Travaux du Muséum National d'Histoire Naturelle «Grigore Antipa»	Vol. XLVIII	pp. 531–540	© 30 Dec. 2005

CHROMATIN ARHITECTURE IN SPERMATOGENESIS OF SOME SPECIES OF AMPHIBIANS

LILIANA BURLIBAȘA, LUCIAN GAVRILĂ

Abstract. Using transmission electron microscopy (TEM) and molecular investigations, some peculiar aspects of chromatin and chromosome organization and evolution in spermatogenesis of amphibians were investigated. The investigations were focused on electrophoretic fractioning of sperm nuclear basic proteins from *Triturus cristatus*, *Xenopus laevis* and *Rana ridibunda*.

Résumé. En employant la microscopie électronique de transmission (TEM) et les techniques moléculaires, ont été étudiées quelques aspects particuliers de l'organisation de la chromatine et du chromosome et de l'évolution de la spermatogenèse des amphibiens. Les investigations ont été concentrés sur le fractionnement électrophorétique des protéines nucléaires basiques du tissu testiculaire de *Triturus cristatus*, *Xenopus laevis* et *Rana ridibunda*.

Key words: spermatogenesis, amphibians, sperm nuclear basic proteins, chromatin.

INTRODUCTION

During spermatogenesis, chromatin undergoes several dynamic transitions, which are often associated with important changes not only in its physical conformation but even in its composition and structure. In most instances, the compositional changes also alter the structure of chromatin. As a result, chromatin becomes highly compacted and gene expression is completely shut down in the spermatozoon. The ways in which all these molecular events are achieved can be mediated by a wide spectrum of apparently diverse chromosomal proteins (Bloch, 1969). The structure of chromatin conformation as a result of protein-DNA interactions in each particular situation is poorly understood in most instances, and the evolutionary relationship amongst these proteins remain obscure (Ausio, 1995).

Sperm nuclear basic proteins (SNBPs) that bind to DNA in animals and plants are highly diverse, in contrast to the evolutionary conservative nucleosomal histones that characterize all other cell types.

Further progress on the chemical characterization of the SNBPs revealed a large degree of compositional variability and structural heterogeneity (Subirana, J. A. et al., 1973), which contrasts with the evolutionarily conserved chemical nature and low structure variability of histones from somatic cells (Isenberg, 1978).

An early attempt to classify the SNBPs was carried out by Bloch (1969) who described among the following types: *Salmo* type (arginine rich proteins), mouse-grasshopper type (proteins containing – SH groups), *Mytilus* type (intermediate between histones and protamines), *Rana* type (histones) and crab type (no basic proteins, such that DNA appears essentially naked) (Fig. 1).

Bloch's classical paper underlined that "the variability (non-conservatorism) of the protein reflects an evolutionary indifference to a relatively unimportant protein in an inert nucleus" (Bloch, 1969). In the same paper, he also indicated that "...although a phylogenetic relationship is often apparent from the similarity of

proteins within tightly defined taxonomic groups (e.g. the clupeids, or the Eutheria), there seems to be no evolutionary trend. Most of the classes of sperm proteins are represented within most of the broad taxa" (Bloch, 1969). In fact, analyses of the distribution of SNBPs in animals indicates that the mode of fertilization acts as a constraint on SNBP diversity, such that either protamines, keratinous protamines or protamine-like SNBPs are present in sperm of taxa exhibiting interne fertilization (Tab. 1).

Table 1

Distribution of SNBPs in animal kingdom (modified from Kasinsky, 1995).

