Regulated hyperacetylation of core histones during mouse spermatogenesis: involvement of histone-deacetylases

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Here we report a detailed analysis of waves of histone acetylation that occurs throughout spermatogenesis in mouse. Our data showed that spermatogonia and preleptotene spermatocytes contained acetylated core histones H2A, H2B and H4, whereas no acetylated histones were observed throughout meiosis in leptotene or pachytene spermatocytes. Histones remained unacetylated in most round spermatids. Acetylated forms of H2A and H2B, H3 and H4 reappeared in step 9 to 11 elongating spermatids, and disappeared later in condensing spermatids. The spatial distribution pattern of acetylated H4 within the spermatids nuclei, analyzed in 3D by immunofluorescence combined with confocal microscopy, showed a spatial sequence of events tightly associated with chromatin condensation. In order to gain an insight into mechanisms controlling histone hyperacetylation during spermiogenesis, we treated spermatogenic cells with a histone deacetylase inhibitor, trichostatin A (TSA), which showed a spectacular increase of histone acetylation in round spermatids. This observation suggests that deacetylases are responsible for maintaining a deacetylated state of histones in these cells. TSA treatment could not induce histone acetylation in condensing spermatids, suggesting that acetylated core histones are replaced by transition proteins without being previously deacetylated. Moreover, our data showed a dramatic decrease in histone deacetylases in condensing spermatids. Therefore, the regulation of histone deacetylase activity/concentration appears to play a major role in controling histone hyperacetylation and probably histone replacement during spermiogenesis.

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Introduction

Acetylation of specific lysines within the N-terminal domains of histones reduces their positive charges and is believed to destabilize their interactions with DNA, affect nucleosome-nucleosome contacts and modify chromatin higher order structure (Tse and Hansen, 1997; Tse et al., 1998; Luger and Richmond, 1998; Hansen et al., 1998; Wolffe and Hayes, 1999). It is known that histone acetylation is linked to several cellular processes, including transcription (Wade et al., 1997; Kuo and Allis, 1998; Workman and Kingston, 1998; Imhof and Wolffe, 1998) and chromatin assembly (Sobel et al., 1995; Roth and Allis, 1996; Grunstein, 1997; Wade et al., 1997; Adams and Kamakaka, 1999).

Spermatogenesis is a uniquely suitable model system in which to study the roles of acetylation in cellular functions associated with cell division and differentiation. It represents the differentiation stages of the germinal cells from spermatogonia to spermatozoa. Spermatogonia enter meiosis by the formation of preleptotene primary spermatocytes, which replicate DNA and subsequently go through the leptotene, zygotene, pachytene and diplotene stages of the first meiotic division prophase. Meiotic I division yields secondary spermatocytes which then rapidly go through meiotic II division, generating haploid round spermatids. The spermatid differentiation process that follows, also called spermiogenesis, includes elongation of the nucleus followed by chromatin condensation, and produces mature spermatozoa (Hess, 1999).

During spermiogenesis, two sets of events are of major importance. One is the extinction of gene transcription and the other is the replacement of histones by protamines. Gene transcription is active in spermatogonia, pachytene spermatocytes and round spermatids. It is thought to stop shortly after, in elongating spermatids (Kierszenbaum and Tres, 1975, 1978; Soderstrom and Parvinen, 1976; Hecht, 1998). Displacement of histones occurs during spermiogenesis halfway through the spermatid development when the nucleus is condensing.

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Histones are replaced by more basic proteins, the transition proteins, which will in turn be replaced by the major sperm proteins, the protamines (Meistrich, 1989).

The mechanisms of histones 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 in condensing spermatids. Indeed, a hyperacetylation of histones in post-meiotic cells was observed in the testis of various animal species including fly (Wolf et al., 1993), trout (Christensen and Dixon, 1982; Christensen et al., 1984), rooster (Oliva and Mezquita, 1982) and rat (Grimes and Henderson, 1984a, b). In the rat testis, a hyperacetylation of histone H4 was observed in situ by Meistrich et al. (1992), which appeared in spermatids beginning elongation (steps 9–10) and showed a maximum in late elongating spermatids (steps 11 and 12). In these latter, intense staining was observed at the caudal area of the spermatid nucleus. In human spermiogenesis, where 85% of histones are replaced by protamines, the remaining histones have been shown to be highly acetylated in spermatozoa (Gatewood et al., 1990). In contrast, in winter flounder and carp, where somatic histones are completely retained in spermatozoa, H4 remains unacetylated in spermatids (Kennedy and Davies, 1980, 1981).

