in the sample buffer of SDS-PAGE (Prieto et al., 2002). The monomeric subunit can only be visualized after extensive (*ca.* 10 min) boiling in the presence of the SDS (1%) sample buffer. We exploited this characteristic property in order to analyze the oocyte extracts from *X. laevis*, *B. marinus* and *R. catesbeiana*. Figure 22 shows the results of unboiled

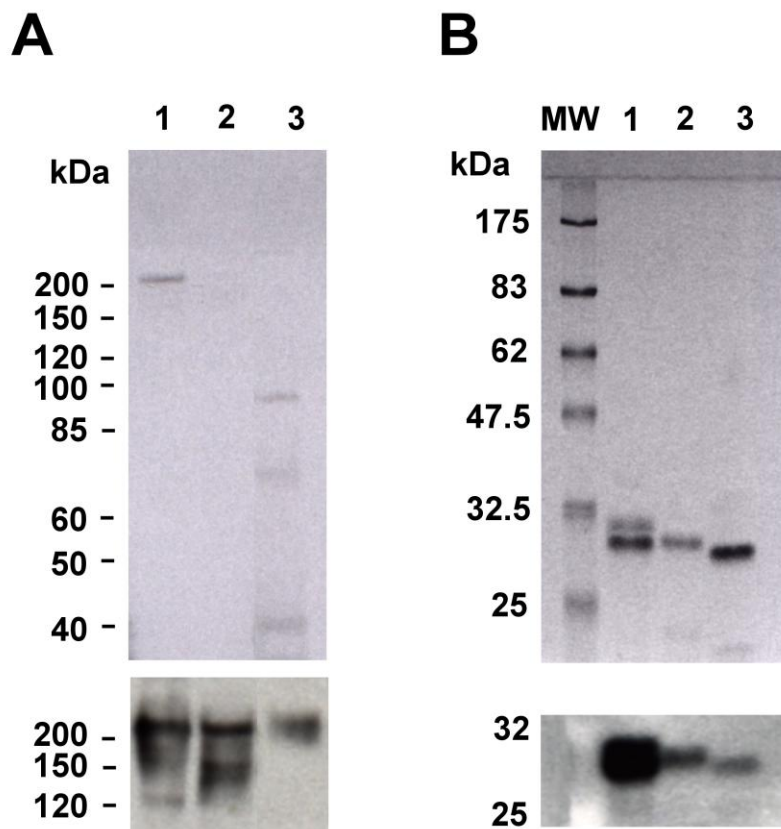


Figure 22. Western blot analysis of egg extracts and purified nucleoplasmin proteins.

A) SDS-PAGE of heat soluble oocyte extracts from: Lane 1, *X. laevis*; lane 2, *B. marinus*; lane 3, *R. catesbeiana*. Oocyte extract aliquots were mixed with an equal volume of 2x SDS sample buffer and loaded in the gel without any previous boiling. Under these conditions the nucleoplasmin protein retains its pentameric conformation (Prieto et al., 2002). Molecular weights are a PageRuler Protein Ladder (Fermentas Life Sciences, Burlington, ON) **B)** SDS-PAGE of nucleoplasmin proteins purified from the extracts of: Lane 1, *X. laevis*; lane 2, *B. marinus*; lane 3, *R. catesbeiana*. Samples were boiled in SDS (0.1%) sample buffer for 10 minutes before loading on the gel to separated nucleoplasmin proteins into their monomeric forms. MW is a prestained broad range molecular weight protein marker (New England Biolabs, Ipswich, MA). Western blot analysis was done using a polyclonal antibody elicited against recombinant *X. laevis* nucleoplasmin (Burglin et al., 1987) and the results are shown in the lower panels of both A) and B) below the corresponding gels.

(Fig. 22A) and boiled (Fig. 22B) extracts separated by SDS-PAGE (upper panels).

The presence of nucleoplasmin was assessed by Western blot analysis using an antibody against *X. laevis* recombinant nucleoplasmin (Fig. 22, lower panels) (Dingwall et al., 1982). For all three extracts a higher molecular weight form corresponding to the pentamer was seen in the unboiled samples (Fig. 22A) which dissociated into monomers following boiling in SDS sample buffer (Fig. 22B). Note that *X. laevis* and *B. marinus* exhibited a smeared band in the region of the SDS-PAGE corresponding to the pentameric form, a fact that can be attributed to either differences in the extent of phosphorylation or the incorrect folding of the pentamer. In addition, due to its highly charged nature (polyglutamic acid tracts and phosphate residues), nucleoplasmin is known to have aberrant migration on SDS-PAGE gels.

