FOLIA HISTOCHEMICA
ET CYTOBIOLOGICA
Vol. 45, No. 4, 2007
pp. 367-374

Cytochemical and immunocytochemical studies of the localization of histones and protamine-type proteins in spermatids of *Chara vulgaris* and *Chara tomentosa*

Katarzyna Popłońska, Agnieszka Wojtczak, Maria Kwiatkowska, Andrzej Kaźmierczak

Department of Cytophysiology, University of Łódź, Poland

Abstract: Spermiogenesis in *Chara* algae, which has been divided into 10 phases (sp I-X), is similar to spermiogenesis in animals. The most important process during spermiogenesis in animals is remodeling of chromatin leading to "sleeping genome", being the result the exchange of histone proteins into protamine-like proteins. Cytochemical studies showed in both *Chara* species (*C. vulgaris*, *C. tomentosa*) that at spI-IV phases only histones were present, at spV-VIII phases - the amount of nuclear protamine-type proteins progressively increased and that of histones decreased while at spIX-X only protamine-type proteins were present. This was also confirmed with capillar electrophoresis. In order to localize more precisely both histones and protamines the immunocytochemical studies with the use of anti-protamine antibodies (protamine-type proteins were obtained from *C. tomentosa* antheridia) and anti-histone H3 antibodies, have been carried out. More specific immunocytochemical studies confirmed cytochemical results including the exchange of histones into protamine-type during spermiogenesis (spV-VIII) in both *Chara* species. At phase V spermiogenesis these strong strand-like anti-protamine signals were observed in cytoplasm which might suggest that protamine synthesis took place in ER.

Key words: *Chara* - Chromatin remodeling - Cytochemical studies - Histones - Immunocytochemical studies - Protamines - Spermiogenesis

---

Introduction

The most important process during spermiogenesis in animals is remodeling of chromatin from nucleosomal structure, characteristic of somatic cells, to lamellar which results in sleeping genome in mature spermatozoids [1].

This process leading to extreme condensation of chromatin (six times more than metaphase chromosomes) [2,3] is the effect of replacement of histones by protamines or other more basic proteins [4,5].

Protamines, strongly alkaline proteins connected with DNA, rich in arginine and cystein, are present in sperm cells. They are a final product of remodeling of proteins which accompanies spermiogenesis in fish, mammals and other animals [6]. During this time in many animal species exchange of proteins consists of two stages. First, somatic histones are replaced by more alkaline transition proteins (TP) and then they are replaced by protamines.

It must by pointed out that the exchange of histone proteins into protamine-type proteins during condensation of chromatin in the course of spermiogenesis does not occur in all organisms, e.g. echinoderms (sea urchin) [6,7], frog *Rana tigerina* [8] and big American frog *Rana catesbeiana* which do not have protamines [9]. In *Rana tigerina* chromatin reorganization occurs by the replacement of histone H1 with histone H1V during differentiation of spermatids and all core histones are still present in sperm chromatin [8].

Protamines were first observed in fish sperm and were believed to be characteristic only of them. However, it is now known that they are present in the sperm of higher vertebrates *e.g.* rooster [10,11], man [12-14] and other mammals as well as in many lower vertebrates and invertebrates [15-17].

Correspondence: K. Popłońska, Dept. of Cytophysiology, University of Łódź, Pilarskiego 14, 90-231 Łódź, Poland;
tel.: (+4842) 6354513, fax.: (+4842) 6354514,
e-mail: popkat@biol.uni.lodz.pl

In lower plants producing free-moving male gametes which belong to *Bryophyta* [18] and *Pteridophyta* [19] protamine-type proteins were also identified. Research on mature spermatozoids of higher algae *Chara* also revealed proteins with electrophoretic mobility similar to that of protamines [20]. A similar phenomenon was described in *Chara vulgaris* [21] and *Chara tomentosa* [22,23].

Spermatogenesis in *Chara* - haplobiont alga - is divided into two stages: first - proliferative stage, during which antheridial filament cells multiply as a result of synchronous mitotic divisions; second - spermiogenesis during which remodeling of spermatids takes place leading to the appearance of fully mature spiral moving spermatozoids.

Light and electron microscopy analyses revealed 10 phases of spermiogenesis (spI-X; selected phases Fig. 1A) which were distinguished on the basis of the size, shape, structure and location of a nucleus in a spermatid, the process of formation of microtubullar manchette and 2 flagellae as well as transformation of proplastids into an amyloplast [21,22].

Earlier comparative cytochemical analyses [24] of the exchange of histone-type proteines (Alfert's and Geschwind's method [25] modified by Sandritter for spermatozoids [26]) into protamine-type ones (Bloch's *et al.* method [27]) showed in both *Chara* species that in the early phases (spI-IV) only histones were present while at the final stages only protamine-type proteins were observed. In *C. tomentosa* spermatids a color product of the reaction characteristic of protamines appeared in phase spV, while in *C. vulgaris* in phase spVI [24]. The results we obtained intrigued us since ultrastructural analyses of spermatids did not show significant differences between the testes species [21,22].

The aim of the present research was to repeat cytochemical reactions with special emphasis put on phases spV and VI to define the moment of protamines appearance in both *Chara* species and conduct modern immunocytochemical analysis of both *Chara* species in order to localize histones and protamines more specifically and to compare the results of this more specific method with those obtained earlier with cytochemical analysis.

