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CHANGES IN PROTEIN COMPOSITION IN THE SPERMATIC CHROMATIN OF THE CEPHOLOPOD *Sepia officinalis*

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Abstract

Much variation has been characterized among species at different levels in the maturation process of male haploid germ cells known as spermiogenesis. In the cuttle-fish, two protein transitions occur to displace histones causing morphological changes in the nucleus, from a round nuclear form with granular chromatin to an elongated nucleus with fibrillar chromatin, which eventually merges to a condensed, homogeneous mass. A basic nuclear protein called precursor protamine, which later converts into protamine, displaces histones playing a role in the morphological changes in nuclear form and chromatin condensation that occur in the male germ cells of *Sepia officinalis*. By using immunolocalization techniques observed in the Transition Electon Microscope, it appears that amounts of histone H4 begin to diminish once fine fibers form, around stage three of spermiogenesis. Precursor protamine appears significantly from the second phase of spermiogenesis, playing an important role in the formation of fine chromatin fibers from granular chromatin. In this investigation, results show that in the phases of chromatin maturation in spermatogenesis, histone H4 is present from the initial stages until thick fibers are formed; precursor protamine is present in the transition from granular to fine fibers of chromatin, and its presence increases greatly once clearly defined fibers are formed in the process of spermiogenesis in *Sepia officinalis*.

Key words Spermiogenesis, protamine, histone, immunolocalization.

INTRODUCTION

In spermiogenesis, morphological changes have been observed in the male haploid gamete, leading to an elongated and chromatin dense nucleus reduced in volume. The granular chromatin from the round nucleus converts into long, dense fibers in an elongated nucleus, which later changes into a pole shaped nucleus which has completely dense and compacted homogenous chromatin (Ribes *et al.*, 2001). These changes in nuclear form and chromatin condensation can be attributed to a progressive change in the composition of the proteins which interact with the DNA in the sperm nucleus (Ribes *et al.*, 2004). Among organisms, there is a great variability in nuclear protein transitions during spermiogenesis. The general case is that histones are substituted by sperm nuclear basic proteins, or SNBP (Ribes *et al.*, 2004). Many forms of SNBP's exist. For example, a direct replacement of histones by protamines occurs in the spermatic chromatin of vertebrates such as trout (Iatrou and Dixon, 1978). In other species of invertebrates, both histone and histone-like proteins are present in the sperm chromatin, such as that of some echinoderms like sea urchin (Sellows and Kmeicik, 1985). Cases of multiple protamine variants also exist, as in octopus spermatic chromatin (Giménez-Bonafé *et al.*, 2004) while in other organisms protamine-like proteins exist in chromatin of late spermatozoa, increasing in quantity throughout the stages of spematozooan chromatin development, such is the case in *Haliotis asinina* (Suphamungmee *et al.*, 2005). Cases also exist of linker histone vari-

ants replacing similar linker histones during spermatid differentiation (Manochanter *et al.*, 2005).

Two major protein transitions occur in the chromatin composition during spermiogenesis in *Sepia officinalis* (Wouters-Tyrou *et al.*, 1991), acting to replace the presence of the histones in the nucleosome. First, a protamine precursor, existing of two protein variants and comprised of highly basic amino acids and rich in arginines appears to promote a progressive condensation of chromatin (Wouters-Tyrou *et al.*, 1991). In its initial stages, the precursor is tri-phosphorylated at serine and threonine residues, as well the carboxy-terminal domain which, following cleavage of the amino terminal domain, comprises the protamines derivative of the corresponding precursor form. This second protein transition occurs at the end of elongation of the nucleus (Rousseaux-Prévost *et al.*, 1988) and is found in only the most mature and compact spermatozoid chromatin (Wouters-Tyrou *et al.*, 1991).

Our intent in this investigation is to study the stage of chromatin maturation associated with these protein transitions, which seem to function in causing the morphological changes and chromatin condensation observed in the nucleus of the male haploid germ cells during spermiogenesis. *Sepia officinalis* was chosen as a model to study these changes in nuclear protein composition in which there occur two protein transitions (Rousseaux-Prévost *et al.*, 1988). The differentiated neuroendocrine organ, internal fertilization, and the presence of all types of germinal cells in the testes of this animal make the cuttle-fish a good model to study, as it is suggested that there exists a close relationship of *Sepia officinalis* to other cephalopod and higher invertebrates (Rousseaux-Prévost *et al.*, 1988).

MATERIALS

Mature and immature male cuttle-fishes were obtained from of the Mediterranean coast of Cataluña, Spain. Gonads were dissected from fresh animals, and treated according to future use of the tissues, either frozen directly or fixed in the proper fixing buffer.

METHODS

Nuclear purification and protein extraction

Epidiydimus and testes tissues of *Sepia officinalis* were homogenized in ice-cold buffer, containing 10 mM Tris, 0.15 M NaCl, and 25 mM benzamidine chloride, a protease inhibitor, and then centrifuged for 5 minutes at 5000 rpm. The pellets were rehomogenized in a buffer containing 0.3 M sucrose, 20 mM Tris/HCl pH 7.5, with 2.5 mM calcium chloride, 25 mM benzamidine chloride, with 0.5% Triton-X 100. The solution was settled 10 minutes on ice, centrifuged again, and the pellets homogenized a third time in the same buffer without Triton X, and then centrifuged. The nuclei were washed with 1 mM Tris/HCl pH 7.5 and 1 mM EDTA. The solution was centrifuged and homogenized with 5 volumes of 0.4 N HCl on ice. The solution was centrifuged again 5 minutes at 10,000 rpm at 4° C. The supernatant was precipitated with 6 volumes of acetone at −20° C. The solution was centrifuged at 8,000 rpm at 4° C. The proteins were recovered in the precipitated sediment and washed in acetone and dried in a concentrator (Speedvac).