In somatic cells, the extent of histone acetylation is known to be the result of two enzymatic activities, histone acetyl transferases (HATs) and histone deacetylases (HDACs) (Kuo and Allis, 1998). Among the HDACs, HDAC6 (or mHDA2) was shown to be over-expressed in the testis compared to other tissues (Verdel and Khochbin, 1999).

In order to investigate the position of histone acetylation during meiosis and post-meiotic remodeling of the chromatin, the chronology of the appearance of acetylated forms of histones was detected in situ for the first time throughout mouse spermatogenesis. The treatment of spermatogenic cell suspension with histone deacetylase inhibitor, TSA, as well as the analysis of class I and II histone deacetylases in fractionated spermatogenic cells, allowed us to propose a critical role of histone deacetylases in the control of histone hyperacetylation, and probably replacement, in condensing spermatids.

Materials and methods

Antibodies

Polyclonal antibodies raised in rabbits against acetylated forms of H3 (H3ac) and H4 (H4ac) (Upstate Biotechnology), H4 acetylated on lysine 5, 8, 12 or 16* (H4ac5 or H4ac8, H4ac12 or H4ac16), H2A acetylated on lysine 5* (H2Aac5) and acetylated H2B* (H2Bac), were used to localize these proteins in the seminiferous epithelium of the adult mouse. These antibodies (*) had been tested and described previously (Turner and Fellows, 1989; Turner et al., 1989). Rabbit preimmune sera were used as controls. Polyclonal antibodies were also raised in rabbits against histone deacetylases HDAC1 and HDAC6.

A monoclonal antibody raised in mouse against the proliferating cell nuclear antigen (PCNA) (Dakopatts Society) and a polyclonal transition protein 2 (TP2) antibody raised in rabbit, kindly provided by Stephen Kistler (Alfonso and Kistler, 1993, Kistler et al., 1996) were also used.

Immunohistochemistry

Tissue preparation. Adult male mice were killed and their testes were removed and immersed in Bouin's fixative. Immersion-fixation was accomplished by pricking the tunica albuginea with a thin needle and placing the testis into the fixative for 24 hours. The testis was then sliced into three equally sized parts and put back into the fixative for another day, after which it was dehydrated and embedded in paraffin.

Immunoperoxidase staining. The different antibodies were applied to serial testis sections in order to precisely define the chronology of their staining pattern. Immunoperoxidase staining of testicular sections was carried out according to a protocol which was adapted in our laboratory. All incubations took place at room temperature (RT) and without coverslips.

Six to ten-micrometer serial paraffin sections of Bouin fixed mouse testis were deparaffinized in xylene (3×5 min) and hydrated through graded series of ethanol (100%, 90%, 70%; 3×3 min each). Sections were incubated overnight in sodium citrate 10 mM (pH = 6) at RT for a better penetration of antibodies.

Prior to immunostaining, sections were incubated for 30 min in 1% hydrogen peroxide (Sigma) to abolish endogenous peroxidase activity. The nonspecific binding of antibody was blocked with TBS (Tris-buffered saline: 0.05 M Tris-HCl/0.15 M NaCl, pH = 7.4) containing 3% goat serum for 1 hour. Then, sections were incubated for two hours with the appropriate primary antibody (rabbit anti-histones, rabbit anti-TP2 or mouse anti-PCNA) diluted in TBS containing 3% goat serum. The dilutions were as follows: H3ac (1:50), H4ac (1:100), H4ac5 (1:400), H4ac8 (1:500), H4ac12 (1:200), H4ac16 (1:500), H2Aac5 (1:1000), H2Bac (1:200), TP2 (1:2000) and PCNA (1:25). After washes in TBS (3×3 min at RT), a secondary antibody (biotinylated goat anti-rabbit diluted 1:600 or biotinylated goat anti-mouse diluted 1:200, Sigma) was applied for 30 min at RT. Sections were then incubated for another 30 min in extravidin conjugated to peroxidase (diluted 1:50, Sigma) and then washed in TBS.

The final detection step was performed by incubating the slides for 10 min in DAB/H_2O_2 peroxidase substrate (diamino-benzidine, Sigma Fast DAB) at RT. Slides were then washed in tap water and stained with PAS (5 min in Periodic acid and 15 min in Schiff reagent at RT, Periodic Acid Schiff-Sigma kit), which specifically stained acrosomes in red. The sections were subsequently dehydrated (3 min each in ethanol 90% and 100%), cleared in xylene (2 min), and mounted with coverslips using Eukitt (Prolabo).