Although the presence of nucleoplasmin in *Bufo* had already been well documented (Ohsumi and Katagiri, 1991), the presence of a nucleoplasmin-like equivalent protein in *Rana* had never been demonstrated, despite the fact it was known that *Rana* egg extracts had the ability to decondense demembrated sperm nuclei from *Xenopus* (Lohka and Masui, 1983). The results of Figure 22 encouraged us to pursue the isolation of such a protein and also to determine the hitherto unknown primary structure of *Bufo* nucleoplasmin. To this end, nuclear extracts were purified as described in the experimental section and the extent of purity of the fractions was monitored by SDS-PAGE Western analysis. Bands for the *B. marinus* and *R. catesbeiana* nucleoplasmins, such as those shown in Figure 22, were excised and analyzed by mass spectrometry to obtain partial protein sequence information. This information, together with the nucleotide sequence available from the genome draft of *X. tropicalis* for the

nucleoplasmin gene [assigned name: ESTEXT_FGENESH1_PG.C_340042 at (JGI, 2005)], was used to generate primers to be used in conjunction with mRNA prepared from both *R. catesbeiana* and *B. marinus* oocyte extracts.

The amphibian nucleoplasmin gene contains multiple exons and encodes highly conserved proteins

As it has been mentioned in the previous section, the current availability of the *X. tropicalis* genome draft has proven to be very useful in achieving some of the objectives pursued in this work. Figure 23 A shows the coding region corresponding to the nucleoplasmin gene of *X. tropicalis* [GenBank:NM_001016938] and the corresponding organization of the gene, which contains of 9 introns, is shown in Figure 23B. A very similar organization was found in the case of the *X. laevis* nucleoplasmin gene when it was analyzed using the genomic DNA of this species and primers designed based on the cDNA sequence (results not shown).

We also established the complete cDNA sequences for one of the nucleoplasmin genes from each *B. marinus* [Genbank:DQ340657] and *R. catesbeiana* [Genbank:DQ340656] which were submitted to GenBank December, 2005 (Fig. 24A-B). The precise number of nucleoplasmin genes in each genome has not yet been determined. However, two different cDNAs have been isolated from *X. laevis* that encode for two almost identical forms of the protein, except for the fact that one of them is 9 amino acids shorter (Burglin et al., 1987) than the other (Dingwall et al., 1987). It is thus possible that at least one other cDNA sequence besides those shown in Figure 24 is present in each of these species. Nevertheless, mass spectroscopic determination of the nucleoplasmin proteins purified by us from the oocyte extracts, confirmed that the

A

```

1 CTCTTGCTCCCTGCTGTAGACTTGCAGAGTGTGCTGTAATGATG M
A S T A S N T S K L E K P V S
46 GCCTCTACAGCCAGTAACACCAGCAAGCTTGAGAAACCAGTGTCT
L I W G C E L N E Q N K T F V
91 CTTATATGGGGTGTGAGCTGAACGAGCAGAACAAGACCTTTGTG
F K V S D E D K S E H Q L A L
136 TTTAAAGTGTCCGATGAAGACAAGAGCGAGCACCAGCTGGCCCTG
R T V C L G D K A K D E F H V
181 AGAACTGTATGCTGGGGACAAGGCCAAAGATGAATTTTCATGTG
V E I V P Q V E G S D V Q P V