Electrophoresis

For analysis of proteins a 15% acrylamide-aceticacid-6M urea gel was used, due to insolubility of protamines in SDS-containing buffers. Protein samples were dissolved in samle buffer containing 8 M urea, 5% acetic acid, 20 mM beta-mercaptoethanol, and 1% methylene green. Electrophoresis was performed at 150 V at constant voltage, with the cathode at the bottom of the gel, for 2 hours, using 5% acetic acid as the running buffer. The gel was stained with 0.25% Coomasie Brilliant Blue R-250 in 50% methanol and 10% acetic acid until clearly stained bands appeared, followed by destaining in 5% methanol and 7% acetic acid until the contrast between gel and protein bands was easily apparent.

Western Blot analysis

Western blotting was performed to insure antibody specificity for the three proteins being studies. Proteins were separated by gel electrophoresis as previously described. Proteins were transferred to a nitrocellulose membrane at 400 mA for 45 minutes at 4° C. using 7% acetic acid, 30% methanol as the transfer buffer. The membranes were blocked with 3% powdered condensed skim milk, or with 5% milk, 1% BSA (as was the case in the immunoblot for precursor protamine detection) diluted in PBS pH 7.4 for 1.5 hours, and then incubated with the primary antibody overnight at 4° C in the same block that was used to block the membrane. The dilutions of the antibodies were 1 ug/ml for anti-H4, 6 ug/ml for anti-H1, and 1:1000 for the serum containing anti-precursor protamine. The membranes were washed several times in distilled water, then incubated with the appropriate HRP-conjugated antibody, either anti-mouse, in the case of anti-H1, or anti-rabbit antibody, in the cases of anti-H4 and anti-precursor

protamine. Secondary antibody incubations, in dilutions of 1:5000 were for 2.5 hours at room temperature, followed by extensive washing of the membrane in distilled water, then a was with PBS pH 7.4 containing 0.005% Tween-20, and final washing with distilled water before revealing the membrane with ECL in a dark room.

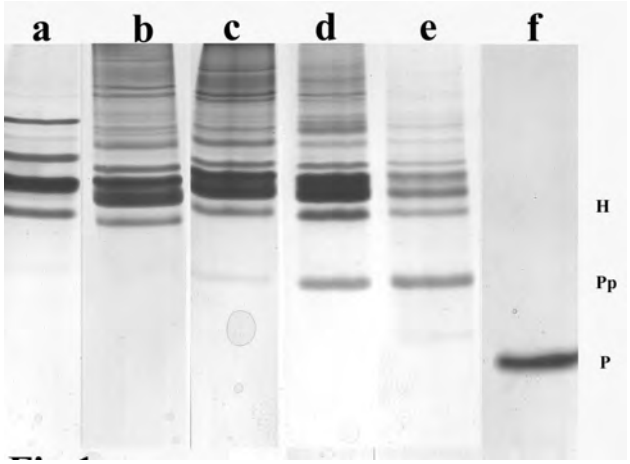

Figure 1 Change in nuclear proteins during spermiogenisis of *S. officinalis* on 15% polyacrylimide/ acetic acid/ urea gels. **Lane *a*:** Histone standard from lamprey. Electrophoretic pattern of nuclear proteins contained in the nuclei of male gonads from cuttlefish, of increasing maturity (lanes *b*-*e*). **Lane *b*:** The most immature testes show this pattern of histone proteins, and no band of precursor protamina. The comparison of lanes b trough e shows that the histone content develops from being mostly histone proteins, to a lesser protein density of histones with the appearance of precursor protamine. The intermediate phase of testes maturity shows a band of more dense precursor protamine, which continues to intensify in the most mature phases, while the histone bands fade in density. **Lane *c*:** The thick histone bands indicate the nuclei from this male gonad are immature, and the faint precursor protamine band (Pp) indicates that the nuclei from which these proteins were extracted are more mature than those shown in lane *b*. **Lane *d*:** The gonad is a bit more mature than that used for extracts shown in lane *c*. The amount of histones is similar based on the intensity of the bands, yet there is noticeably a higher quantity of precursor protamine, due to the more intense band at the migration point of this protein. **Lane *e*:** nuclear extract from testes shows lesser contents of histones (H), and a strong apparent band of precursor protamine (Pp), marking the testes from which the nuclear proteins were extracted as the most mature, due to its protein pattern. **Lane *f*:** Nuclear extract from the epididymus of *Sepia officinalis*, showing only protamine (P). The protamine protein migrates further in the gel, corresponding to the lower molecular weight once the amino terminus has been cleaved from the precursor.

Tissue preparation for immunolocalization

Testes were obtained from sexually mature cuttle-fish. Pieces of testes 1.5 mm in diameter were fixed in a 1% glutaraldehyde plus 4% paraformaldehyde buffer and embedded in Lowicryl resin.

Immunolocalization

Lowicryl embedded ultrathin sections of the testes were obtained and mounted on 300-meshnickel grids. The applications of various solutions to the tissue sections were done by floating the grids, tissue-side down, on drops of relevant solutions. The sections were first blocked for 30 minutes in 0.1 M PBS pH 8.1 with 1% BSA, in the case of localizing H4, or with 0.1 M PBS pH 8.1 with 4% BSA, 1% goat serum, and 20 mM glycine for localization of precursor protamine. The primary and secondary antibodies were diluted in 0.1 M PBS pH 8.1 with 1% BSA in the case of localizing H4 and with 0.1 M PBS pH 8.1 with 4% BSA, 1% goat serum, containing 0.01% Tween-20 for H1 and precursor localizations. The primary antibodies were applied to at concentrations of 4, 6, and 9 ug/ml in the case of H4 and at dilutions of 1:600 and 1:1000 for the precursor antibody. Negative controls were done on trials with each of the three antibodies used to in the localizations with the step of the primary antibody omitted and incubation of this stage was with the antibody diluting solution alone. Further negative controls were done with the same treatments, but with epididymus tissues instead of testes being mounted on the grid. Following primary antibody incubation, the grids were washed several times with the solution used to dilute the antibodies. The secondary antibodies, goat-anti rabbit in the case of precursor and H4, conjugated with gold of 15 nm, was applied at a dilution of 1:25. Following incubation with the secondary antibody, a series of extensive washing was done with 0.1 M PBS pH 8.1, followed by several washes with distilled water before being dried. The sections were counterstained with uranyl acetate and lead citrate and then washed in distilled water and dried. Ultrathin sections were examined for immunogold labelling by TEM.