Materials and Methods

Apical parts of *Chara vulgaris* thalli were obtained from plants grown in an artificial pond located in the Rogów Arboretum (Poland), and *Chara tomentosa* from the Powidzkie lake situated close to the village Powidz near Konin (Poland).

Antheridia were taken from III-V node pleuridia counting from the apical buds. Before the onset of the experiment, the plants were cultivated for a few days in tanks containing water from natural environment at the photoperiod similar to natural, *i.e.* L:D=14:10. Antheridia of *C. vulgaris* and *C. tomentosa* were studied.

Cytochemical staining of histones and protamines. In order to reveal histones whole plants were fixed in 4% paraformaldehyde/Sörensen's phosphate buffer (0.125 M, pH=7.2) for 1h at room temperature (RT). Alfert's and Geschwind's method [25] modified by Sandritter for spermatozoids [26] was used for staining. Following hydration the material was hydrolysed in 5% trichloroacetic acid at 95°C for 15 min. The plants were rinsed three times in 70% ethanol and after a short hydration they were stained in 0.1% Fast Green FCF (BDH Chemicals Ltd) in Michaelis buffer (pH=8.18) for 30 min at RT. Then they were rinsed in the buffer alone and finally shortly in water.

In order to reveal protamines the material was fixed in 3% glutaraldehyde in 0.1M cacodylate buffer (pH=7.3) for 3 h at RT, stored in 70% ethanol and subjected to deamination with the use of Van Slyke's method for ε-amino-lysine groups [26] followed by staining with Bloch's *et al.* method [27]. After 1 h hydration the material was deamined in 0.5% $NaNO_2$ for 12 h at RT. The plants were rinsed twice in water then hydrolysed in a saturated water solution of picric acid in a water bath with constant stirring at 60°C for 3 h. After rinsing in water whole plants were stained in Tris/HCl buffer (pH=8.3) with 0.05% eosin yellow (POCH Gliwice) for 1 h at RT then rinsed in the buffer alone and finally shortly in water. In both cases isolated antheridia were squashed on uncovered slides and embedded in canada balsam.

Immunocytochemical studies of histones and protamines. Isolated antheridia of both *Chara* species were fixed in 10% formalin and 4 μm paraffin sections were prepared. The paraffin sections deparaffinized with xylene, were gradually hydratated in alcohol series and in distilled water. The antigenic sites were unmasked by microwave treatment (700W, 0.01M citrate buffer, pH=6.0, 12 min), the slides were cooled and rinsed with distilled water.

Immunocytochemical localization of histone H3. Histone H3 antibodies were used since electrophoretic analyses showed that this histone persisted during spermiogenesis for the longest time [22]. Sections were placed in Tris/HCl buffered saline (TBS pH=7.6, DAKO) for 10 min at RT. Then they were permeabilized with 0.1% Triton X-100 in TBS for 14 min at RT. The sections were washed three times for 2 min each with the mixture TBS and 0.2% Tween 20 (the washing buffer) and then blocked in 5% BSA in TBS for 1 h and in the washing buffer for 5 min. After this, the sections were incubated overnight at 4°C with a primary antibody to histone H3 (rabbit polyclonal antibody (Cell Signaling) at a 1:25 dilution) diluted in TBS containing 5% BSA and 0.5% Tween 20.

The material was washed three times for 5 min each with the washing buffer and the sections were incubated with secondary antibodies (anti-rabbit IgG conjugated with FITC (Sigma) diluted 1:70 in TBS containing 5% BSA and 0.5% Tween 20 for 1 h at RT in darkness. The sections were washed for 10 min in the washing buffer and two times for 10 min in TBS.

The slides were stained using DAPI (1 μg/1 ml) for 15 min in darkness. Then the sections were embedded in PBS/glycerol mixture (9:1) with 2.3% DABCO (1,4-diazabicyclo-[2,2,2] octane, Sigma). The cells were analysed using an Optiphot-2 epifluorescence microscope (Nikon), equipped with UV-2A (excitation - λ =360-460 nm) for DAPI and with B-2A blue light filter (excitation - λ =450-490 nm).

Immunocytochemical localization of protamines. The sections were placed in Phosphate Buffer Saline (PBS, containing 0.14 M NaCl, 3 mM KCl, 8 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH=7.4) for 6 min at RT. Then they were permeabilized with 0.1% Triton X-100 in PBS for 14 min at RT. The sections were washed three times for 2 min each with the mixture of PBS and 0.2% Tween 20 (the washing buffer) and then blocked in PBS containing 10% (w/v) non-fat dried milk in the washing buffer for 5 min at RT.

Next the material was rinsed for 3 min in the washing buffer. After this, the sections were incubated for 90 min at RT with a primary antibody (rabbit polyclonal antibody) (Dept. of Immunology, Institute Microbiology and Immunology University of Łódź) to protamines isolated from antheridia of *C. tomentosa* at a 1:500 dilution) diluted in PBS containing 1% BSA and 0.5% Tween 20.

The material was washed three times for 2 min each with the washing buffer and the sections were incubated with secondary antibodies (anti-rabbit IgG conjugated with FITC (Sigma) diluted 1:70 in PBS containing 1% BSA and 0.5% Tween 20 for 1 h at RT in darkness. The remaining procedure followed that described for immunocytochemical localization of histone H3.