226 GTAGAAATAGTTCCTCAGGTAGAAGGAGCGATGTACAACCTGTT
P I A S L K P S I L P M A T M
271 CCCATAGCCAGTCTAAAGCCATCTATTCTGCCTATGGCTACAATG
V G I E L T P P V T F R L K A
316 GTGGGCATTGAGTTAACTCCCTCCAGTTACTTTCCGGCTGAAAGCT
G S G P V Y I S G Q H I A L E
361 GGCTCGGGACCAGTATATATAGCGGCCAGCATATCGCATTGGAA
E D Y S W A E E E G E E E V
406 GAAGACTACTCATGGGGGAGGAAGAAGGTGAGGAGGAAGTAGAA
E E E E E E D P E S P P K A V
451 GAGGAAAGAGGAGGAAGATCCTGAATCCTCCAAAAGCAGTA
K R P A A S K K G S Q A K K K
496 AAAAGGCCAGCAGCCTCCAAAAAGGGGAGTCAGGCGAAGAAGAAG
K M D K D E E E S S E E D S P
541 AAAATGGACAAGATGAGGAAGAAAGTTCTGAAGAAGACAGTCCA
V K K G V Y I S G Q H I A L E
586 GTGAAAAAGGGCAAAGGCGCGGACGAGGAGGAAAGCCGGCTGCT
K K *
631 AAGAAATGAGGTGCTGGATCGTCTTGTCTAGGTGTATTTGGCTGT
676 ATTTAAATATCCAGTCCACCATCCTGTAGAATACTGACATTTGT
721 GGGCTACGTACCCATCATGGCCGCTGTCTTCTTGCACCATG
766 TTCCTCCTATGATGATGGCAGGCTGGATATCGTCTTTATGTAATG
811 TAATATATATGAATCAATGTTCTGCTTGTAACTTGTAAAATAA
856 AGTCCCTTTGATGGGAAAAAAAAAAAAAAAAAAAAAAAAA

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B

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1 AAAACTCTTGTGCGGCAACTCTGGAAGTAGTGTAGCAAGATGGAT M D
S T I S N T S K L D K P V S L
46 TCTACAATCAGTAACACCAGCAAGTTGGACAAACCAGTGTCTCTC
I W G C E L N E Q T K T F T F
91 ATATGGGGTGTGAGCTCAATGAACAGACCAAGACATTCACATTT
K V S D E D K S E H Q L A L R
136 AAAGTGTGAGTGAAGATAAGAGTGAGCATCAACTGGCGTTAAGG
T V C L G N A A K D E F H I V
181 ACAGTATGCTTTGGGAATGCAGCAAAGGATGAGTCCACATAGTA
E I V P Q A V E G S D A K P V
226 GAAATAGTCCCTCAGCAGTAGAAGGAGTACGCAAAACCGGTT
P I A T L K P S I L P M A T M
271 CCTATTGCCACTAAAGCCATCTATTCTACCAATGGTACCATG
V G I E L T P P V T F Q L K S
316 GTGGCATTGAATGACTCCCTCCAGTCACATTCAGTGAAGTCT
G S G P I Y I S G Q H V A L E
361 GGAICTGGGCAATTTATATTAGTGGTGCAGCATGTTGCATTGGAA
A D Y S W A E E E G E E E V
406 GCAGACTACTCCTGGGGGAGGAGGAAGAAGGTGAGGAGGAGGTG
E E E E E E D P E S P P K P
451 GAAGAGGAAGAGGAGGAGGATCCAGATCCTCCAAAACCT
V K R P A A S K K A G Q A K K
496 GTAAAGAGGCCAGCAGCTTCCAAGAAGGACAGGCAAGAAAG
K K M E K D E E S S E E E D
541 AAGAAAATGAAAAAGATGAAGAGGAAAGTTCTGAAGGAGGAGC
S P A K K G K G R G R K P S K
586 AGTCCTGCCAAAAGGGCAAAGGAGGAGGAAAGCCCTCTAAG
K *
631 AAATGACTGCATGAAGTCTACCTTTTTTGTATTGCTCCAGTCT
676 GTATTTAATGCCAGGCTGTACTTGTATCCTTATACCTCACCA
721 CCTGCCACATGGACTACACAAGCAATGGAGATCTGTCCAGCTCA
766 AGATCAGTCTAAGATCTGTATTGTATCTTTTTTTGGCAATGT
811 ATTCGTAAAATAAAGCTTTTCTGTAGGAAAAAAAAAAAAA

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Figure 24. Coding nucleotide sequences of *B. marinus* and *R. catesbeiana* nucleoplasmin.

The nucleotide sequences determined in this study and translated protein sequences of nucleoplasmin cDNAs from A) *B. marinus* [GenBank:DQ340657] and B) *R. catesbeiana* [GenBank:DQ340656] are shown.

proteins encoded by the cDNAs shown in Figure 24 correspond to the main translated mRNA forms (results not shown).

The comparison of the amino acid sequences for the different amphibian nucleoplasmin proteins is shown in Figure 25. As it can be seen, the protein sequence is extremely conserved (approximately 75 % identity) among the different amphibian species analyzed. Hence, it is to be expected that these proteins all have a common closely related function across these species.