TAXON	SNBP type	ANIMAL GROUPS	FERTILIZATION	HABITAT
Phylum Chordata				
Subphylum Vertebrata				
Class Mammalia	KP (P1)	Infraclass Eutheria	Int	Ter, Mar
	KP (P1+P2a+P2b)	Order Primate	Int	Ter
	PL	Order Marsupalia	Int	Ter
Class Aves	P	Superorder Paleognathae	Int	Ter
Class Reptilia	PL	Order Squamata	Int	Ter
	PL	Order Testudines	Int	Ter
Class Amphibia	PL	Genus *Xenopus* + *Siluriana*	Ext	Ter
	PL	Genus *Bufo*	Ext	Ter
	PL	Genus *Litoria*	Ext	Ter
	H1	Genus *Ran*a	Ext	Ter
Class Osteichthyes				
Division Teleostei	PL	Genus *Gasterosteus*	Ext	Mar
	P	Subfamily Salmoninae	Ext	Mar
	H	Family Sparidae	Ext	Mar
Class Chondrichthyes				
Subcls.		Family	Int	Mar
Elasmobranchii	KP, P	Scyliorhinidae		
Subphylum	PL (P1)	Suborder	Ext	Mar
Urochordata		Phlebobranchiata		
	PL (P1,P2)	Genus *Styelidae*	Ext	Mar
Phylum Echinodermata				
Class Holothuroidea	H1, Φo	Genus *Holothuria*	Ext	Mar
Class Echinoidea	H1, H2B	Order Echinoida	Ext	Mar
Subcls. Asteroidea	H1	Order Forcipulata	Ext	Mar
Phylum Mollusca				
Class Polyplacophora	PL (P1, P2)	Genus *Mopalia*	Ext	Mar
Class Gastropoda				
Ord. Archaeogastropoda	PL (P2)	Family Trochidae	Ext	Mar
Ord. Patellogastropoda	PL (P1)	Family Lottiidae	Ext	Mar
Ord. Mesogastropoda	PL (P3)	Family Littorinidae	Int	Mar
Ord. Neogastropoda	PL (P3)	Family Nucellidae	Int	Mar
Class Bivalvia				
Subcls. Pteriomorphia	PL	Family Mytilidae	Ext	Mar
Subcls Heterodonta	PL	Family Tridacnidae	Ext	Mar
Class Cephalopoda	P	Order Teuthodea	Int	Mar

Abbreviations: Ext - external; Int - internal; Mar - marine; Ter - terrestrial

Based on current information, SNBPs can be grouped in three main categories: histone type (H type); protamine type (P type) and protamine-like (PL type) (Figs 1, 2).

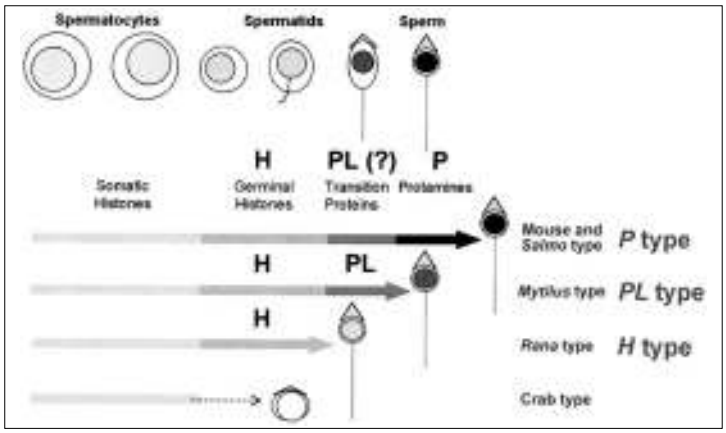

Fig. 1 – A diagram to illustrate the major SNBP types and SNBP transitions during spermatogenesis in different groups of metazoans (Ausio, 1999).

