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# Molecular and ultrastructural studies of the sperm chromatin from *Triturus cristatus*

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## Summary

The study of nuclear molecular architecture during gametogenesis represents one approach towards the deciphering of the molecular organization of eukaryotic chromatin. 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. Dynamic alterations in chromatin structure mediated by postsynthetic histone modification and DNA methylation constitute a major regulatory mechanism of gene function of eukaryotes. Using transmission electron microscopy and molecular investigations, some peculiar aspects of chromatin organization and evolution in spermatogenesis of the crested newt *Triturus cristatus* were investigated. We focused our investigations on the dynamics of chromatin structure after treatment with TSA (a histone deacetylase inhibitor).

Keywords: Chromatin, DNA methylation, Histone acetylation, Sperm nuclear basic proteins (SNBPs)

## Introduction

The DNA of eukaryotic organisms is associated with a number of basic proteins in a macromolecular complex termed chromatin. At the structural level, the most important function of this assembly is to compact the lengthy DNA molecule inside the limited available nuclear space. In somatic cells, chromatin is a dynamic structure, because DNA must be accessible for replication, transcription and repair. DNA is packaged in these cells via assembly of core histones, linker histones and non-histone proteins. However, during spermiogenesis (a complex process in which spherical spermatids become mature spermatids, which are released at the luminal free surface as spermatozoa) there is a dramatic remodelling of chromatin that is characterized by considerable cellular morphological change, concurrent with modifications in the nature and content of the nuclear basic proteins. In most instances, the composition of the chromosomal proteins at the onset and in the final stages of spermatogenesis is quite different. As a result, chromatin becomes highly compacted and gene expression is completely shut off in the spermatozoon.

Sperm nuclear basic proteins (SNBPs) exhibit a very complex evolutionary pattern resulting from the combination of a discrete number of protein motifs that seem to repeat themselves within different phylogenetic taxa (Ausio, 1995; Chiva *et al*., 1995; Rocchini *et al*., 1996). These protein motifs can be basically represented by three different types: H type (histone), P type (protamine) and PL type (protamine-like) (Bloch, 1969). The last type consists of SNBP with compositional and structural characteristics intermediate between the H and P types (Ausio, 1995). These three types of proteins are sporadically but non-randomly distributed throughout the animal kingdom (Saperas *et al*., 1994; Ausio, 1999) with the protamine type being predominantly distributed among the organisms at the upper

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phylogenetic levels of the deuterostome and protostome branches (Kasinsky, 1989; Ausio, 1995; Rocchini *et al*., 1996).

The information about SNBP composition from organisms occupying a lower position in the phylogenetic tree of tetrapods is still scarce. In the present paper we analyse the type of SNBP of *Triturus cristatus* (order Urodela, class Amphibia, supraclass Tetrapoda) by polyacrylamide gel electrophoresis (PAGE) electrophoresis.

Chromatin is organized in discrete nucleosome subunits. In the nucleosome, about 200 base pairs of DNA are wrapped in approximately two negative superhelical turns about a histone core octamer (consisting of 'core histones' H2A, H2B, H3 and H4). Thus, DNA is stabilized by nucleosomes in negative supercoiled state, which poises eukaryotic chromatin for genetic activity (replication, transcription). In addition to the 'core histones', 'linker histones' (proteins of the H1 histone family) bind to the linker DNA regions connecting the neighbouring nucleosome subunits and condense chromatin into higher-order structure fibres of about 30 nm in diameter. Although electrostatic interactions play an important role in maintenance of this organization, only about half of the negatively charged DNA phosphates are neutralized by the arginine/lysine side chains. This makes the nucleohistone complex very sensitive to environmental ionic conditions and amenable for interaction with other regulatory proteins (Ausio, 1995).

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 nucleosomal organization of the somatic chromatin type, as can be visualized 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.

The mechanisms of histone displacement are not known in detail, but there could be a link between the extensive acetylation of histones in elongating spermatids and the subsequent replacement of histones by protamines or protamine-like proteins in condensing spermatids. In order to gain an insight into the mechanisms controlling histone hyperacetylation/histone replacement during spermiogenesis, we treated spermatogenic cells with a histone deacetylase inhibitor, trichostatin A (TSA).

## Materials and methods

### Living organism

The male newts (*Triturus cristatus*) were obtained from Bucharest Botanical Garden Pool, University of Bucharest. As control the males of mouse *Mus musculus* were used.