Amphibian nucleoplasmin is closely related to mammalian NPM2

It is possible to use the sequence information shown in Figure 25 in the context of all the nucleophosmin/nucleoplasmin metazoan sequences to determine the phylogenetic relationships of their main families: NPM1, NPM2, and NPM3 (Fig. 26). NPM1 includes a set of nucleoplasmin-like proteins, also referred to as nucleophosmin, which localize to the nucleolus of somatic cells and have been implicated numerous cellular processes including ribosome biogenesis (Busch et al., 1982), centrosome duplication (Okuda, 2002) and nuclear chaperoning (Okuwaki et al., 2001b; Szebeni and Olson, 1999). NPM1 variants have an important role in embryonic development and mutations of the genes encoding these proteins have been shown to be involved in acute myeloid leukemia (Grisendi et al., 2005). NPM2 and NPM3 are nuclear chaperone proteins. The latter is ubiquitously expressed in many tissues (Shackleford et al., 2001) in contrast to NPM2 which is only found in oocytes and eggs (Burns et al., 2003). Mammalian NPM2 has been shown to play a critical role in nucleolar and nuclear organization and plays an important role in the maintenance of the perinuclear heterochromatin which is usually observed in eggs and early embryos (Burns et al., 2003). Although *Npm3* antisense

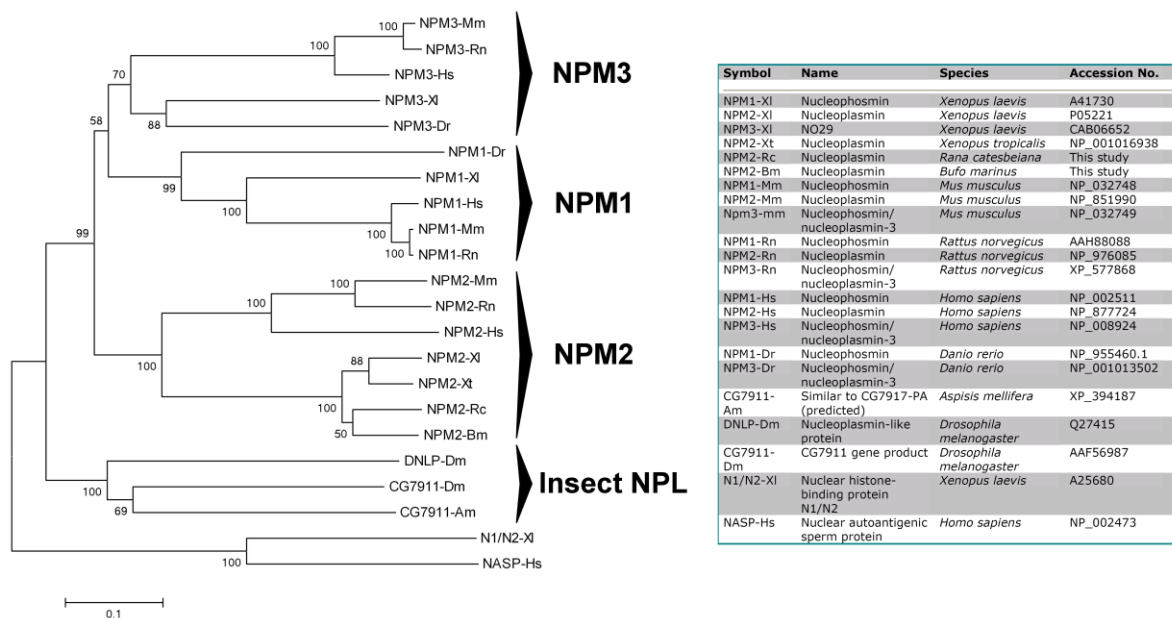


Figure 26. Phylogenetic tree of nucleophosmin/nucleoplasmin family members from various metazoans.

Amino acid sequences were aligned with CLUSTAL W (Thompson et al., 1994), and the tree was produced with MEGA 3.1 (Kumar et al., 2004) using the neighbor-joining method. Two other histone-binding proteins, NASP (Welch et al., 1990) and N1/N2 (Kleinschmidt et al., 1986), which are closely related to each other (Welch et al., 1990) but unrelated to the nucleophosmin/nucleoplasmin family were used to root the tree. Bootstrap significance values are shown at the corresponding internal nodes after 1000 replications.

oligonucleotides injected into mammalian oocytes significantly prevented histone assembly and male pronuclear formation (McLay and Clarke, 2003), the molecular components involved in the sperm chromatin remodelling after fertilization in mammals still remains largely unknown.