Electron microscopy

Testes and epididymus from *Sepia officinalis* were pre-fixed in 2.5% glutaraldehyde-paraformaldehyde in 0.1% acodylate buffer (pH 7.3) and then post fixed in 1% osmium tetroxide (in 0.1 M cacodylate buffer). After fixation the tissues were dehydrated and embedded in Spurr's resin. Ultrathin sextions were stained with uranyl and lead citrate and examined in a Hitachi

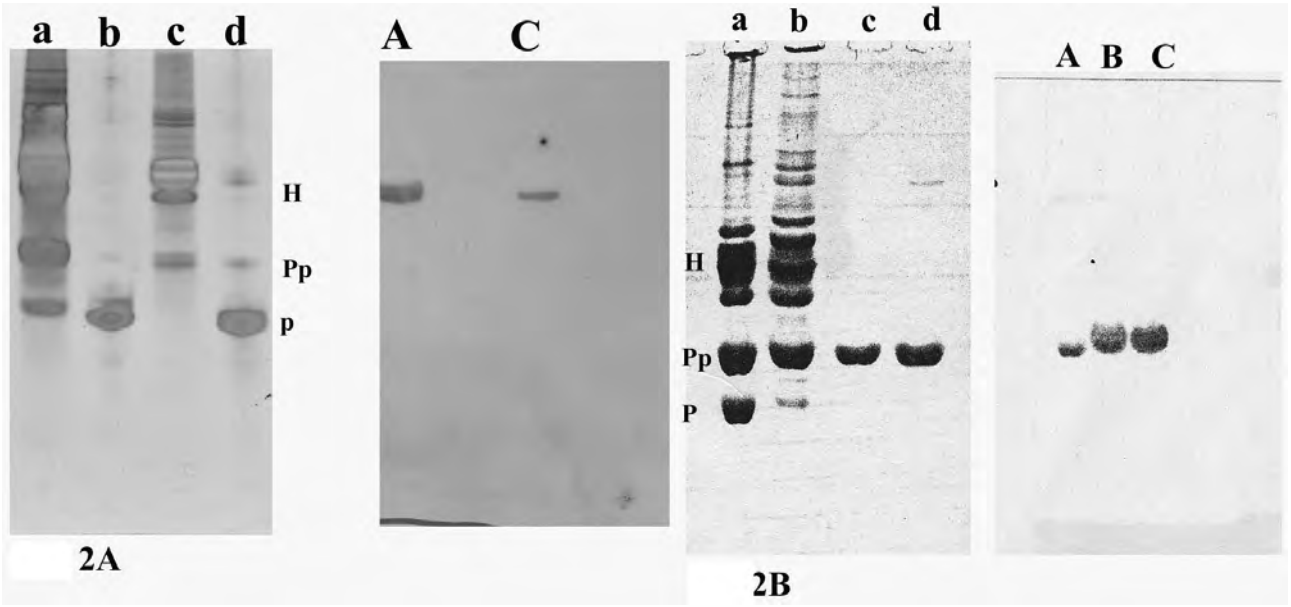

Figure 2 **Figure 2A.** Western Blot analysis of anti-histone H4. **Lanes *a-d***: 15% polyacrylamide/ acetic acid/ urea gel transferred to nitrocellulose membrane used for blot. **Lane *a*:** nuclear extracts from mature gonad of cuttlefish. **Lane *b*:** nuclear extracts from epididymus of cuttlefish. **Lane *c*:** nuclear extracts from mature gonad of sepiola. **Lane *d*:** nuclear extracts from epididymus of sepiola. **Lane *A*:** revealed film, showing antibody binding to the site on the membrane corresponding to the migration of histone H4 from sepia. **Lane *C*:** Label from anti-H4 antibody binding to the site of migration of the histone H4 in the sepiola. The marking of the antibody at these two points only shows specificity of the antibody for only histone H4. **Figure 2B.** Western Blot analysis of anti-precursor. **Lanes *a-d*:** Acetic acid urea gel of sperm nuclear proteins. **Lane *a*:** sperm nuclear extracts from squid. **Lane *b*:** Sperm nuclear extracts from cuttlefish. **Lane *c*:** Precursor protamine, isoform P2. **Lane *d*:** Precursor protamine, isoform P1. **Lanes *A-B*:** Revealed film from Western Blot with anti-precursor protamina. **Lane *A*:** Antibody reactivity to squid precursor protamine. **Lane *B*:** anti-precursor activity to precursor from cuttlefish. **Lane *C*:** Anti-precursor antibody reactivity to precursor protamine extract, isoform P1. Western blotting proves that the antibody specificly recognizes the precursor protamina, in its P1 isoform.