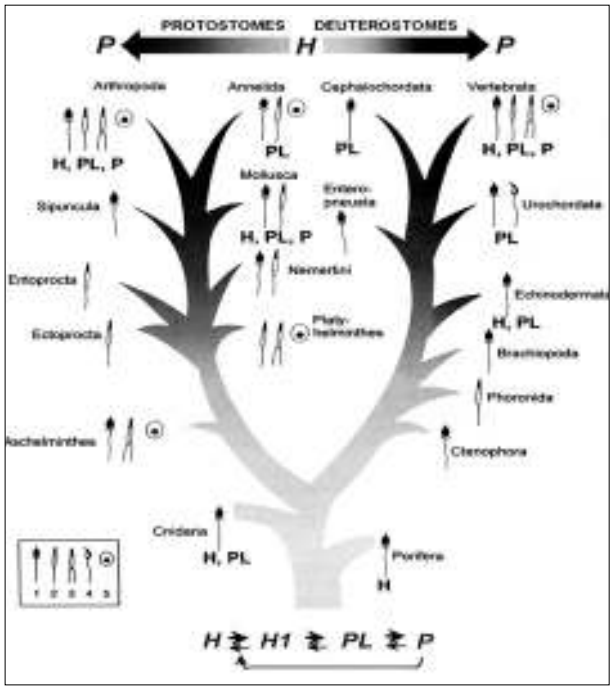

Fig. 2 - Schematic representation of the evolution of the major SNBP types. The basic pattern of evolution among the different SNBP types is shown at the base of the tree with the bold arrows. H, primitive histone protein precursor; H1 primitive sperm histone H1 precursor; P, arginine-rich protamine. The revers arrows indicate the existence of reversions among the different major SNBP types. 1-primitive sperm type; 2-modified sperm; 3-biflagellate sperm; 4-ascidian sperm type and 5-aflagellate sperm. The arrows at the top indicate the direction of the evolutionary trend from primitive histone protein to arginine-rich protamine in the protostome and deuterostome branches (Ausio, 1999).

The first group of SNBPs in this classification, histone type, basically corresponds to Bloch's *Rana* type. It consists of histones that are compositionally and structurally related to the histones that are found in the nuclei of somatic cells (Ausio, 1999). The protamine type is the second group of SNBPs, consists in arginine-rich highly basic proteins (Ausio, 1999). During spermiogenesis, these proteins replace the majority of the germinal somatic-like histones, and they are the main SNBPs found in mature sperm.

The third group of proteins of this classification is the protamine-like (PL) type, and is the most structurally heterogenous group. All of the proteins from this group are closely related to molecules from the histone H1 family (Ausio, 1995).

The information about the SNBP compositions from organisms occupying a lower position in the phylogenetic tree of tetrapods is still scarce. In this regard, in the present paper we analyze the type of SNBP of *Triturus cristatus* (Ord. Urodela, Cls. Amphibia, Supracls. Tetrapoda) by Urea - Acetic Acid-PAGE electrophoresis.

Chromatin organization resulting from the interactions between the PL-P type proteins with DNA is quite different. Although PL and P proteins usually coexist with a small amount of histone in the sperm nucleus (Avramova et al., 1984; Ausio, 1986; Gatewood et al., 1987), the structure of the nucleoprotein complexes arising from the interaction of these proteins with DNA lacks the nuclosomal organization of the somatic chromatin type, as can be vizualized by X-ray diffraction (Ausio & Subirana, 1982; Ausio & Suau, 1983). The overall negative superhelicity of DNA is lost, most likely as a result of the topoisomerase II activity associated with the histone displacement/replacement by these PL or P proteins. Thus, the nucleohistone-nucleoprotamine (protamine-like) transition leads to a complete reorganization of chromatin, while possibly maintaining the specific three dimensional organization of DNA and its DNA loop domain structure (Ausio, 1995). The detailed molecular structures of the nucleoprotein (P, PL) complexes are still unknown.

Our research was focused on chromatin organization and evolution in spermatogenesis of crested newt.

MATERIAL AND METHOD

Living organism

Male newt (*Triturus cristatus*), male *Rana ridibunda* and male *Xenopus laevis*.

Electron microscopy technique

Testes were dissected and fixed in 2.5%-5% glutaraldehyde in 0.1 M sodium cacodylate buffer and post-fixed in 1% osmium tetraoxide in the same buffer. They were then dehydratated in a graded ethanol series and embedded in Epon 812. The samples were sectioned on an ultramicrotome, stained in 4% aqueous uranyl acetate, post-stained with lead citrate and examined with a Philips 201 electron microscope.

Extraction of SNBPs

For extraction of SNBPs from *T. cristatus, Rana ridibunda* and *Xenopus laevis*, frozen testes from each animal were resuspended in aproximately 0.5 ml of 0.15M NaCl, 10mM Tris-HCl pH 7.5 buffer containing 0.2mM phenylmethyl-sulfonylfluoride PMSF. The samples were then homogenized in this buffer in a homogenizer. The homogenate was spun down for 10 min. at 16,000 g at 40C in a centrifuge. The pellets resuspended in the same buffer were spun down on a BSA gradient, according to a method described by Bellvé, 1993; Romrell et al., 1976. This technique allows germ cell separation according to their respective sizes. The sedimentation carried out on one adult newt testis allowed the separation of three cell suspensions enriched respectively in pachytene spermatocytes (P), in round and early elongating spermatids (RES) and in condensing spermatids and residual bodies (CS). The cell suspension was then filtered and centrifugated at 1000 rpm for 10 min. The cells were re-suspended in 18 ml of HAM F12/DMEM containing 0.5%