As shown by Figure 26 and not surprisingly, the four amphibian sequences cluster together in this analysis. They are in turn grouped with NPM2 opposed to family members in NPM1 and NPM3 or the insect nucleoplasmin-like proteins. Although amphibian nucleoplasmins group with mammalian NPM2 relative to other family members, they are in distinct clusters of the evolutionary analysis. In addition, broader evolutionary analyses (manuscript in preparation) reveal a polyphyletic origin for the NPM2 lineage, which maybe due to the differentiation between amphibians (SNBPs of H, PL, and P-types) and mammals (SNBPs of the P-type).

R. catesbeiana nucleoplasmin is mainly expressed in oocytes

In order to put the phylogenetic implications into a functional perspective, we made RNA extracts from different tissues of *R. catesbeiana* and the levels of expression of nucleoplasmin and nucleophosmin were assessed using dot blot Northern hybridization (Fig. 27). Of the three genera of amphibians studied here, we chose *Rana* for this analysis because we reasoned that the presence of canonical histones in the mature sperm of this organism makes it unlikely that the major function of nucleoplasmin is that of remodelling of the male pronucleus chromatin.

The results obtained are similar to those obtained with other vertebrate species. As expected from its generic participation in ribosome biogenesis, nucleophosmin was

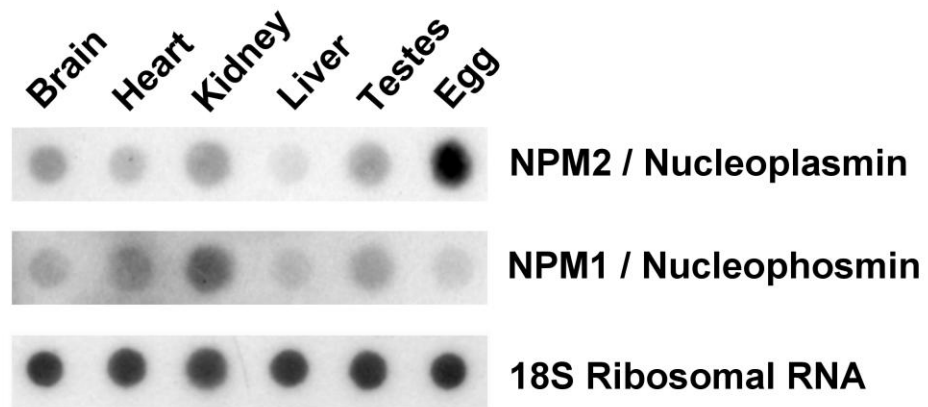


Figure 27. Northern dot blot hybridizations comparing Npm2 and Npm1 mRNA levels in different *R. catesbeiana* tissues.

In the top two rows 7.5 μg of total RNA was loaded per well and the blot was probed with P^{32} labelled *X. laevis* *Npm1* or *R. catesbeiana* *Npm2* cDNA amplified from PCR. In the bottom row 1.5 μg of total RNA was loaded per well and the blot was probed with a P^{32} labelled 18S ribosome cDNA probe which was used as a loading control.

widely distributed throughout the different tissues analyzed (Fig. 27) with different levels of expression which likely reflect their ribosome assembly nucleolar activity. In contrast, nucleoplasmin (Fig. 27) mRNA appeared to be primarily transcribed in the oocyte, a result that is consistent with the results reported for other species (Burns et al., 2003; Prado et al., 2004). Although some background is observed in the lane of dots corresponding to the nucleoplasmin tissue distribution, this appears to follow the same pattern of distribution observed for nucleophosmin and it is likely the result of the some cross hybridization of the nucleoplasmin and nucleophosmin probes.

Thus, like mammalian nucleoplasmin, amphibian NPM2 is predominantly expressed in the oocytes where it likely accumulates and persists throughout the first stages of early development (Burns et al., 2003). In the case of mammals, NPM2 participates in different roles from nucleolar organization to chromatin remodelling (Burns et al., 2003).