To check for non-specific staining, the primary antibodies to both histone H3 and protamines the two control probes have been done. The first one was prepared with non-immune rabbit IgG and the second - without pre-immune rabbit IgG. Positive immunosignals were observed only in the cells treated with the specific primary antibodies.

Photography. Images were taken using a Nikon color camera attached to a Nikon microscope.

Extraction of protamines from mature antheridia of *Chara tomentosa*. Protamines were extracted form antheridia of *C. tomentosa* L. whose antheridial filament cells were at the last phase of spermiogenesis described as a terminal phase [22]. Developmental phases were randomly estimated in the selected antheridia by light microscopy.

During preparation, the male specimens of *C. tomentosa* were cultivated in the laboratory at about 20°C under light using white fluorescent tubes (5 W·m-2). Antheridia were rinsed with distilled water and then gently crashed in cold glass mortar with 10 mM Tris/HCl saline buffer (TBS) at pH=8.0 supplemented with 10 mM NaCl, 5 mM $MgCl_2$, 0.25 M sucrose, 2 mM DTT and cocktail protease inhibitor (Sigma; added immediately prior to use). To separate antheridial filaments from shield cells the crashed antheridia were repeatedly (8-10 times) washed (4°C) with TBS buffer, using Pasteure pipette. During the last washing, the antheridial filaments were collected by centrifugation at 1 000 × g for 15 min (4°C).

Then the pellet was digest by DNase I (EC 3.1.21.1; Sigma) at 37°C during 20 min at 10 units per ml at DNase digestion medium (0.25 M sucrose, 10 mM Tris, 10 mM NaCl, 5 mM $MgCl_2$, coctail inhibitor). After digestion the extract was chilled on ice and treated with H_2SO_4 at a final concentration of 0.2 M and then stirred using the magnetic stirrer for 12 h (4°C). Insoluble material was precipitated by centrifugation at 12 000 × g for 40 min (4°C). The supernatant was withdrawn and the pellet was reextracted with 0.2 M H_2SO_4 for additional 8 h. The acid extracts were combined and proteins were precipited for 1 h with cold (-20°C) acetone at a final concentration of 80%. The precipitate was collected by centrifugation (12 000 × g for 30 min at 4°C), washed twice with cold acetone and dried in vacuo with CentiVap Concentrator (Labconco). Proteins were resuspenced in PBS buffer (pH=7.4) and samples were filtered by centrifugation (5 000 × g) through 30 kDA cut-off membrane filter (Amicon). Then the lower solution was filtered by centrifugation (1 000 × g) through 3 kDA cut-off membrane filter. The upper solution was precipited for 1 h with cold (-20°C) acetone, centrifugation at 12 000 x g for 30 min (4°C), washed twice with cold acetone, dried in vacuo and resuspended in PBS buffer (pH=7.4) and treated with H_2SO_4 (0.2 M), then stirred using the magnetic stirrer for 2 h (4°C), centrifuged at 12 000 x g for 40 min (4°C). Then precipited 1 h with cold (-20°C) acetone (80%), centrifuged at 12 000 x g for 40 min (4°C) and dried *in vacuo*. The protein concentration used to rabbit immunisation was determined by Bradford method [28] with BSA as the standard and was 800 µg per ml.

Results

Cytochemical studies

In the repeated cytochemical analyses concerning histone-type and protamine-type proteins in spermatids of *Chara vulgaris*, special attention was put to phases spV and spVI. In spermatid nuclei in phases spI-IV of spermiogenesis only strong color reaction to histones was observed (Fig. 1IIIB). It was show that both in *C. vulgaris* and *C. tomentosa* starting from phase spV decrease in histones was accompanied by gradual increase in protamine-type proteins (Fig. 1VB, C). Thus at this stage the replacement of nuclear proteins from histones with protamine-type proteins started and lasted till phase spVIII (Fig. 1VIIIB, C). Moreover, in phase spV (Fig. 3a) more strongly stained strands of cytoplasm were observed. During the final phases of spermiogenesis spIX-X (Fig. 1XC) only protamine-type proteins were present in spermatozoid nuclei, while no color reaction revealing histone presence was observed (Fig. 1XB).

Immunocytochemical studies

Immunocytochemical studies were carried out in order to localize more specifically both types of proteins.

The use of histone H3 antibodies revealed positive antigene reaction in spermatid nuclei during phases I-IV (Fig. 2IIIA). During phases spV-VIII (Fig. 2V-VIIIA) this reaction was less intensive than in earlier phases. At the final spermiogenesis phases (spIX-X) (Fig. 2XA) no positive antygene signals were revealed.

The use of protamin antibodies did not give positive reaction in spermatid nuclei during phases spI-IV (Fig. 2IIIC), however revealed such signals later spV-X (Fig. 2V-VIIIC). In phase spV (Fig. 3b) strong signals in the form of cytoplasm strands near a nucleus were also present. Later (spVI-VIII) the reaction was observed both in the cytoplasm and in the nucleus (Fig. 2VI-VIIIC) while towards the end (spIX-X) strong signals were present at the nucleus periphery and slightly weaker inside the nucleus (Fig. 2XC).

Discussion

In *Chara* during spermiogenesis numerous ultrastructural and biochemical changes lead to the formation of two-flagellae spermatozoids ready to move in water.