BSA and 1 g/ml DNase. The cell suspension was allowed to sediment through a 2-4% BSA gradient at 4°C during 70 min. Thirty-six fractions were collected, numbered 1 to 36 from the top of the gradient. Each sample was centrifuged at 1000 rpm for 10 min. Condensing spermatids (CS) were usually found in fractions 5-7. These fractions were re-suspended in the buffer containing 0.5 ml of 0.15M NaCl, 10mM Tris-HCl, 0.2mM phenylmethylsulfonylfluoride PMSF, 0.5% Triton X-100 and were homogenized and centrifuged for 10 min. at the 16,000g at 4°C. This step was usually repeted twice. The final pellets were resuspended in aproximately 0.8ml of 0.4 N HCl, homogenized and kept at 4°C with occasional stirring. The HCl extracts were next spun down at 16,000g in the Eppendorf microfuge for 10 min. The supernatants thus obtained were then precipitated with 5 volumes of acetone for 1-2 hr at –20°C. The tubes were centrifuged using the above procedure and the pellets were dried and then re-suspended in distilled water.

Electrophoretic fractioning of SNBPs

Urea (6.25M)-polyacrylamide gel electrophoresis (PAGE) was carried out on 15% slab gels, pH 3.2, with 0.9N acetic acid running buffer, according to Panyim & Chalkley (1969). Unfractionated SNBPs (1mg/ml) were disolved in tray buffer containing 2.5M urea and 5% glacial acetic acid (v/v). The gel was run at a constant voltage 10V/cm. After electrophoresis, gels were stained with 0.2% (w/v) Comassie blue in 25% (v/v) methanol/10% (v/v) acetic acid and destained in 10% (v/v) methanol/15% acetic acid (v/v). The gels were scanned with Jencons-PLS UVB Bioimaging System.

RESULTS AND DISCUSSIONS

Using TEM analysis, some unusual aspects of chromatin and chromosome organization and evolution in spermatogenesis of the amphibian *Triturus cristatus* were evidenced.

The DNA within the sperm nucleus is organized into a genuine three-dimensional conformation due to replacement of histones with another class of nuclear proteins, namely nuclear sperm-specific proteins. Comparing the nucleosomal binding pattern of somatic histones with the binding pattern of nuclear sperm-specific proteins localized in the minor groove of the DNA double helix, one can notice that nuclear sperm-specific proteins ensure an almost complete covering of the DNA, with very few uncovered areas. Thus, in contrast to the somatic cell nucleus, in the sperm nucleus the nucleosomal structure as well as the negative superhelicity are lost, the nucleoprotein complex acquiring a special physical conformation with very important functional consequences (Nishi et al., 1994).

The chromatin condensed stage at the level of spermatids and spermatozoon is the highest known stage in nature, and actually, it represent an adapting process regarding the fulfilment of genetic material transport function of spermatozoon avoiding the loos of genetic information (Figs 3, 4).

Chromatin becomes highly compacted and gene expression is completely shut down in the spermatozoon. Chromatin compactation and the replacement of histones with sperm specific proteins may be gradual, involving discrete steps evidenced by transmission electron microscopy (Figs 5, 6, 7). Chromatin condensation helps to streamline by reducing volume. It also serves a protective function, reducing the susceptibility of the DNA to mutation or physical damage.

Fig. 3 – Transverse sections through spermatids, showing urodele features of a longitudinal juxta-axonemal fibre, a long undulating membrane, and mitochondria (X18 430).

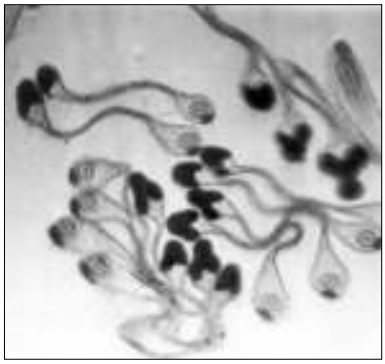

Fig. 4 – The ultrastructural image of sperm head (X 23214).