H-600 transmission electron microscope (Ribes *et al.*, 2001)

RESULTS AND DISCUSSION

Basic nuclear proteins

The observed proteins from nuclear extracts of testes from cuttle-fish at various stages of maturity show that in the most initial stages of spermiogenesis, containing mostly spermatogonia, primary and secondary spermatocytes, and round spermatids, only histones in the nuclei of the cells are present in the electrophoretic pattern (see figure 1). The migration of the histones can be observed from top (anode) to bottom (cathode). A slight trace of precursor protamine was observed in the nuclear extract of immature gonads. In intermediately mature gonads, the precursor band is slightly more intense. Finally, in mature gonads, a heavy precursor band is noticed along with the histones. According to the maturity of the male gonad from the sepia used to extract the nuclear proteins, the quantity of precursor protamine in the nuclear extracts vary. Gonads with more nuclei in elongation begin to demonstrate precursor protamines, and elongated and electodense nuclei will show protein extracts of precursor, although it is unclear if the cleavage of the precursor into the protamina occurs in the gonad, or in the epididymus.

Western Blot analysis

Western Blotting analysis proved that the antibody to histone H4 was very specific in recognizing its antigenic site (see figure 2*a*). Since H4 is the most evolutionarily conserved histone, it makes sense that this antibody easily recognizes the antigenicity of H4 and easily maintains the specificity it has for its epitope on this histone.

The precursor antibody was generated against the most initial phase of the precursor protamine, the phosphorylated form. Therefore, this antibody recognizes the most initial phases of the first protein transitions that take place in the cuttle-fish male germ cell nucleus

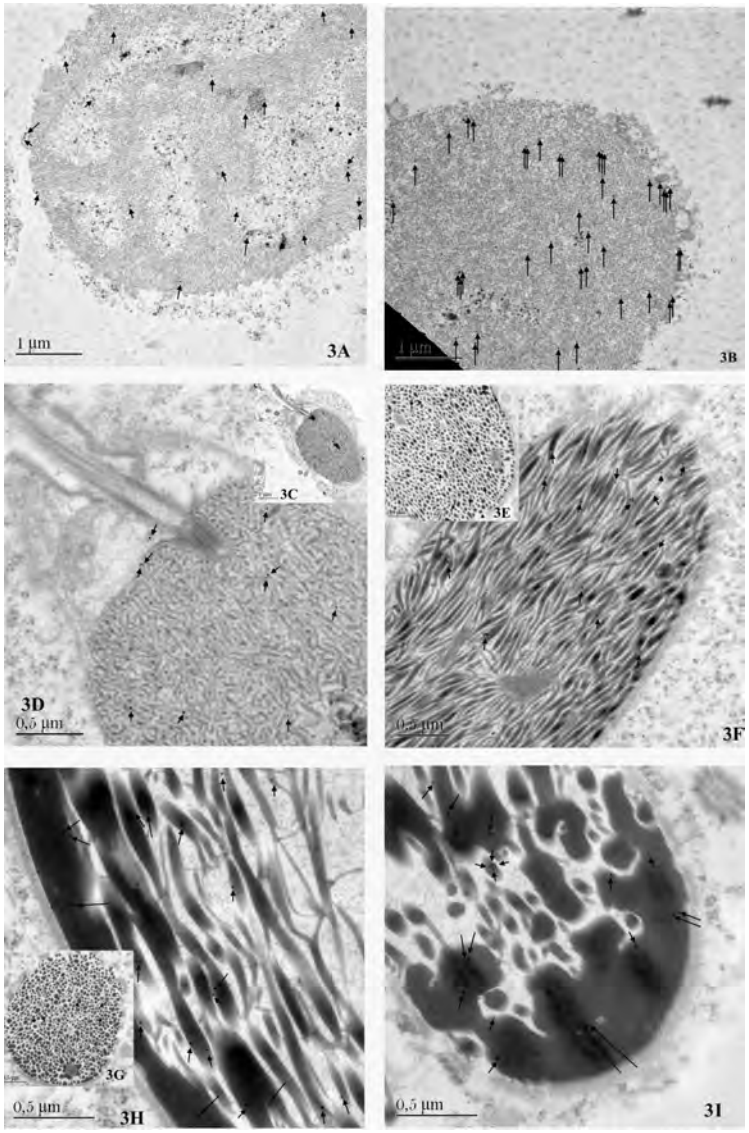

Figure 3 Transmission electron microscopy images from *Sepia officinalis* spermiogenesis, demonstrating the H4 immunolabeling at six of the phases described in spermiogenesis. **3A:** spermatocite, the most initial phase of spermiogenesis, where euchromatin and heterochromatin coexist in the nucleus. Immunogold labels of 15 nm are difficult to see without the use of amplification of the image by using tools of a computerized program. The arrows were placed on labels identified with the help of Photoshop to amplify the image, allowing the ability to distinguish where the labels were located in the nucleus. **3B:** Early round spermatid, phase 2 of spermiogenesis. The chromatin is a homogenous mass of granular heterochromatin, and the nucleus maintains it round shape. **3C:** Spermatid nucleaus at phase 3, showing the morphology of the fine chromatin fibers which have begun to form from the granules of chromatin it the previous phase. The nucleus is beginning to elongate in form. The invagination of the developing flagella is another identifying characteristic of this phase. **3D:** Detail of the nucleus in phase 3 around the base of the invagination of the flagella where mitochondria have accumulated, a characteristic of a nucleus in this phase. The thin fibers show a comparable amount of labeling in relation to a similar small surface area a nuclei of phase 2. **3E:** Inset showing the transversal view of a nucleus at the fourth stage of sperm maturation. Notice the thicker chromatin fibers. Less labelling is noticed, due to the lesser exposition of surface area of chromatin fibers in comparison to that of a lateral view. **3F:** Lateral cut of an elongating spermatid in phase 4 of spermiogenesis. The chromatin is now organized in long thick fibers and the nucleus is stretching out in shape. Initiating from this phase there are noticeably fewer labels marking antibody binding once negating the unspecific binding of the secondary antibody observed in this phase (see figure 3.1 *A*). When subtracting the unspecific labeling on dense chromatin, it is evident that the labeling from phase 4 to the end of spermiogenesis is due to the tendency of the secondary antibody to unspecificly bind to electrodense chromatin, and the lableing does not actually signify the presence of H4 (see figure 3.1). **3G:** Transversal view of the thick fibers, characteristic of a nucleus in stage 5. Little marking is shown. **3H:** Detail of an elongated spermatid. The thick fibers from the previous phase have merged into fewer, thicker and denser fibers, and the nucleus has further elongated in form. **3I:** Elongated spermatid with homogenously condensed chromatin. The chromatin dense, thick fibers from the previous phase have now begun to attach to the surrounding nuclear envelope for further diminution of the nuclear volume; the nuclear form is now very elongated.

during spermiogenesis, and immunoblotting indicates that it is specific for the precursor protamines (see figure 2*b*).