On the basis of light and electron microscope analyses the sequence of structural changes in spermatids was observed, starting from spermatid formation (phase spI) till full maturation (phase spX). The structure of phases spI-IV exhibits many features characteristic of meristematic cells. Phase spV presents very extensive endoplasmic reticulum and traces of

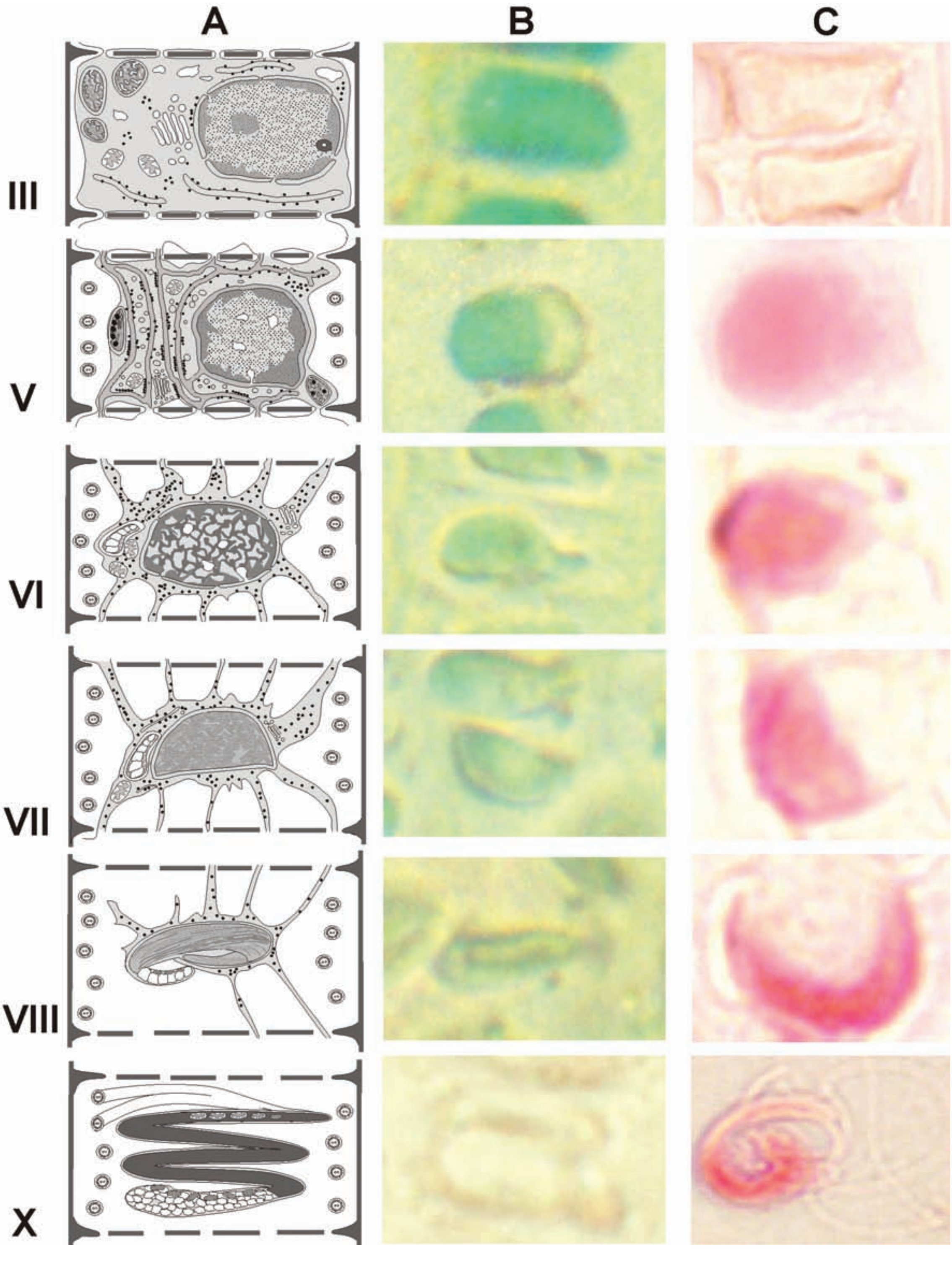

Fig. 1. The selected phases of *Chara vulgaris* spermiogenesis. Ultrastructural changes during different phases of spermiogenesis (**A**) according to [21], modified. The antheridial filament cells of *C. vulgaris* cytochemically stained to reveal histones (green **B**) and protamine-type proteins (red **C**) during successive phases of spermiogenesis (magnification × 1900).

starch grains in plastids and microtubular manchette as well as 2 elongating flagellae. Phase spVI is characterized with a net-like nucleus structure, phases spVII-VIII with the appearance of fibrillar chromatin, phase spIX with lamellar chromatin and phase spX with extremely condensed chromatin and polar localization of organelles [21,22].