Discussion

The specificity of nucleoplasmin, within the functional context of its participation in the remodelling of sperm chromatin with different types of SNBPs (see Fig. 28), is unclear. The results described in the previous section show that within amphibians, nucleoplasmin is a highly conserved protein (Fig. 22 and Figs. 24-25) in contrast to the structural and compositional heterogeneity of the SNBPs (Fig. 21). This suggests that the removal of SNBPs is a highly non-specific process, a notion that is further reinforced by

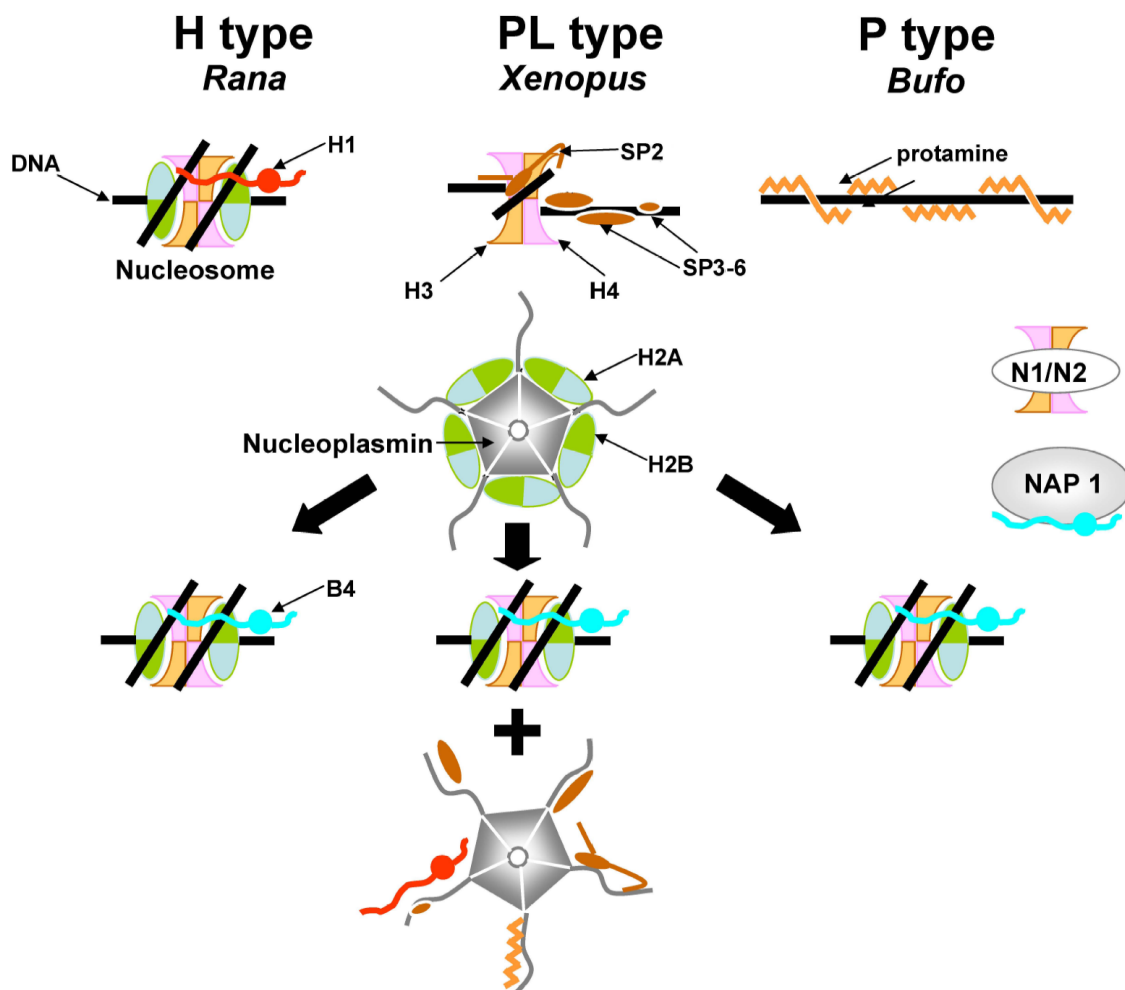


Figure 28. Schematic representation of the amphibian sperm chromatin remodeling by nucleoplasmin.

The chromatin structures corresponding to each of the different SNBP-types is schematically shown in the upper part of the figure. Upon fertilization of the egg, nucleoplasmin (shown here as a pentamer) stereo-specifically bound to histone H2A-H2B dimers exchanges these dimers with the SNBP components that become non-specifically (electrostatically) bound to the polyglutamic tracts of the unstructured C-terminal tails of the molecule. Note that only chromosomal proteins associated with the “linker-like” (non-helically constrained) domains of the sperm chromatin are extracted by nucleoplasmin which is highly phosphorylated at this stage of development (Ramos et al., 2005). Other nuclear chaperones are likely involved in the transition from the sperm chromatin to the male pronuclear chromatin. In this regard N1/N2 would be responsible for the assembly of H3/H4 (Kleinschmidt et al., 1985) and NAP-1 for the assembly of egg/early embryo-specific histone B4 like histone H1 molecules (Shintomi et al., 2005).