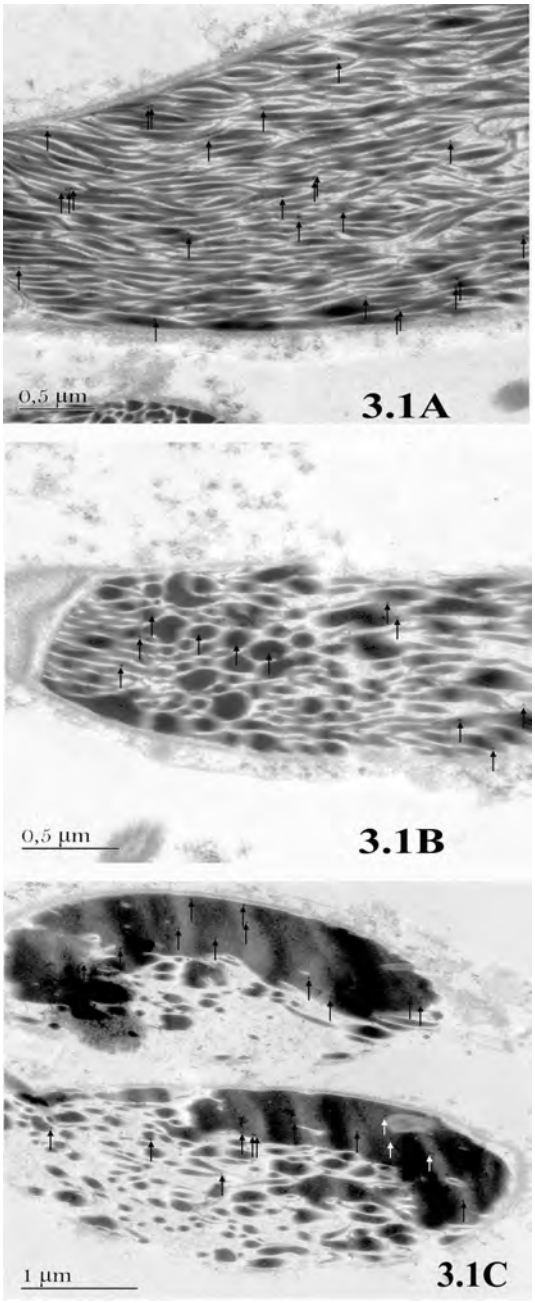

Figure 3.1 Transmission electron microscopy from immunolocalizations of cuttlefish testes, negative controls. The primary antibody was omitted, and samples were only treated with goat anti-rabbit secondary antibody. Labeling of the secondary antibody is comparable to that demonstrated in samples incubated with anti-H4 antibody. The labeling appears to be due to a high affinity of the goat-anti rabbit secondary antibody to chromatin of high density. **3.1A:** Detail of elongating spermatid in phase 4 of spermiogenesis. **3.1B:** Detail of elongated spermatid in phase 5 of sperm maturation. **3.1C:** Spermatid nucleus at phase 6, with hightly condensed chromatin, homogenously compacted at the lamelle.

Localizations of histones H4 and protamine precursor in male germ cells by immunoelectron microscopy

Anti-histone H4 Immunolabeling with anti-histone H4 show that in the initial stage, in which exist both euchromatin and heterochromatin, a large amount of labelling was demonstrated, with a notable increase in the label in the areas of heterochromatin than in euchromatin (see figure 3*a*). Higher condensation of the chromatin means a higher concentration of the histones and the exposition of their epitopes for recognition by the primary antibody; there is not actually an increase in the amount of histone H4 between the two classes of chromatin.

The second stage of spermiogenesis, characterized by homogenous granular heterochromatin, was the stage with the largest amount of immunolabeling (see figures 3*b* and 3*c*). At this point the chromatin is highly organized in nucleosomes, therefore there is a uniform granular state at this phase, in which many H4 histones would have epitopes exposed in relation to the size of the nucleus, which is still large in volume, and round in shape, providing a large surface for the presence of the protein-antibody union.

Phase 3, characterized by the initial formation of fibers in which the granular chromatin begins to unite into fine chromatin fibers of 20-25 nm, also demonstrated a large amount of labelling, though a slight decrease was observed (see figures 3*d* and 3*e*). Similar amounts of labelling were observed at this nuclear stage as that demonstrated in the first stage. It is proposed that the slight decrease in labelling signals the initiation of the protein transfer between nucleosomal histones and precursor protamine. This important change in the chromatin composition, as thin fibers form, is most likely due to the displacement of the granular state, into the stage of fiber formation, at which different proteins function in the process.