The results of cytochemical [24 and actual] and regarded more specific immunocytochemical studies aiming at revealing histones and protamines in the

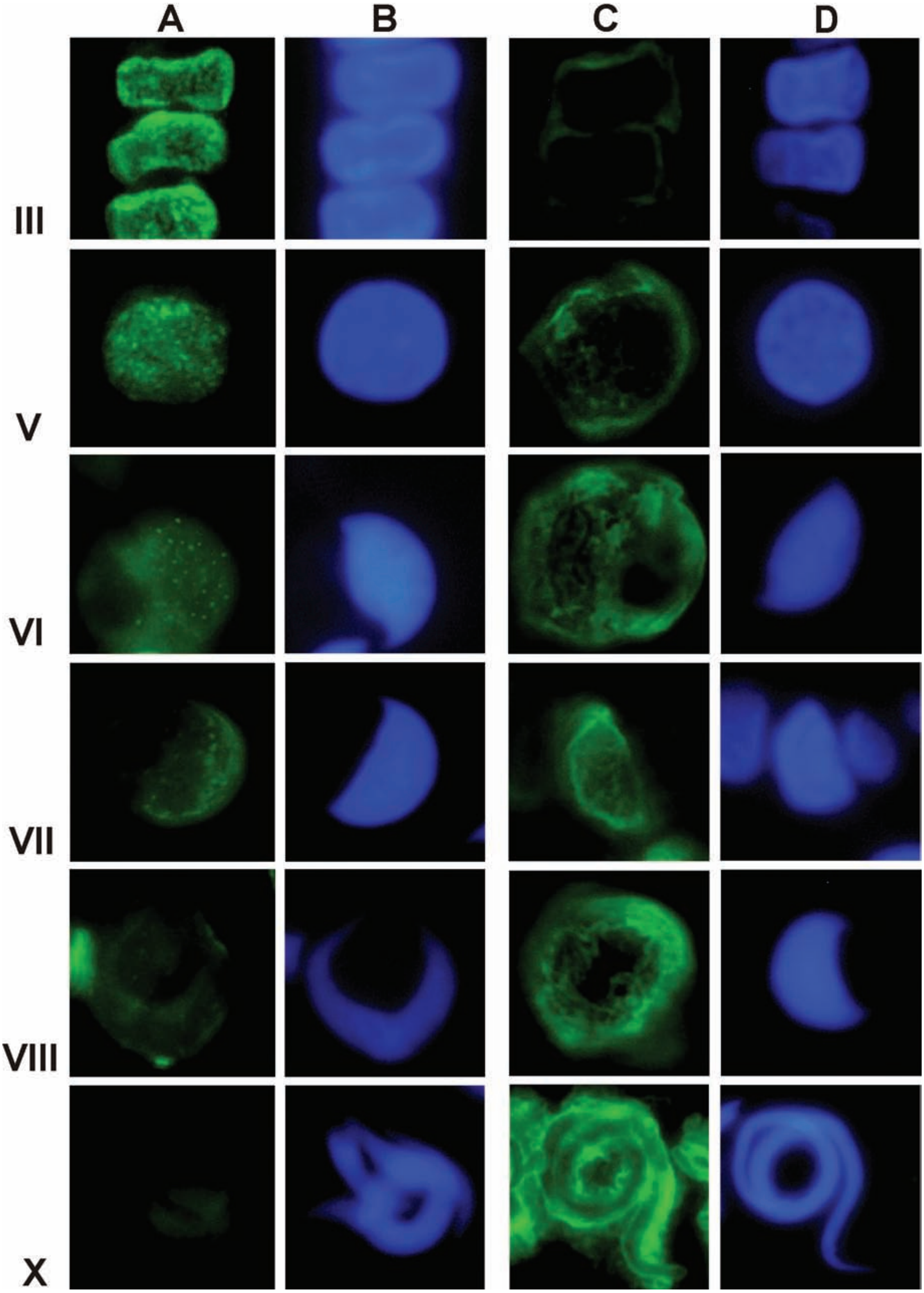

Fig. 2. Immunocytochemical localization of histone H3 (**A**) and protamine-type proteins (**C**) in spermatids of *C. vulgaris*. Nuclei staining with DAPI (**B,D**) (magnification × 1750).

spermatids of both *Chara* species were in agreement. They showed that during early phases of spermiogenesis (spI-IV) only histones were present which were gradually (spV-VIII) replaced by protamine-type proteins and finally (spIX-X) only the latter ones were observed.

Protamines were identified in *Chara* for the first time by Robert [20] who conducted electophoretic analyses of spermatozoids that revealed that these strongly alkaline proteins with mobility comparable to salmon protamines co-existed with somatic histones. More precise analyses with the use of capillary electrophoresis were conducted on *C. tomentosa* four stages of spermiogenesis: early, mid, late and terminal [22]. They confirmed the exchange of somatic proteins into generative protamine-type ones during the medium phases of spermiogenesis which was shown earlier by cytochemical analyses [24]. In early spermiogenesis there were only core and linker histones while in the mid phase protamine-type proteins appeared. In mature spermatozoids there were no histones which were replaced by three fractions of alkaline proteins (9.1; 9.6 and 11.2 kDa) exhibiting electrophoretic mobility similar to that observed in salmon protamines. Disappearance of linker histones following their modification preceded disappearance of core histones. In *C. tomentosa* spermiogenesis no transition proteins (TP) were observed [22].

After the use of different methods it was shown that immunocytochemical analyses revealing the presence of histone H3 and protamine-type proteins confirmed the earlier results obtained by cytochemical analyses and capillary electrophoresis.