### 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 tetroxide in the same buffer. They were then dehydrated 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.

### TSA treatment

Spermatogenic cells from the whole testis were incubated in TSA (100 ng/ml, diluted in Ham F12/DMEM) for 3 h at 35 °C (Hazzouri *et al*., 2000).

Male adult of *Triturus cristatus* was intraperitoneally injected with TSA (100 IU/10 g body weight from stock solutions 10 mg/ml).

### Extraction of SNBPs

For extraction of SNBPs from *T. cristatus*, as from the control mammal *Mus musculus*, frozen testes from a single animal were resuspended in aproximately 0.5 ml of 0.15 M NaCl, 10 mM Tris-HCl pH 7.5 buffer containing 0.2 mM phenylmethylsulfonylfluoride (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 4 °C in a centrifuge. The pellets resuspended in the same buffer were spun down on a bovine serum albumin (BSA) gradient, according to a method previously described (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 (respectively, mouse) 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 centrifuged at 1000 rpm for 10 min. The cells were resuspended in

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18 ml of Ham F12/DMEM containing 0.5% BSA and 1 mg/ml DNase. The cell suspension was allowed to sediment through a 2–4% BSA gradient at 4 °C for 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 resuspended in buffer containing 0.5 ml of 0.15 M NaCl, 10 mM Tris-HCl, 0.2 mM PMSF, 0.5% Triton X-100 and were homogenized and centrifuged for 10 min at 16 000 *g* at 4 °C. This step was usually repeated twice. The final pellets were resuspended in aproximately 0.8 ml of 0.4 N HCl, homogenized and kept at 4 °C with occasional stirring. The HCl extracts were next spun down at 16 000 *g* in an Eppendorf microfuge for 10 min. The supernatants thus obtained were then precipitated with 5 volumes of acetone for 1–2 h at –20 °C. The tubes were centrifuged using the above procedure and the pellets were dried and then resuspended in distilled water.

### Electrophoretic fractionation of SNBPs

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

### DNA isolation from testes

Each sample (100 mg of *T. cristatus* testicular tissue) was suspended in microfuge tubes with 300 ml of digestion buffer (100 mM NaCl, 50 mM Tris-HCl, 1% SDS, 50 mM EDTA, pH 8.0). Proteinase K (10 mg/ml) was added to a final concentration of 100 mg/ml and the samples incubated for 2 h at 50 °C, then overnight at 37 °C. After digestion was complete, an equal volume of 5 M LiCl was added to each tube. The sample was mixed thoroughly by inversion for 1 min, then 600 ml of chloroform was added and the samples placed on a rotating wheel for 30 min. The samples were spun for 15 min at high speed in a benchtop microfuge and the supernatant was carefully removed to a new microfuge tube. Exactly 2 volumes of room temperature absolute ethanol were added and the tube inverted several times until the DNA precipitated. The samples were then resuspended in TE buffer.

### DNA digestion

DNA was digested with isoschizomere endonucleases *Msp*I and *Hpa*II. After DNA purification, *Msp*I, *Hpa*II, enzyme buffer, BSA (stock concentration 5 mg/ml) and distilled water were added to a final volume of 45 ml. Each of the samples was incubated overnight at 37 °C. The samples were run in 2% agarose gel electrophoresis.

## Results and discussion

During spermatogenesis, chromatin from gonadal stem cells undergoes several dynamic transitions, which are often associated with important changes in its physical conformation, composition and structure. Chromatin becomes highly compacted and gene expression is completely shut down in the spermatozoon. Condensation is facilitated by the formation of specific DNA–protein complexes. Proteins that may be involved include sperm nuclear basic proteins (SNBPs). Chromatin compaction and the replacement of histones with sperm-specific proteins may be gradual, involving discrete steps evidenced by transmission electron microscopy (Fig. 1).

The chromosomal proteins in spermatozoa – SNPBs – have great structural variability. Significant progress has been made in the last 10 years in the physical and chemical description of these proteins in a variety of organisms, although this aspect has never been studied in certain groups of animals, such as amphibians of the order Urodela.