Sections were observed with a transmission light microscope (Axioskop MC80, Zeiss), and spermatids were staged according to the criteria of Russell (1990). In the mouse, the study of serial testis sections allowed the detection of different antibodies on several sections of the same tubule at exactly the same stage.

Spermatogenic cell suspensions

Testicular cells containing cells of all stages were recovered from the testes of an adult mouse (T), whereas testicular cells containing only spermatogonia and Sertoli cells were recovered from the testes of 6–7 days old mice (G). Cell suspensions enriched in mouse spermatogenic cells of specific stages were obtained by velocity sedimentation of testicular cells at unit gravity on a BSA gradient, according to a method described by Bellvé and co-workers (Bellvé, 1993; Romrell et al., 1976). This technique allows germ cell separation according to their respective sizes. The sedimentation carried out on one adult 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). Briefly, after the removal of the tunica albuginea, the mouse testis was incubated in Krebs-Ringer bicarbonate medium (EKRB) containing 1 mg/ml collagenase for 30 min at 37 °C and then with 2 mg/ml trypsin in EKRB for 15 min at 37 °C. The cell suspension was then filtered and centrifuged at 1000 rpm for 10 min. The cells were re-suspended in 18 ml of HAM F12/DMEM containing 0.5% BSA and 1 μg/ml DNase. The cell suspension was allowed to sediment through a 2–4% BSA gradient at 4 °C during 70 min. Thirty-six 10 ml fractions were collected, numbered 1 to 36 from the top of the gradient. Each sample was centrifuged at 1000 rpm for 10 min, re-suspended in 0.5 ml of HAM F12/DMEM medium and examined by phase-contrast microscopy, allowing cell identification. Condensing spermatids (CS) were usually found in fractions 5–7, round and early elongating spermatids (RES) in

fractions 10–12, and spermatocytes at the pachytene stage (P) in fractions 22–23. The different spermatogenic cell suspensions were then either used for immunoblotting or immunofluorescence experiments.

TSA treatment

Spermatogenic cells from the whole testis (T) or from enriched fractions (P, RES or CS) were incubated in TSA (100 ng/ml, diluted in HamF12/DMEM) for three hours at 35 °C, before being spread onto slides for immunofluorescence.

Immunofluorescence

Slides preparation. Testis dissected from the mouse was cut into several parts which were placed into tissue culture medium at room temperature. The slides were prepared according to a protocol which was adapted from the "microspreading" technique described by Hulten et al. (1992). Cell suspensions were made by gently stroking the tubules with tweezers in order to tease out the cellular contents. One drop of the cell suspension was placed on each vectabond-coated slide, and six drops of 2% formaldehyde (freshly prepared from ultrapure formaldehyde, TAAB 16%, Jose Delville Technology) were then added. The slide was allowed to stand for 10 min at RT. All fluid was then poured away, and the slide was gently dipped three times in distilled water.

Immunofluorescence staining. The slides were immediately submitted to the immunofluorescence procedure. A 0.1 mg/ml RNase (Sigma) treatment was applied for 1 hour at 37 °C. The slides were then blocked in three changes of PBT (PBS 1× (Neurobio)/1.5% BSA (Sigma)/0.1% Tween20 (Sigma)) in a coplin jar for at least 30 min each to minimize the background. The cells were incubated overnight with the primary antibody (rabbit anti-histone antibodies) in PBT/0.13% sodium azide at the same dilutions as for immunohistochemistry, in a moist chamber at RT. They were then washed in PBT with agitation and incubated with a mouse anti-rabbit antibody conjugated to FITC (diluted 1:100, Jackson Interchim) for two hours at RT. After being washed again in PBT, the slides were counterstained with 250 ng/ml of the DNA-specific dye PI (propidium iodide).

Microscopy. Immunofluorescence preparations were first screened with an epifluorescence microscope (Axiophot, Zeiss). For 3D analysis of fluorescently labeled specimen, confocal laser scanning microscopy (LSM 410, Zeiss) was applied. Following excitation with the 488 nm line of an argon ion laser, PI and FITC serial optical sections distant of 0.1–0.2 µm were acquired simultaneously using a 63× magnification oil immersion objective with a zoom of 3.9×. The optical sections were then transferred to a Unix workstation (Silicon Graphics) where signals were analyzed by adequate softwares (Edit3D and Ana3D, Parazza et al., 1993) and three-dimensional reconstruction of objects could take place.