Phase spV is crucial during the process of *Chara* spermatid differentiation. Cytochemical and immunocytochemical studies showed that protamine-type proteins in both *Chara* species appeared during this spermiogenesis phase, and the exchange of nucleohistones into nucleopratamines which then started enabled proper condensation of chromatin thus leading to the appearance of extremely condensed chromatin in mature spermatozoids.

Ultrastructural studies of *C. vulgaris* and *C. tometosa* showed that in phase spV an extensive ER system filled with dark fine-granular substance was observed and the intermembranous space of the nuclear envelope was filled with a similarly looking homogenous substance [21-23,29]. Similar pictures resembling ER cysterne were also observed during both cytochemical (Fig. 3a) and immunocytochemical (Fig. 3b) analyses: distinct colors reaction and strong antigene signals against protamine-type proteins were revealed as parallel strands in the cytoplasm near spermatid nuclei. The obtained results seem to suggest that the synthesis of protamine-type proteins takes place in ER [21-23,29] and that they are transported through outer nuclear envelope space via endocytosis. In order to prove our hypothesis we are soon going to carry out analyses with the use of immunogold technique.

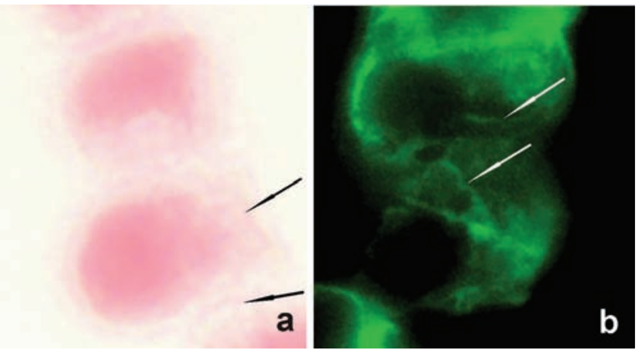

Fig. 3. Pictures of *C. vulgaris* spermatids in phase spV of spermiogenesis with strong signals in the form of cytoplasmic strands (arrows) near a nucleus, during both cytochemical (**A**) and immunocytochemical (**B**) analyses (magnification × 1900).

Above assumption correspond with the results obtained in mammals: protamines first appear at the periphery of a nucleus and it was suggest that a nuclear envelope might play a role during the replacement of transition proteins by protamines during spermiogenesis [30]. Analyses of spermiogenesis in mice showed that protamine 1 (P1) bound with lamin B receptor (LBR), an inner nuclear membrane protein, due to the fact that protein p32 left LBR allowing P1 to bind [31]. Similar pictures proving the presence of protamine-type proteins in the spermatids of both *Chara* species in phase spV were observed (Fig. 2VC). During final stages of spermiogenesis (spIX-X) the strongest anti-protamine signals were revealed also at the periphery of a nucleus (Fig. 2XC). We suppose that this may be caused by the appearance of protamines both in mid (an analogue of protamine1 in mice) and late (an analogue of family of protamine 2 in mice) spermiogenesis [32]. Weaker antigene reaction in spermatozoid nuclei during phases spIX-X, in comparison with earlier phases, may be due to the extreme condensation of the sperm chromatin. This chromatin state decreased the number of the sites which were accessible for the antibody-binding as shown with the use of the immunogold technique [17,33].

Exchange of histone proteins into protamine-type proteins results in disappearance of nucleosomes and appearance of a completely different structure which, magnified about × 220 000, reveals fine spirals [21,Wojtczak unpublished results.] being DNA particles joined with protamines as Ward suggests [34].

Contrary to *Chara*, in some organisms a small amount of somatic histones is still present in late spermatids and mature spermatozoids [35]. During late mouse spermiogenesis five new histone variants, for example H2AL1 and H2AL2, were discovered specifically marking the pericentric regions in condensing spermatids and participating in the formation of new nucleoprotein structures [36]. In man the exchange of nucleohistones into nucleoprotamines is approximately 85% complete during late spermiogenesis [37,38]. Most mammals have only one form of protamines, however in a few species including man and mouse there are two: protamine 1 (P1) and the family of protamine 2 (P2) proteins (P2, P3, P4) [13] while in *Chara* there are three fractions of protamine-type proteins of the following masses: 9.1; 9.6 and 11.2 kDa [22].

Analyses of the disturbances in functioning of protamine genes (*Prm1* and *Prm2*) in mice showed that mutation in one allele in either *Prm1* or *Prm2* resulted in deficiency in protamines [39,40]. Sufficient amount of protamine 2 is crucial for the normal process of nuclear chromatin condensation. Lack or shortage of this protamine make spermatozoids unable of fertilization [13,41-44].

Replacement of histones by protamines leads to transcription inhibition which is accompanied by chromatin condensation and recognition of methylated residues on histone tails by the protein HP1 (heterochromation protein 1) which is involved in reorganization of chromatin structure [45,46].

In *C. vulgaris*, similarly as in mice [47], transient demethylation of DNA [48] was observed which correspond with intensive ^{3}H-lysine and ^{3}H-arginine incorporation [21]. On this basis a hypothesis can be put forward that decondensation of chromatin in early spermiogenesis in *C. vulgaris*, similarly as in animals, is connected with activation (by demethylation) of numerous genes involved in reorganization and differentiation and, in preparation for next phases, crucial changes in chromatin structure resulting from the replacement of histones by protamines take place. This is followed by increasing DNA methylation reaching maximum at final phases of spermiogenesis [48].