Despite their enormous structural variability, the nuclear sperm-specific proteins can be grouped, from the compositional point of view, into three basic categories. Type H (histone type) consists of lysine-rich proteins with amino acid compositions that, although specific for the germ line, are structurally and compositionally similar to those of the somatic histone type. This grouping is equivalent to the *Rana* type of Bloch's classification. (Bloch, 1969). At the other end of this classification, type P (protamine type) consists of proteins of low molecular mass (4000–10 000 daltons) that are arginine-rich (arginine content 60%). The PL type (protamine-like type) consists of proteins with an amino acid composition intermediate between the H and P types. The arginine and lysine content usually amounts to at least 35–50% of their amino acid composition, but occasionally it can be higher. Although the ratio of these two amino acids may vary over a wide range, it usually stays constant for a given taxonomic group (Ausio, 1995). In most instances, these proteins can be structurally related to histone H1 (Ausio, 1995). In the final stages of spermatogenesis, PL proteins exhibit a significant degree of structural heterogeneity,

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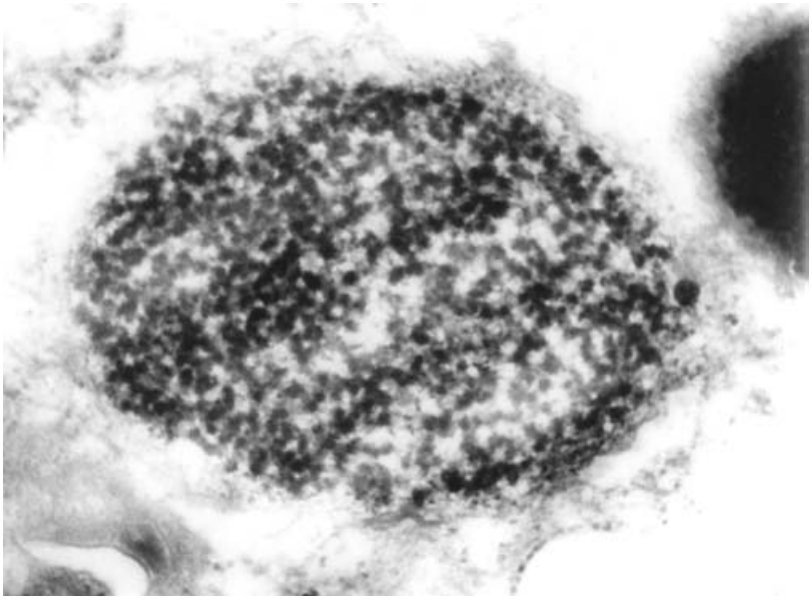

**Figure 1** Intermediate stage of chromatin condensation in the transition from telophase II to spermatids, in *Triturus cristatus* (magnification × 23 214).

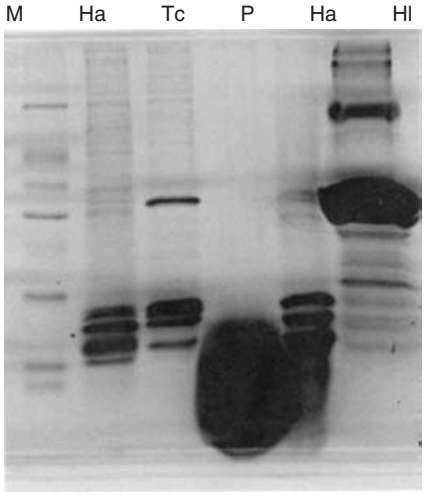


**Figure 2** Urea (6.25 M)–polyacrylamide gel electrophoresis (PAGE) of SNBP from *Triturus cristatus* (Tc). M, molecular weight marker; P, protamine marker; Ha, arginine-rich histone marker; Hl, lysine-rich histone marker.

with molecular masses in the 5000–30 000 dalton range. This group corresponds to the intermediate proteins (*Mytilus* type) of Bloch's classification (Bloch, 1969).

One of the major objectives of our research was the isolation and electrophoretic fractionation of SNBP in *Triturus cristatus* (Fig. 2). For electrophoretic analysis we uses a lysine-rich histone marker (f1 fraction), an arginine-rich histone fraction (f3 fraction) and protamine (P type from salmon sperm – salmine).