At the transition from the third to the fourth stage of chromatin condensation, when the fine fibers further organize into thicker and condensed fibers, it is highly probable that the complete substitution of histone H4 by the precursor protamine occurs. Many biochemical changes must be occurring molecularly to bring about the disassociation of H4. For example, it has been demonstrated that H4 is acetylated in meiosis, and this acetylated form disappears in condensing spermatids (Govin *et al.*, 2004). It is proposed that in order to drive the unbinding of H4, this histone is acetylated at its tail domain in order to lower the

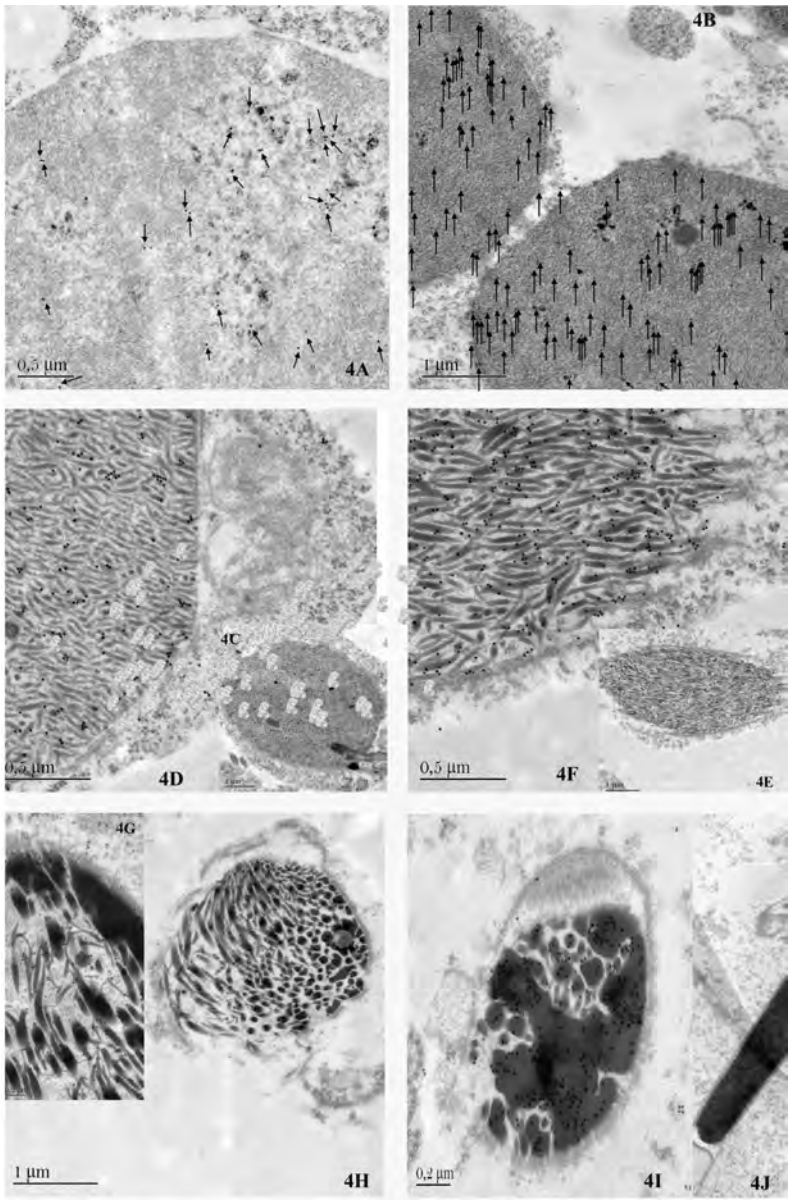

Figure 4 Transmission electron micrographs of sections of testes of *Sepia officinalis*, stained with polyclonal goat anti-rabbit antibody labeled with immunogold following incubation with anti-precursor protamina antibody. **4A:** Initial phase of spermiogenesis, where the the heterochromatin and the euchromatin are still present. Notice the fair amount of labeling which was found to be similar to that demonstrated as background labeling, and is therefore not significant to represent the presence of the precursor protamine. **4B:** Granular chromatin is homogeneous. In this nucleus there is an increase in the labeling from that demonstrated by background, and shows that there is a significant amount of precursor protamine present at the second phase of maturation. **4C:** Phase 3 of spermiogenesis, showing fine fibers. In the small area shown of the nucleus in 4D, it is evident that the density of labeling is high; when applying this heavy labeling to the whole nucleus shown here, the labeling is superior to that of the nucleus in phase 2 of spermiogenesis. **4D:** Detail showing the intense labeling within the fine fibrilar chromatin of phase 3. Notice the mitochondria accumulated around the outer nuclear membrane, characteristic of a nucleus in phase 3. **4E:** Phase 4 of spermiogenesis, demonstrated by the long, thick chromatin fibers, and the nucleus more in elongation, with large amounts of immunolabeling, similar to that of phase 3. **4F:** Detail of a spermatocite nucleus at phase 4 of maturation. The fibers are densely covered with immunolabeling for the precursor protamina. When applying this labeling to the whole nucleus shown in 4*E*, the labeling much superior than that of phases 1 or 2 of spermiogenesis, but similar to that of the fine fibrilar stage 3 of spermiogenesis. **4G:** Detail of longitudinal cut of nucleus in phase 5. Heavy labelling is notice throughout, especially in the area of the homogenously dense chromatin mass. **4H:** Nucleus in phase 5 of sperm maturation, showing lateral and transversal cuts of the chromatin fiber. The fibers are beginning to coalesce toward becoming a large chromatin mass. High amounts of labelling are still observed. **4I:** Phase 6 of spermiogenesis. The chromatin is accumulating toward the same point, becoming a dense mass. **4J:** Final phase of spermiogenesis; the spermatozoon. The elongated, pole-shaped nucleus and homogenously condensed chromatin mark the final stage of sperm maturation. The spermatozoon cells of this degree of maturation are found in the center of the gonad tubule, just before being translocated to the epididymus.

charge, and hence lower its affinity to the DNA (Iizuka *et al.*, 2001). The increase in binding affinity of the precursor protein could also be occuring at this phase to further drive this protein transition in chromatin with dense fibers.