Process of gamet formation in *Chara* is more similar to that in animals than in higher plants. This is indicated both by ultrastructural analyses and by the exchange of histones into protamines. To our knowledge this process of exchange in *C. vulgaris* and *C. tomentosa* has not been described so precisely so far.

Acknowledgements: The authors thank Prof. H. Długońska, dr D. Kierońska, dr J Gatkowska from Dept. of Immunology Institute Microbiology and Immunology University of Łódź for rabbit polyclonal antibody to the protamines isolated from the antheridia of C. tomentosa. This study was partly supported by the National Committee of Scientific Research (MNiSW), grant no. 3 PO4C 033 25.

References

[1] Ward WS. The structure of the sleeping genome: implications of sperm DNA organization for somatic cells. *J Cell Biochem*. 1994;55:77-82.

[2] Pogany GC, Corzett M, Feston S, Balhorn R. DNA and protein content of mouse sperm. Implications regarding sperm chromatin structure. *Exp Cell Res*. 1981;136:127-136.

[3] Ward WS, Coffey DS. DNA packing and organization in mammalian spermatozoa: comparison with somatic cells. *Biol Reprod*. 1991;44:569-574.

[4] Dadoune J-P. Expression of mammalian spermatozoal nucleoproteins. *Microsc Res Tech*. 2003;61:56-75.

[5] Steger K. Transcriptional and translational regulation of gene expression in haploid spermatids. *Anat Embryol*. 1999;199: 471-487.

[6] Wouters-Tyrou D, Martinage A, Chevaillier P, Sautičre P. Nuclear basic proteins in spermiogenesis. *Biochimie*. 1998;80:117-128.

[7] Wouters-Tyrou D, Sautičre P, Biserte G. Covalent structure of the sea urchin histone H4. *FEBS Lett*. 1976;65:225-228.

[8] Manochantr S, Sretarugsa P, Chavadej J. Chromatin organization and basic nuclear proteins in the male germ cells of Rana tigerina. *Mol Reprod Dev*. 2005;70:184-197.

[9] Itoh T, Ausio J, Katagiri C. Histone H1 variants as sperm-specific nuclear proteins of Rana catesbeiana, and their role in maintaining a unique condensed state of sperm chromatin. *Mol Reprod Dev*. 1997;47:181-190.

[10] Oliva R, Dixon GH. Chicken protamine genes are intronless. The complete genomic sequence and organization of the two loci. *J Biol Chem*. 1989;264:12472-12481.

[11] Oliva R, Dixon GH. Expression and processing of the rooster protamine mRNA. *Ann NY Acad Sci*. 1991;637:289-299.

[12] McKay DJ, Renaux BS, Dixon GH. Human sperm protamines. Amino-acid sequences of two forms of protamine P2. *Eur J Biochem*. 1986;156:5-8.

[13] Oliva R. Protamines and male infertility. *Hum Reprod Updat*. 2006;12:417-435.

[14] Yoshii T, Kuji N, Komatsu S, Iwahashi K, Tanaka Y, Yoshida H, Wada A, Yoshimura Y. Fine resolution of human sperm nucleoproteins by two-dimensional electrophoresis. *Mol Hum Reprod*. 2005;11:677-681.

[15] Gimenez-Bonafe P, Ribes E, Sautiere P, Gonzalez A, Kasinsky H, Kouach M, Sautiere P-E, Ausio J, Chiva M. Chromatin condensation, cysteine-rich protamine, and establishment of disulphide interprotamine bonds during spermiogenesis of Eledone cirrhosa (Cephalopoda). *Eur J Cell Biol*. 2002;81: 341-349.

[16] Ma J, Katz E, Belote JM. Expression of proteasome subunit isoforms during spermatogenesis in Drosophila melanogaster. *Insect Mol Biol*. 2002;11:627-639.

[17] Suphamungmee W, Apisawetakan S, Weerachatyanukul W, Wanichanon C, Sretarugsa P, Poomtong T, Sobhon P. Basic nuclear protein pattern and chromatin condensation in the male germ cells of a tropical abalone, Haliotis asinina. *Mol Reprod Dev*. 2005;70:211-221.

[18] Reynolds WF, Wolfe SL. Changes in basic proteins during sperm maturation in a plant, Marchantia polymorpha. *Exp Cell Res*. 1978;116:269-273.

[19] Reynolds WF, Wolfe SL. Protamines in plant sperm. *Exp Cell Res*. 1984;152:443-448.

[20] Robert D. Le noyau du gaméte mâle chez les végétaux. *Ann Sci Nat Bot Paris*. 1984;6:151-164.

[21] Kwiatkowska M, Popłońska K. Further ultrastructural research of Chara vulgaris spermiogenesis: endoplasmic reticulum, structure of chromatin, ^{3}H-lysine and ^{3}H-arginine incorporation. *Folia Histochem Cytobiol*. 2002;40:85-97.

[22] Kwiatkowska M, Kaźmierczak A, Popłońska K. Ultrastructural, autoradiographic and electrophoretic examinations of Chara tomentosa spermiogenesis. *Acta Soc Bot Pol*. 2002;71: 201-209.

[23] Kwiatkowska M, Popłońska K. RER and protamine-type proteins during Chara tomentosa L. spermiogenesis. *Acta Soc Bot Pol*. 2003;72:5-9.