The electrophoregram indicates the presence of *Triturus cristatus* SNBP (Tc line) in the form of three bands having the same electrophoretic mobility as the arginine-rich histones, similar to the f3 (H3) fraction (with molecular weights ranging from 14 200 Da to 20 100 Da), and a band of lower mobility, corresponding to the lysine-rich histone marker, similar to the F1 (H1) fraction, with a molecular weight close to 26 000 Da. The reference literature presents the PL-I (or H1 sperm-specific proteins) proteins as deriving from the lysine-rich H1 linker histone. Arginine-rich histone proteins are also present, which are equivalent to the H3 histone – a histone that is not replaced in the final stages of spermatogenesis. The electrophoregram results indicated that, in *Triturus cristatus*, SNPBs are represented by PL-I proteins, similar to the histone fraction H1, the same SNBP type also being found in the genus *Rana* of the order Anura. Considering the information in the reference literature (Kasinsky, 1995) regarding the SNBP types present in certain representative taxa (Table 1), it was determined that the representative SNBP types in taxa with external fertilization include H, H1 (PL-I) and PL.

The precise mechanisms of histone replacement and the signals triggering such replacement in advanced phases of spermatogenesis are still uncertain. One possibility is the generation of a more hydrodynamic sperm head that speeds the transit through the female reproductive tract and across the zona pellucida surrounding the egg (Braun, 2001). It may also be that the nucleoprotamine/nucleoprotamine-like structures protect the genetic material in the sperm head from physical and chemical damage. Alternatively, packing of sperm chromatin may serve to reprogramme the paternal genome (Braun, 2001). Developmental reprogramming of the parental genomes occurs during egg

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**Table 1** The SNBP types present in certain representative taxa, their fertilization type and habitat

| 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 (PL-I) | Genus *Rana* | Ext | Ter |
| Class Osteichthyes | | | | |
| Division Teleostei | PL | Genus *Gasterosteus* | Ext | Mar |
| | P | Subfamily Salmoninae | Ext | Mar |
| | H | Family Sparidae | Ext | Mar |
| Class Chondrichthyes | | | | |
| Subclass Elasmobranchii | KP, P | Family Scyliorhinidae | Int | Mar |
| Subphylum Urochordata | PL (P1) | Suborder Phlebobranchiata | Ext | Mar |
| | PL (P1,P2) | Genus *Styelidae* | Ext | Mar |
| **Phylum Echinodermata** | | | | |
| Class Holothuroidea | H1, $\Phi_0$ | 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 | | | | |
| Order Archaeogastropoda | PL (P2) | Family Trochidae | Ext | Mar |
| Order Patellogastropoda | PL (P1) | Family Lottiidae | Ext | Mar |
| Order Mesogastropoda | PL (P3) | Family Littorinidae | Int | Mar |
| Order Neogastropoda | PL (P3) | Family Nucellidae | Ext | Mar |
| Class Bivalvia | | | | |
| Subclass Pteriomorphia | PL | Family Mytilidae | Ext | Mar |
| Subclass Haterodonta | PL | Family Tridacnidae | Ext | Mar |
| Class Cephalopoda | P | Order Teuthodea | Int | Mar |

Modified from Kasinsky (1995).
Int, internal; Ext, external; Ter, terrestrial; Mar, marine.

and sperm formation. However, the direct relationship between chromatin repackaging in sperm and developmental reprogramming is unknown. Fluorescence *in situ* hybridization indicates that chromosomes are not packaged haphazardly into sperm, and in humans, where about 15% of the DNA remains packaged in nucleohistones, it seems that specific sequences remain bound by histones (Gatewood *et al*., 1987). Are the transition proteins (PL) and protamines directly involved in developmental reprogramming?

Researchers have analysed the genomic DNA methylation level in spermatogonia and spermatids (Gama-Sosa *et al*., 1983; Grunau, 1999), obtaining variable, even contradictory, results that can be explained by the limitations imposed by such an approach and by the extremely low amounts of DNA extracted and analysed by the HPLC method. Stanford *et al*. applied the method of restriction with isoschizomere enzymes (Msp/Hpa) and reached a more accurate conclusion regarding the high methylation of mature sperm. Monk (1990; Zuccotti & Monk, 1995), using the same method to determine the level of methylation in mouse oocytes, sperm and embryo, confirmed that the level of methylation in sperm DNA is lower than in somatic tissues.

Apart from the methylation of DNA, histone acetylation is another epigenetic modification that could modulate the chromatin conformation and, as a result, the genetic activity.

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All these chemical changes in the chromatin components (histones and DNA) are important for chromatin remodelling, which is closely related to gene expression.

As the information in the literature regarding the epigenetic modifications in advanced stages of spermatogenesis is unclear and contradictory, we considered it appropriate to analysis the effect of a histone deacetylase inhibitor, trichostatin A (TSA), at the cytological level (by transmission electron microscopy, TEM) and at the molecular level (DNA methylation level, presence of SNBP).