the observation that nucleoplasmin binds equally well to protamines (Prieto et al., 2002; Rice et al., 1995), protamine-like proteins (Prado et al., 2004; Ramos et al., 2005; Rice et al., 1995) and somatic linker histones (Ramos et al., 2005) *in vitro*. The non-specificity of the sperm chromatin remodelling process (Fig. 28) is also supported by the observations that different egg extracts appear to be interchangeable in terms of their ability to decondense sperm chromatin. (Philpott et al., 2000). In addition, the normal progression of sperm DNA decondensation in NPM2-null embryos in mice suggests that other related proteins (either NPM1 or NPM3) may even be able to compensate in this process (Burns et al., 2003). It is possible that the polyglutamic tracts of nucleoplasmin in conjunction with phosphorylation play a critical role in out-competing the electrostatic interaction between the highly positively charged SNBPs and DNA in sperm chromatin (Prado et al., 2004).

The lack of specificity with which nucleoplasmin removes SNBPs during the formation of the male pronuclei (Fig. 28) contrasts with the specificity implicit in the binding of H2A and H2B histones during the early stages of development of the zygote. While the molecule has a conserved binding site highly specific for histones (Dutta et al., 2001), which is most likely stereo-specific (Arnan et al., 2003) and involves the highly structured N-terminal part of the molecule (Dutta et al., 2001), the binding of SNBPs is quite non-specific and it probably involves the glutamic acid rich domains that become overexposed when the molecule becomes phosphorylated during oocyte maturation (Banuelos et al., 2003). Indeed, nucleoplasmin is found associated with H2A-H2B in the egg (Laskey et al., 1978), a property that originally led to the first time coining of the term molecular chaperone to describe this molecule (Dingwall and Laskey, 1990; Laskey et al., 1978; Philpott et al., 2000). Our results suggest that in addition to *X. laevis*, whose

sperm chromatin is deficient in H2A-H2B (Philpott et al., 1991), nucleoplasmin contributes the H2A-H2B dimers during the formation of the male pronuclei in other vertebrate organisms regardless of the initial SNBP composition of the male chromatin during fertilization. Other assembly factors such as N1/N2 (Dilworth et al., 1987; Kleinschmidt et al., 1990; Zucker and Worcel, 1990) and NAP1 (Shintomi et al., 2005) provide the H3-H4 and egg-specific linker histones (such as B4 (Smith et al., 1988) in *Xenopus*) respectively. This process continues throughout the assembly of chromatin that takes place during the cell divisions preceding the mid blastula transition (Philpott et al., 2000). Nucleoplasmin phosphorylation, which remains high until this stage, may facilitate the histone exchange between nucleoplasmin and the newly replicated chromatin and does not interfere with the H1 deposition that takes place downstream of the replication fork long after the nucleosomes have been assembled (Bavykin et al., 1993).

Finally, we have shown that nucleoplasmin shares a close phylogenetic relationship (Fig. 26) and similar tissue distribution (Fig. 27) to mammalian NPM2 (Burns et al., 2003). Not only this, but the genes encoding for these two proteins exhibit a highly complex similar organization which in the case of the amphibian counterpart spans over eight translated exons (Fig. 22). Thus, it is highly possible that like mammalian NPM2, amphibian nucleoplasmin has some additional chromatin remodelling functions during these early stages of development. All these observations (Fig. 22 and Fig. 26-27) also confirm that mammalian NPM2 is the genuine mammalian equivalent of amphibian nucleoplasmin.

Conclusion

Our results clearly show that nucleoplasmin is conserved between different amphibian species and its presence is independent of the SNBP-type. Although amphibian nucleoplasmin clusters with mammalian NPM2 in phylogenies, broader evolutionary analyses (Eirin-Lopez et al., 2006a) reveal a polyphyletic origin for the NPM2 lineage, which maybe due to the differentiation of SNPBs between amphibians (H, PL, and P-types) and mammals (P-type). Thus, whereas histone storage and exchange in early development represents a critical function of nucleoplasmin, the appearance of SNPBs of the H-type early in metazoan evolution, as well as their subsequent differentiation towards PL and P-types, could have been a strong enough functional constraint to recruit SNBP removal as an acquired non-specific function. This additional role became critical in animals as the evolution of SNPBs progressively led to the incorporation of more specialized PL and P proteins in sperm chromatin. In closing, nucleoplasmin can be defined as a non-specific ATP-independent sperm chromatin remodeller with a specific chromatin assembly activity that plays a critical role in chromatin metabolism during the early stages of development.