[24] Popłońska K. Cytochemical studies on histone-type and protamine-type proteins during spermiogenesis in Chara vulgaris and Chara tomentosa. *Folia Histochem Cytobiol.* 2002;40: 233-234.

[25] Alfert M, Geschwind I. A selecive staining method for the basic proteins of cell nuclei. *Proc Natl Acad Sci.* 1953;39: 991-998.

[26] Myśliwski A. Histochemia histonów. In: Krygier-Stojałowska A, Godlewski H, eds *Topochemiczne Metody Badań Komórek i Tkanek*. PWN, Warszawa; 1982:361-376.

[27] Bloch DP, Howard YC, Hew MS. Methods for the cytochemical characterization of nuclear basic proteins and their application to problems of development. *Ann Histochime.* 1961;7: 497-500.

[28] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976;72:248-254.

[29] Kwiatkowska M. Changes in ultrastructure of cytoplasm and nucleus during spermiogenesis in Chara vulgaris. *Folia Histochem Cytobiol.* 1996;34:41-56.

[30] Biggiogera M, Muller S, Courtens JL, Fakan S, Romanini MG. Immunoelectron microscopical distribution of histones H2B and H3 and protamines in the course of mouse spermiogenesis. *J Elect Microsc Tech.* 1992;20:259-267.

[31] Mylonis I, Drosou V, Brancorsini S, Nikolakaki E, Sassone-Corsi P, Giannakouros T. Temporal association of protamine 1 with the inner nuclear membrane protein lamin B receptor during spermiogenesis. *J Biol Chem.* 2004;279:11626-11631.

[32] Zhao M, Shirley CR, Mounsey S, Meistrich ML. Nucleoprotein transitions during spermiogenesis in mice with transition nuclear protein Tnp1 and Tnp2 mutations. *Biol Reprod.* 2004;71:1016-1025.

[33] Courtens JL, Kistler WS, Plően L. Ultrastructural immunolocalisation of histones (H2B, H3, H4), transition protein (TP1) and protamine in rabbit spermatids and spermatozoa nuclei. Relation to condensation of the chromatin. *Reprod Nutr Dev.* 1995;35:569-582.

[34] Ward WS. Deoxyribonucleic acid loop domain tertiary structure in mammalian spermatozoa. *Biol Reprod.* 1993;48:1193-1201.

[35] Dadoune J-P, Siffroi J-P, Alfonsi M-F. Transcription in haploid male germ cells. *Int Rev Cytol.* 2004;237:1-56.

[36] Govin J, Escoffier E, Rousseaux S, Kuhn L, Ferro M, Thévenon J, Catena R, Davidson I, Garin J, Khochbin S, Caron C. Pericentric heterochromatin reprogramming by new histone variants during mouse spermiogenesis. *J Cell Biol.* 2007;176:283-294.

[37] Barone JG, de Lara J, Cummings KB, Ward WS. DNA organization in human spermatozoa. *J Androl*. 1994;15:139-144.

[38] Prigent Y, Muller S, Dadoune JP. Immunoelectro microscopical distribution of histones H2B and H3 and protamines during human spermiogenesis. *Mol Hum Reprod.* 1996;2:929-935.

[39] Braun RE. Packaging paternal chromosomes with protamine. *Nat Genet.* 2001;28:10-12.

[40] Cho C, Willis WD, Goulding EH, Jung-Ha H, Choi YC, Hecht NB, Eddy EM. Haploinsufficiency of protamine-1 or -2 causes infertility in mice. *Nat Genet.* 2001;28:82-86.

[41] Carrell DT, Liu L. Altered protamine 2 expression is uncommon in donors of known fertility, but common among men with poor fertilizing capacity, and may reflect other abnormalities of spermiogenesis. *J Androl.* 2001;22:604-610.

[42] Cho C, Jung-Ha H, Willis WD, Goulding EH, Stein P, Xu Z, Schultz RM, Hecht NB, Eddy EM. Protamine 2 deficiency leads to sperm DNA damage and embryo death in mice. *Biol Reprod.* 2003;69:211-217.

[43] O'brien J, Zini A. Sperm DNA integrity and male infertility. *Urology.* 2005;65:16-22.

[44] Steger K, Pauls K, Klonisch T, Franke FE, Bergmann M. Expression of protamine-1 and -2 mRNA during human spermiogenesis. *Mol Hum Reprod.* 2000;6:219-225.

[45] Hoyer-Fender S, Singh PB, Motzkus D. The murine heterochromatin protein M31 is associated with the chromocenter in round spermtids and is a component of mature spermatozoa. *Exp Cell Res.* 2000;254:72-79.

[46] Sassone-Corsi P. Unique chromatin remodeling and transcriptional regulation in spermatogenesis. *Science.* 2002;296: 2176-2178.

[47] del Mazo J, Prantera G, Torres M, Ferraro M. DNA metylation changes during mouse spermatogenesis. *Chromosome Res*. 1994;2:147-152.

[48] Olszewska MJ, Gernand D, Godlewski M, Kunachowicz A. DNA methylation during antheridial filament development and spermiogenesis in Chara vulgaris (Charophyceae) analysed by in situ nick-translation driven by methylation-sensitive restriction enzymes. *Eur J Phycol.* 1997;32:287-291.

Submitted: 13 March, 2007
Accepted after reviews: 25 May, 2007