The ultrastructural analysis of newt (*Triturus cristatus*) testis tissue incubated in TSA (100 ng/ml diluted in Ham F12/DMEM) for 3 h at 35 °C revealed significant chromatin remodelling in sperm nuclei. Decompaction of the sperm nuclei occurs and electron-dense areas appear that can be interpreted as condensations of chromatin for restoring chromosomal structures (Fig. 3). This chromatin remodelling was produced by treatment with TSA, which inhibits histone deacetylases in nanomolar concentrations, the hyperacetylation resulting in chromatin relaxation and modulation of genetic expression.

PAGE in 15% polyacrylamide gel of the SNBPs extracted from testis tissue incubated with TSA, as well as from testis tissue taken from an adult male *T. cristatus* that had been intraperitoneally injected with 100 IU TSA (from the stock solution of 10 mg/ml) revealed the importance of the chemical modification of histones (i.e., deacetylation) in the replacement of histones by sperm-specific proteins. The absence of the band corresponding to PL-I was noted in both cases (Fig. 4).

More than 30 years ago, Vincent Allfrey proposed that histone acetylation was associated with transcriptional activity in eukaryotic cells (Allfrey *et al*., 1964; Pogo *et al*., 1966). Acetylation occurs at lysine residues on the amino-terminal tails of the histones, thereby neutralizing the positive charge of the histone tails and decreasing their affinity for DNA (Hong *et al*., 1993). Histone acetylation changes the structure of chromatin, as demonstrated by changes in chromatin folding (Ausio & Van Holde, 1986; Fletcher & Hansen, 1995; Garcia-Ramirez *et al*., 1995; Tse *et al*., 1998; Wang *et al*., 2001) and susceptibility to nucleases (Litt *et al*., 2001). Recently, Eden *et al*. (1998) proposed that DNA hypermethylation could cause a decrease in the level of histone acetylation. The idea was supported by Nan *et al*. (1998) and Jones *et al*. (1998). Under the circumstances, we also proceeded to study the impact of chemical modifications as the histone acetylation/deacetylation on the DNA.

We started by isolating DNA from the testicular tissue of a normal *T. cristatus*, used as control, as well as from TSA-incubated testicular tissue of *T. cristatus*. After purifying the DNA, we performed restriction with *Msp*I/*Hpa*II isoschizomere enzymes to detect the level of methylation. The analysis of the electrophoretic profiles of DNA restricted with *Msp*I/*Hpa*II revealed a global demethylation of the DNA in the testicular tissue incubated with TSA (Fig. 5). This indicates the

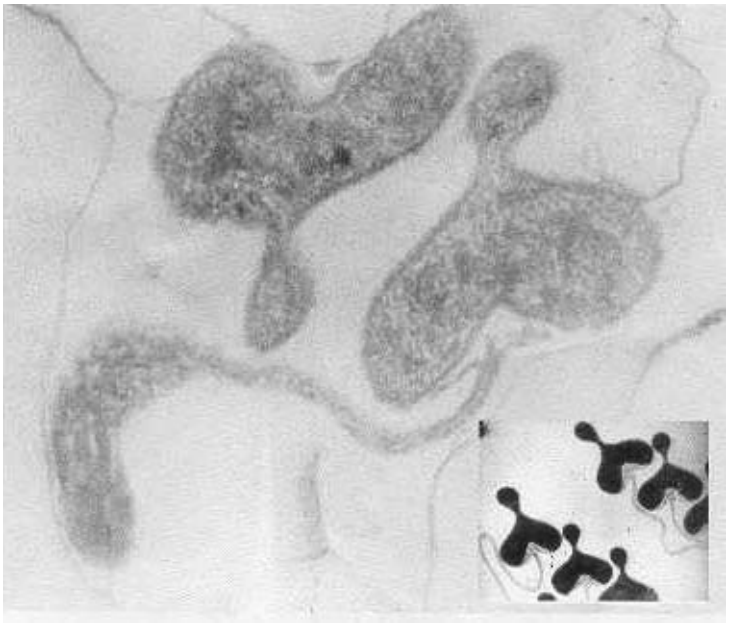

**Figure 3** Transverse section through a *Triturus cristatus* sperm head after the incubation of testes with the histone deacetylation inhibitor TSA. The figure at the bottom right (Gavrila & Mircea, 2001) is an ultrastructural image of a sperm head without TSA incubation. The difference in electron density between the sperm nuclei is clear (magnification × 30 412).