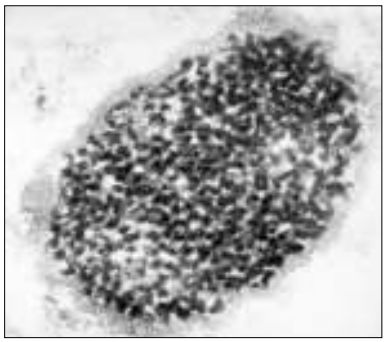

Fig. 5

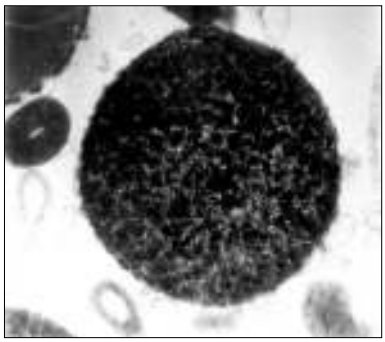

Fig. 6

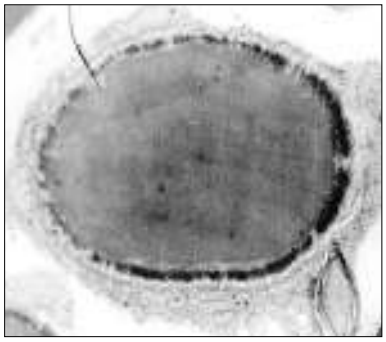

Fig. 7

Figs 5-7 Intermediate stage of chromatin condensation in the transition from telophase II to spermatids, in *Triturus cristatus* (X 73 670).

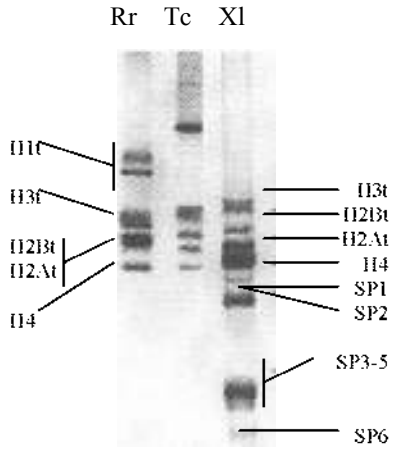

Fig. 8 – Urea (6.25M)-polyacrylamide gel electrophoresis (PAGE) of SNBP from *Rana ridibunda* (Rr), *Triturus cristatus* (Tc) and *Xenopus laevis* (Xl).

Condensation is facilitated by formation of specific DNA-complexes. Proteins that may be involved include protamines (small, highly basic, arginine-rich proteins) protamine-like proteins or other sperm-specific proteins. The type of sperm-specific protein on *Triturus* is unknown.

One of the major objectives of our research was to isolate and electrophoreze fractioning of SNBP in the species *Triturus cristatus* comparing with the *Rana* type histones and *Xenopus* sperm nuclear basic proteins (Fig. 8).

Based on this electrophoregram, it appears that the *Triturus cristatus* sperm nuclear basic proteins are intermediate between *Rana* histones (Bloch, 1969) and *Xenopus* protamine-like proteins with low molecular weight (Ausio, 1999).

The electrophoregram indicates the presence of *Triturus cristatus* SNBP (Tc line) in the form of four bands, three of them having the same electrophoretic mobility as the Arg-rich histones, similar to *Rana* and *Xenopus* Arg-rich fraction (histone core somatic-like) and a band of lower mobility, indicating a particular sperm nuclear basic protein. The PL-I (or H1 sperm specific proteins) proteins are considered to derive from the Lys-rich H1 linker histone (Ausio, 1995) (Fig. 9). Testes specific histone proteins, with lower molecular weight are also present, which are equivalent to the H3, H2A and H2B histones that are not replaced in the final stages of spermatogenesis. The electrophoretic results indicated that, in *Triturus cristatus*, SNPB are represented by PL-I proteins (protamine-like proteins with high molecular weight).

The PL-type represents an intermediate type both from the structural and functional point of view. Whereas protamines have only been found at the tips of the most evolved groups from both the protostome and deuterostome branches, PL proteins are already found in the eukaryote groups preceding this branching. The intermediate structural features of the PL proteins, must reflect their intermediary positions. *Rana* sp. (Ord. Anura) is a special case to H type reversion (Kasinsky et al., 1999).

It was hypothesized that proteins of the protamine type (P type) have evolved from a primitive somatic-like histone precursor via a PL-type intermediate through a mechanism of vertical evolution (H $\rightarrow$ PL $\rightarrow$ P) (Subirana et al., 1973).