When accounting for the unspecific secondary antibody binding to electrodense chromatin, as that characteristic of nuclei in phases 4-7 of maturation, our results show that there was not significant H4 labeling at the fourth stage of spermiogenesis (see figures 3*f*, 3*e* and 3.1). Fibers are thicker (40-50 nm) and the chromatin is denser at this phase, which marks a crucial point in spermiogenesis and the chromatin protein composition. Due to this thick and condensed nature of the chromatin, negation of some unspecific binding of the secondary antibody was necessary (see figure 3.1), leaving an insignificantly low amount of labelling at this point. The analysis of the absence of significant H4 labeling in dense chromatin indicates that the histones have been displaced, and the first protein transition has already occurred, causing of the morphological chromatin change. The absence of significant labelling remains throughout the final stages of spermiogenesis, as the protein transitions continue to occur in the absence of histones, in order to bring about the full condensation of the chromatin (see figures 3*h-j*).

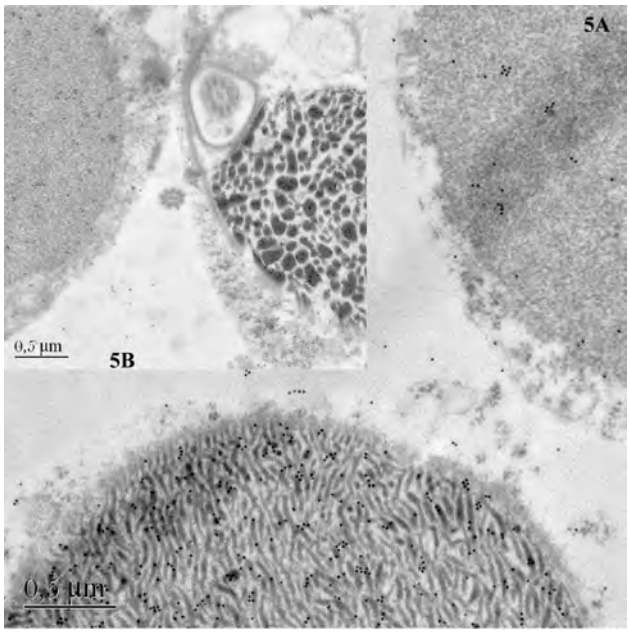

Figure 5 Transmission electron microscopy from immunolocalizations of cuttlefish testes, marking the presence of precursor protamine. Comparison of precursor labeling between nuclei of different stages of maturity in spermiogenesis. **5A:** Comparison of nuclei from phase 2 and phase 3, showing the superior precursor labeling in the nucleus with fibrilar chromatin of phase 3, versus the nucleus with granular chromatin in phase 2 which shows fewer precursor labels. **5B:** Comparison of a spermatocite of the second phase with homogenous granular chromatin, similar in maturity to that shown in 5*A*, to a maturing spermatid of the fifth phase of spermiogenesis. The nucleus with granular chromatin shows much less antibody labeling when compared to that of the nucleus with condensed chromatin masses, which has a very elevated level of labeling.

Anti-precursor protamine In our observations of the immunogold labeling, each phase of spermiogenisis showed some marking of the antibody. When analysing the significance of the labeling demonstrated, it is important to note that the amount of labeling of the precursor protamine is uncomparable to that of H4, due to the different nature of the antibodys used. Although both are polyclonal, the H4 antibody is a commercial antibody (Upstate) and the precursor protamine was generated in the laboratory of Juan Ausió. This antibody demonstrates superior affinity, so more overall labeling was observed in sample incubations with precursor protamin antibody than those incubated with anti H4.

The first stage of spermiogenesis showed only a small amount of labeling in the euchromatin and heterochromatin, which was very similar to the antibody labeling observed as background (see figure 4*a*). Therefore, the labeling noticed in the most immature nucleus was disregarded, as there is no significant difference in antibody labeling between that of the early sperm nucleus and background labeling.

The immunolocalization performed using anti-precursor protamine antibody shows a slight increase in labelling of the antibody in the second phase of spermiogenesis, where homogenous granular heterochromatin is found in the nucleus (see figures 4*b* and 4*c*). This superior labeling compared to that similar to background marking as demonstrating in the more immature nucleus indicates coexistence of the precursor protamines with nucelosomes. The presence of precursor protamine in the second phase could have an important function to bring about chromatin changes in the granular chromatin. It is possible that the precursor plays the role to organize these granules of 20-25 nm into fibers of 25-30 nm. When chromatin fibers begin to form, transitioning from the granular state of the chromatin to fibrilar chromatin, it is important to note that it is quite probable that there is a specific protein which is bringing about this morphological change in chromatin. This change in chromatin condensation, marking the transition from the second to the third phase of spermiogenesis, seems the crucial point in which a change in protein affinity to the DNA begins to occur, making the transition from chromatin

organized by histones to chromatin organized by precursor protamines.

At the third phase of spermiogenesis when defined fibers are observed, a noticeable increase in the precursor labeling was observed (see figures 4*d* and 4*e*). The density of labeling was much more intense and numerous, showing that an important change in the protein-DNA interaction has occurred. Beginning at the fibrillar stages of spermiogenesis, a transition takes place from histones being the dominant protein, to precursor protamine being the dominant DNA interacting protein. In the third stage, however, it is previously mentioned that an extensive amount of histone H4 also exists. It is quite probable that in stage 3 of spermiogenesis changes are occurring to modify the binding affinities of these two proteins to bring about the final protein transition that will be fully brought about by the time stage 4 is reached, where fully elongated and thicker chromatin fibers are formed (see figures 4*f* and 4*g*).