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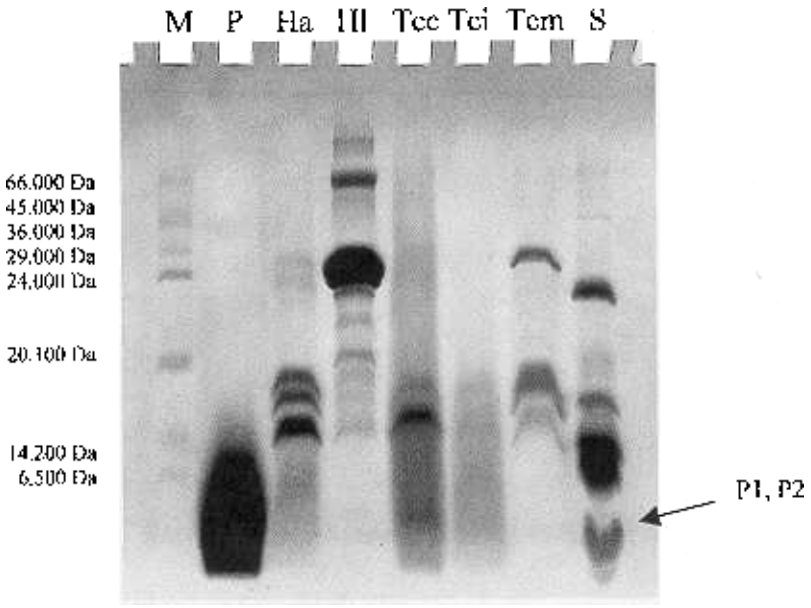


**Figure 4** Urea (6.25 M)–polyacrylamide gel electrophoresis (PAGE) of SNBP from *Triturus cristatus* (Tcm) martor (without TSA treatment); Tcc, SNBP from *T. cristatus* testes after incubation with TSA; Tci, SNBP from *T. cristatus* injected with TSA; S, SNBP from mouse testes without TSA treatment; P1 and P2, the two protamines from mouse testes; M, molecular weight marker; P, protamine marker; Ha, arginine-rich histone marker; Hl, lysine-rich histone marker.


**Figure 5** Electrophoregram of restricted DNA isolated from control newt testes (A, B) and from newt testes after TSA treatment (C, D).

close connection between the chemical modification of histones and the variations in DNA methylation level.

TSA inhibits the histone deacetylases, producing a hyperacetylated status of histones in the spermatozoon chromatin. This status is likely to prevent the replacement of histones by SNBP. The obvious connection between the acetylation/deacetylation of histones and the methylation/demethylation of the DNA is difficult to explain. Most probably, the histone deacetylase interacts with the methyl-binding proteins (MeCP).

## Conclusions

The results of the sperm-specific protein electrophoregram indicated that, in *Triturus cristatus*, the SNPB are represented by PL-I proteins similar to the histone fraction H1, the same type of SNBP also found in the genus *Rana* of the order Anura.

The ultrastructural analysis of the newt (*Triturus cristatus*) testicular tissue incubated in TSA (a histone deacetylase inhibitor) evidenced major chromatin remodelling in the sperm nuclei. The decompaction of the sperm nuclei was noted at the electron microscopic level, together with the appearance of an electron-dense area that could be interpreted as chromatin condensations meant to restore the chromosomal structures.

The electrophoretic analysis of the SNBPs extracted from the testicular tissue incubated with TSA indicated the absence of sperm-specific proteins. Thus, the regulation of histone deacetylase activity/concentration appears to play a major role in controlling histone hyperacetylation and probably histone replacement during spermiogenesis. Histone deacetylation could be interpreted as an early signal of histone replacement by highly basic sperm-specific proteins and also tightly linked to nuclear condensation.

The analysis of the electrophoretic profiles of DNA restricted with *Msp*I/*Hpa*II isoschizomere enzymes

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showed a global demethylation of the DNA in the TSA-incubated testicular tissue. This is an indication of the close connection between the chemical modification of histones and variations in the DNA methylation level.

Modulation of the chromatin structure during spermatogenesis requires changed patterns of histone proteins and histone modifications which contribute to restructuring of chromatin and to transition towards the inactivation of the genome in the generation of the condensed genome of mature sperm.

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