The detailed molecular structures of the nucleoprotein (protamine P and protamine-like PL) complexes are still controversial. Both PL and P proteins

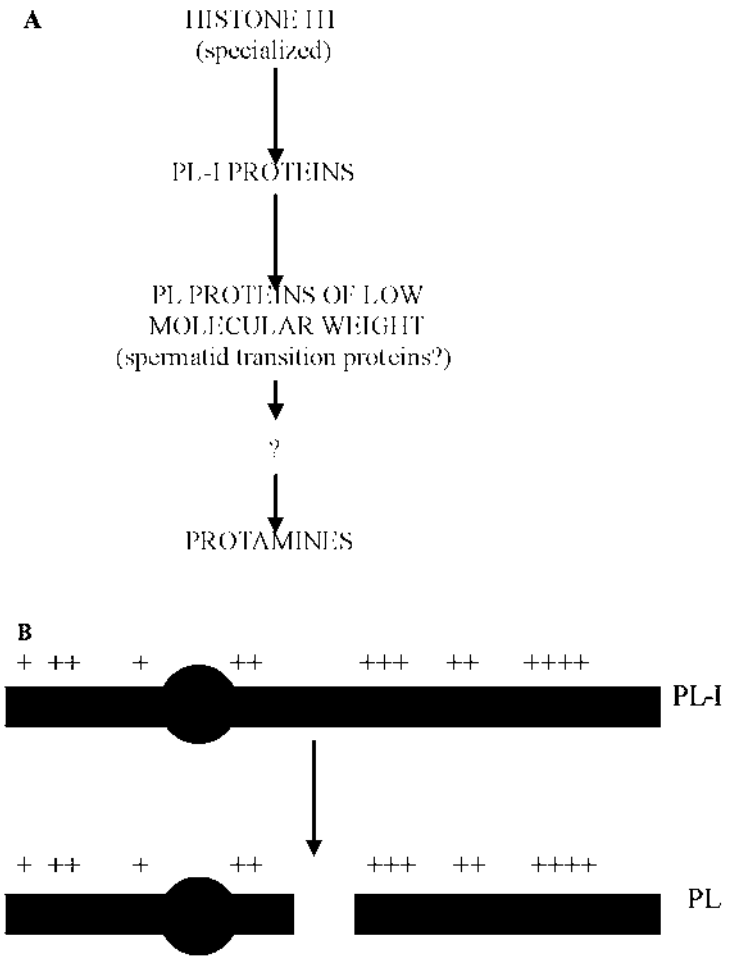

Fig. 9 – A: Proposed evolutionary relationship for the nuclear sperm specific proteins. B: Some of low molecular weight PL proteins might have arisen from post-translational cleavage of a larger PL-I precursor (Ausio, 1995).

interact electrostatically with DNA (which basically retains a B conformation) to form fully saturated complexes, unlike the somatic nucleohistone. In these complexes, the PL and P proteins have been postulated to adopt an - helical like configuration.

At the higher order level of organization, it has shown (Casas et al., 1993) that PL proteins, like H type proteins, can organize the nucleoprotein complexes into 250-500 Å fibres regardless of the particular PL composition and the absence of nucleosome-like structures. This is an important finding because it indicates that the higher order structures of the nucleoprotein complexes are mainly determined by the ionic nature of interaction involved, rather than the particular of the proteins itself.

Conclusions

Spermatogenesis is a complex differentiation process which is characterized, among other features by conspicuous stage-specific nuclear events such as the pairing of homologous chromosomes, the replacement of histones with sperm-specific proteins during spermiogenesis and, as a result, chromatin condensation and its inactivation in sperm cells. TEM analysis has revealed that the chromatin of spermatogenic cells undergoes structural rearrangements upon differentiation from spermatogonia to mature spermatozoa. Our preliminary analysis has revealed that a primitive H1-like-PL-I protein is the major chromosomal protein found in the sperm nucleus of *Triturus cristatus* (Ord. Urodela).

ARHITECTURA CROMATINEI ÎN SPERMATOGENEZA UNOR SPECII DE AMFIBIENI

REZUMAT

Spermatogeneza este un proces complex ce implică multiplicarea celulelor stem germinale, diferențierea lor în spermatocite, diviziunea meiotică şi, în final, transformarea acestora în spermatozoizi maturi. Toate aceste procese necesită remodelarea structurii cromatinei la nivel local şi global, aceasta realizându-se prin intermediul unei cascade de evenimente transcripționale şi reglatorii. În timpul progresiei spermatogenezei are loc o reorganizare a genomului haploid urmată de o compactare extremă a ADN.