We speculate that at the transition point of spermiogenesis, when thin fibers show labelling of both precursor and histone H4, that the charge of the hyperphosphorylated precursor protamines is increased by cleaving the phosphates, also increasing the charge its binding affinity to the DNA, while histone tails undergo acetylation to weaken their binding to the DNA (Wang *et al.*, 2001). With each phosphate cleaved the precursor protamine would have a stronger binding capability to the DNA until it is finally fully dephosphorylated and later cleaved at its amino terminus, leaving the protamine as the sole binding protein to the DNA in the most mature chromatin, as is observed in the highly electrodense chromatin of stages 6 and 7 of spermiogenesis.

The large increase in labelling from the granular to the fine fibrilar state of the chromatin continued to be observed throughout the final stages of spermiogenesis. A high amount of labelling was observed in mature, condensed chromatin as that of phases 5-7 where electrodense fibers coalesce to become a dense chromatin mass reduced in volume (see figures 4*h-k*). It was previously thought that in such a condensed chromatin state as that observed in the final phase of spermiogenesis, that the precursor would have already been cleaved to give way to the protamine. It is possible that the 21 amino acids which were cleaved, making up the amino domain of the precursor and contain the antigenicity recognizable by the precursor antibody, is still maintained and located in the chromatin, exposable to antibody binding. It is also a possibility that the precursor really does exist in such mature chromatin, but that it is in its most dephosphorylated state at this point, and is still recognized as an antigen by the antibody, yet not as bound to the DNA as would be the protamine interacting in the chromatin to bring about its highly condensed state at these last three stages of spermiogeneis.

The last possibility that must be kept in mind when analyzing the results has to do with some unspecific binding demonstrated by the secondary antibody. There was a pattern of increased immunogold labeling in highly condensed chromatin, both in the negative controls, and in the experimental sets. Though the increase in secondary antibody binding in electrodense chromatin was accounted for in the observations in which primary antibody was present, it is still not fully understood if some of the immunolabeling was due to the presence of the primary antibody being bound to the protein of interest, or if some of the labeling mentioned is due to unspecific binding of the secondary antibody to chromatin which is very electrodense.

Our initial results indicate that in the early phases of the germ cell nuclei, there is coexistence of the precursor protamine with the histones in the granular and initial fibrilar stages of spermiogenesis, since both H4 and precursor were observed in the second and third phases of spermiogenesis. At the fourth phase of long, dense chromatin fibers, precursor protamine is the main protein interacting with the DNA to bring about this stage of chromatin orgainization. The results also indicate that there is a coexistence of both precursor protamine, as well as protamine in the most advanced germ cell nuclei in the testes of *Sepia officinalis*.

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BIBLIOGRAPHY

Doenecke, D.; Albig, W.; Bode, C.; Drabent, B.; Franke, K.; Gavenis, K.; Witt, O. (1997). «Histones: genetic diversity and tissue-specific gene expression». *Histochem. Cell. Biol.*, 107:1-10.

Giménez-Bonafé, P.; Soler, F.; Buesa, C.; Sautière, P.; Ausió, J.; Kouach, M.; Kasinsky, H.; Chiva, M. (2004). «Chromatin organization during spermiogenesis in *Octopus Vulgaris* II: DNA-interacting protein». *Molecular Reproduction and Development*, 68:232-239.

GOVIN, J.; CARON, C.; LESTRAT, C.; ROUSSEAUX, S.; KHOCHBIN, S. (2004). «The role of histones in chromatin remodelling during mammalian spermiogenesis». *European Journal of Biochemistry*, 271:3459-3467.

IATROU, K.; KIXON, G. H. (1978). «Protamine messenger RNA: Its life history during spermatogenesis in rainbow trout». *Red. proc.*, 37:2526-2533.

IIZUKA, M.; SMITH, M. M. (2003). «Functional consequences of histone modifications». *Current Opinion in Genetics and Development*, 13:154-160.

RIBES, E.; GIMÉNEZ-BONAFÉ, P.; SOLER, F.; GONZALEZ, A.; SAPERAS, N.; KASINSKY, H.; CHIVA, M. (2004). «Chromatin Orgainization during Seprmiogenisis in *Octopus Vulgaris*. I: Morphological structures». *Molecular Reproduction and Development*, 68:223-231.

RIBES, E.; SANCHES DE ROMERO, L.; KASINSKY, H.; DEL VALLE, L.; GIMÉNEZ-BONAFÉ, P.; CHIVA, M. (2001). «Chromatin Reorganization during spermiogenesis of the mollusc *Thais homostoma* (Muricidae): implication for sperm nuclear morphogenesis in cenogastropods». *Journal of Experimental Zoology*, 289:304-316.

ROUSSEAUX-PRÉVOST, R.; ENGLEHARDT, R.; ROUSSEAUX, J.; WOUTERS-TYROU, D.; SAUTIÈRE, P. (1988). «Nuclear protein transition in cuttle-fish spermiogenesis: immunocytochemical localization of a protein specific for the spermatid stage». *Gamete Research*, 19:277-290.

SELLOS, D.; KMICCIK, D. (1985). «Characterization of the histones and protamines from the sperm of the marine worm *Platynereis dumerilii*.» *Comp. Biochem. Physiol.*, 80:119-126.

SUPHAMUNGMEE, W.; APISAWEKAKAN, S.; WEERACHATYANUKUL, W.; WANICHANON, C.; STRETARUGSA, P.; POOMTONG, T.; SOBHON, P. (2005). «Basic nuclear protein pattern and chromatin condensation in the male germ cells of a tropical abalone, *Haliotis asinina*.» *Molecular Reproduction and Development*, 70:211-221.

WOUTERS-TYROU, D.; CHARTIER-HARLIN, M. C.; MARTIN-PONTHIEU, A.; BOUTILLON, C.; VAN DORSSELAER, A.; SAUTIÈRE, P. (1991). «Cuttlefish spermatid-specific protein t. Molecular characterisation of tow variants T1 and T2, putative precursors of sperm protamine variants Sp1 and Sp2». *The Journal of Biological Chemistry*, 266:17 388-17 395.