Overall Summary and Conclusion

Fish and amphibians are ideal models for the comparative study of chromatin remodelling by sperm nuclear basic proteins (SNBPs) because they include genera with representative of each of the three SNBP types described in Chapter 1. In this dissertation, organisms that contain histones in their sperm were focused on because the least amount of information on sperm chromatin compaction is available for them. The first organism investigated was *Xenopus laevis*, which has sperm containing high and low molecular weight PLs and a complement of the histones H3 and H4. The primary structure and the molecular mass determined for the large *X. laevis* PLs, SP1 and SP2, added to results of previous studies (Ariyoshi et al., 1994; Abe and Hiyoshi, 1991), indicated that the lower molecular weight SP2 protein results from a post translational cleavage at the N-terminal region of SP1 (Chapter 2). It was revealed that SP2 has a winged helix domain with two flanking N- and C- terminal intrinsically disordered regions. Furthermore, SP2 was able to form chromatosomes *in vitro*, a characteristic of the H1 family of proteins. Although SP2 is closely related to vertebrate H1x, its higher arginine content identifies this H1 histone as a highly specialized PL-I SNBP (Ausio, 1999). Further studies should focus on following the SP1-SP2 cleavage *in vivo* with antibodies to the N-terminal tail of SP1 and the core region of the protein. This would determine if the if the cleavage correlates with chromatin compaction as it does in invertebrate organisms. In addition, comparing the ability of SP1 and SP2 to condense reconstituted chromatin fibers *in vitro* would provide direct evidence.

In contrast to *X. laevis*, *Ictalurus punctatus* (Channel catfish) was shown to contain only somatic-like histone in the ripe testes (H-type) (Chapter 3). Despite the lack of specialized SNBPs the sperm chromatin was fully condensed. Nuclease digestion showed the testes chromatin was organized into a repetitive structure indicative of uniformly condensed chromatin. Several histone variants and post-translational modifications (PTMs) present in the testes were assessed by western blotting. Histone H3 was trimethylated at lysine 27, which is a well known marker of facultative heterochromatin, and histone H4 was phosphorylated at serine 1, which has been documented to affect nuclear size and may help stabilize chromatin compaction in mice and yeast. The histone variants, H2A.Z and H2A.X that are retained in mature human sperm were also present. Characterization of the ripe testes chromatin indicates that channel catfish is a good model organism for the study of H-type sperm chromatin. The western analysis expanded upon the small amount of data available on histone acetylation of H-type fish (Kurtz et al., 2009) by determining the presence of other PTMs and variants. However, there are many more histone variants and PTMs that are important for higher order chromatin structure and further studies are needed to assess their presence in Catfish. The impact these proteins and PTMs have on chromatin structure in H-type sperm is also needed.

The full sequence of a nucleoplasmin protein and its expression was determined for the first time in an H-type organism, *Rana catesbeiana* (Chapter 5). It was shown that nucleoplasmin is not only present in *R. catesbeiana* (H-type), *X. laevis* (PL-type) *Bufo marinus* (P-type) but also highly conserved among these three amphibians that represent each of the three SNBP types. The amphibian nucleoplasmins were shown to phylogenetically group with mammalian NPM2. The exact role played by nucleoplasmin

in H-type organisms has not been determined. It is likely involved in histone chaperoning after the first divisions that follow fertilization, although this hypothesis remains to be tested. Another NPM family member, NPM3, was recently shown to have a histone chaperoning function in the testes of mice where it may act as a chaperone for transition protein 2 (TP2) (Pradeepa et. al., 2009). The comparison of NPM histone chaperones could be extended to determine if NPM3 is present in the sperm of these three amphibians and if it acts as a histone chaperone.

In closing, the use of fish and amphibians to compare sperm chromatin remodelling in organisms with different SNBP types showed that despite the heterogeneity in SNBP composition many similarities still exist between example organisms of all three types. For instance, although H-type SNBPs lack the increased arginine content and reduced size seen in P- and PL-types, they are still able to fully compact sperm chromatin in a similar manner. The histone variants and PTMs present in channel catfish testes, which contain H-type sperm, were similar to those present in human sperm which contains protamines and only retains 15% histones. Similarities also exist following the remodelling process after fertilization as the histone chaperone nucleoplasmin is present and conserved at the protein level in amphibians of all three SNBP types.

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