Utilizând microscopia electronică de transmisie şi tehnicile moleculare au fost investigate unele aspecte particulare ale organizării cromatinei în spermatogeneza amfibienilor. Autorii şi-au concentrat cercetările asupra fracționării electroforetice a proteinelor nucleare bazice specifice țesutului testicular la *Triturus cristatus*, *Xenopus laevis* şi *Rana ridibunda.*

LITERATURE CITED

AUSIO, J., 1986 - Structural variability and compositional homology of the protamine-like components of the sperm from the bivalve molluscs. Comparative Biochemistry and Physiology, 85B: 439-449.

AUSIO, J., 1995 – Histone H1 and the evolution of the nuclear sperm-specific proteins. *In:* Advences in Spermatozoal Phylogeny and Taxonomy. Mémoires du Muséum National d'Histoire Naturelle, Paris, 166: 447-462.

AUSIO, J., 1999 - Histone H1 and Evolution of Sperm Nuclear Basic Proteins. Journal of Biological Chemistry, 274 (44): 31115-31118.

AUSIO, J., P. SUAU, 1983 - Structural heterogeneity of reconstituted complexes of DNA with typical and intermediate protamines. Biophysical Chemistry, 18: 257-267.

AUSIO, J., J. A. SUBIRANA, 1982 - Nuclear proteins and the organization of chromatin in spermatozoa of *Mytilus edulis.* Experimental Cell Research, 141: 9-45.

AVRAMOVA, Z. V., A. O. ZALENSKY, R. TSANEV, 1984 - Biochemical and ultrastructural study of the sperm chromatin from *Mytilus galloprovincialis*. Experimental Cell Research, 152: 231-239.

BELLVE, A. R., 1993 - Purification, culture and fractionation of spermatogenic cells. Methods in Enzymology, 225: 84-113.

BLOCH, D. P., 1969 - A catalog of sperm histones. Genetics, suppl., 61: 93-111.

CASAS, M. T., J. AUSIO, J. A. SUBIRANA, 1993 - Chromatin fibers with different protamine and histone compositions. Experimental Cell Research, 204: 192-197.

GATEWOOD, J. M., G. R. COOK, R. BALHORN, E. M. BRADBURY, C. W. SCHMID, 1987 - Sequence-specific packing of DNA in human sperm chromatin. Science, 236: 962-964.

ISENBERG, I., 1978 - The Cell Nucleus, 4, Part A: 135-154. H. Busch (ed), Academic Press, New York.

KASINSKY, H. E., L. GUTOVICH, D. KULAK, M. MACKAY, D. M. GREEN, J. HUNT, J. AUSIO, 1999 - Protamine-like sperm nuclear basic proteins in the primitive frog *Ascaphus truei* and histone reversions among more advanced frogs. Journal of Experimental Zoology, 284: 717-728.

NISHI, N., S. TOKURA, T. ARAMAKI, K. IWATA, K. FUKUE, K. NITTA, S. NISHIMURA, Y. OKAMOTO, M. TSUNEMI, 1994 - DNA-binding mode of protamine: investigation by differential circular dichroism. Peptide Chemistry, 1993: 285-288.

PANYIM, S., R. CHALKLEY, 1969 - High resolution acrylamide gel electrophoresis of histone. Archives of Biochemistry and Biophysics, 130: 337-346.

ROMRELL, L. J., A. R. BELLVE, D. W. FAWCETT, 1976 - Separation of mouse spermatogenic cells by sedimentation velocity. A morphological characterization. Developmental Biology, 49: 119-131.

SUBIRANA J. A., C. COZCOLLUELA, J. PALAU, M. UNZETA, 1973 – Protamines and other basic proteins from spermatozoa of mollusks. Biochimica et Biophysica Acta, 317: 346-379.

Received: March 2, 2005
Accepted: May 9, 2005

Universitatea București, Facultatea de Biologie,
Aleea Portocalelor nr. 1-3, Sector 6,
060101, *București, România*
e-mail: trili_poli@yahoo.com