Immunoblotting

Histones were extracted from the five cell suspensions (T, G, P, RES and CS) by lysis in protein loading buffer. Samples were analysed by SDS-15% polyacrylamide gel electrophoresis. Proteins were then transferred from gel to HybondC+ membrane at 250 mA for 50 min. Non-specific protein binding was blocked by incubating the membrane in PBS containing 10% skimmed milk and 0.3% Tween20 during 1 h at RT. The membrane was then incubated with specific antibodies (H4ac 1:250; H4ac5 1:800; H4ac8 1:1000; H4ac12 1:400; H4ac16 1:1000; HDAC1 1:200; HDAC6 1:400; TP2 1:1000) diluted in PBS containing 0.2% Tween and 10% FCS, for one hour at RT, washed three times in PBS/0.5 M NaCl, incubated in secondary peroxidase-conjugated antibody (diluted 1:5000 in PBS containing 1% milk and 0.03% Tween20) during one hour at RT, and washed three times in PBS/0.5 M NaCl. Detection was carried out using the ECL Kit (Amersham).

Results

Immunodetection of acetylated histones

Immunodetection of acetylated H3 and H4 (H3ac and H4ac, respectively) on histological preparations of mouse testis, revealed a nuclear staining that was restricted to some nuclei. The staining of spermatids with anti-acetylated H3 (H3ac) antibody was confined to elongating steps 10 and 11 spermatids (stages X and XI, Fig. 1a and 1b). Acetylated H3 did not stain spermatogonia or preleptotene spermatocytes. With the antibody against acetylated H4, early elongating step 8 spermatids (of late tubule stage VIII) and elongating step 9, 10 and 11 spermatids (stages IX, X and XI) showed an intense staining which appeared uniformly distributed over the nucleus (Fig. 1c). In addition, acetylated H4 was found in spermatogonia and preleptotene spermatocytes nuclei from stage II–III to stage VIII (Fig. 1d). All other germinal cells were negative.

Antibodies raised against H2A acetylated at lysine 5 (H2Aac5) and acetylated H2B (H2Bac) were also applied. Staining of acetylated H2A was observed in elongating step 9, 10 and 11 spermatids (stages IX, X and XI, Fig. 1e). H2Aac5 also showed intense staining of spermatogonia and preleptotene spermatocytes (Fig. 1f). In spermatids H2Bac was confined to steps 10 and 11 (stages X and XI, Fig. 1g). H2Bac was also detected in spermatogonia and preleptotene spermatocytes (Fig. 1h).

Histone H4 presents four potential sites of acetylation on lysines 5, 8, 12 and 16. It is likely that specific acetylation on each lysine of the amino-terminal domain has a defined and significant role in chromatin structure and function (Turner et al., 1992; Sobel et al., 1995; Kuo et al., 1996; Kuo and Allis, 1998; Turner, 1998). In order to characterize more precisely the acetylation of H4 in elongating spermatids and spermatogonia/preleptotene spermatocytes, we have attempted to determine which lysines of histone H4 were affected by acetylation at these two stages of mouse spermatogenesis.

Antibodies raised against H4 acetylated at lysine 5 (H4ac5), at lysine 8 (H4ac8), at lysine 12 (H4ac12) and at lysine 16 (H4ac16) were successively used on serial mouse testis sections. All four were positive in elongating steps 8, 9, 10 and 11 spermatids (stages VIII, IX, X and XI, Fig. 2b–e). Spermatogonia and preleptotene spermatocytes were also stained with the antibodies against H4 acetylated at lysine 5, 8 and 16 whereas H4 acetylated at lysine 12 was not detected in these cells (Fig. 2g–j).

The results of the immunohistochemical detection of acetylated histones on paraffin sections of mouse testis are summarized in Fig. 3.

These investigations also showed that histones in pachytene cells are underacetylated. In order to confirm this finding and rule out an accessibility problem with in situ detection of acetylated histones, Western blots were prepared with extracts from adult mouse testis (T), or cell fractions enriched in pachytene spermatocytes (P) or in round and early elongating spermatids (RES). They were probed with anti-H4ac, -H4ac5, -H4ac8 and -H4ac16 antibodies. As expected the antibodies used did not efficiently label histones from pachytene spermatocytes (P) while they reacted strongly with histones from testis (T) or from round and elongating spermatids (RES) (data not shown).

Finally, in order to see if a pattern of H4 acetylation with specific acetylated lysines was only observed in the mouse or if

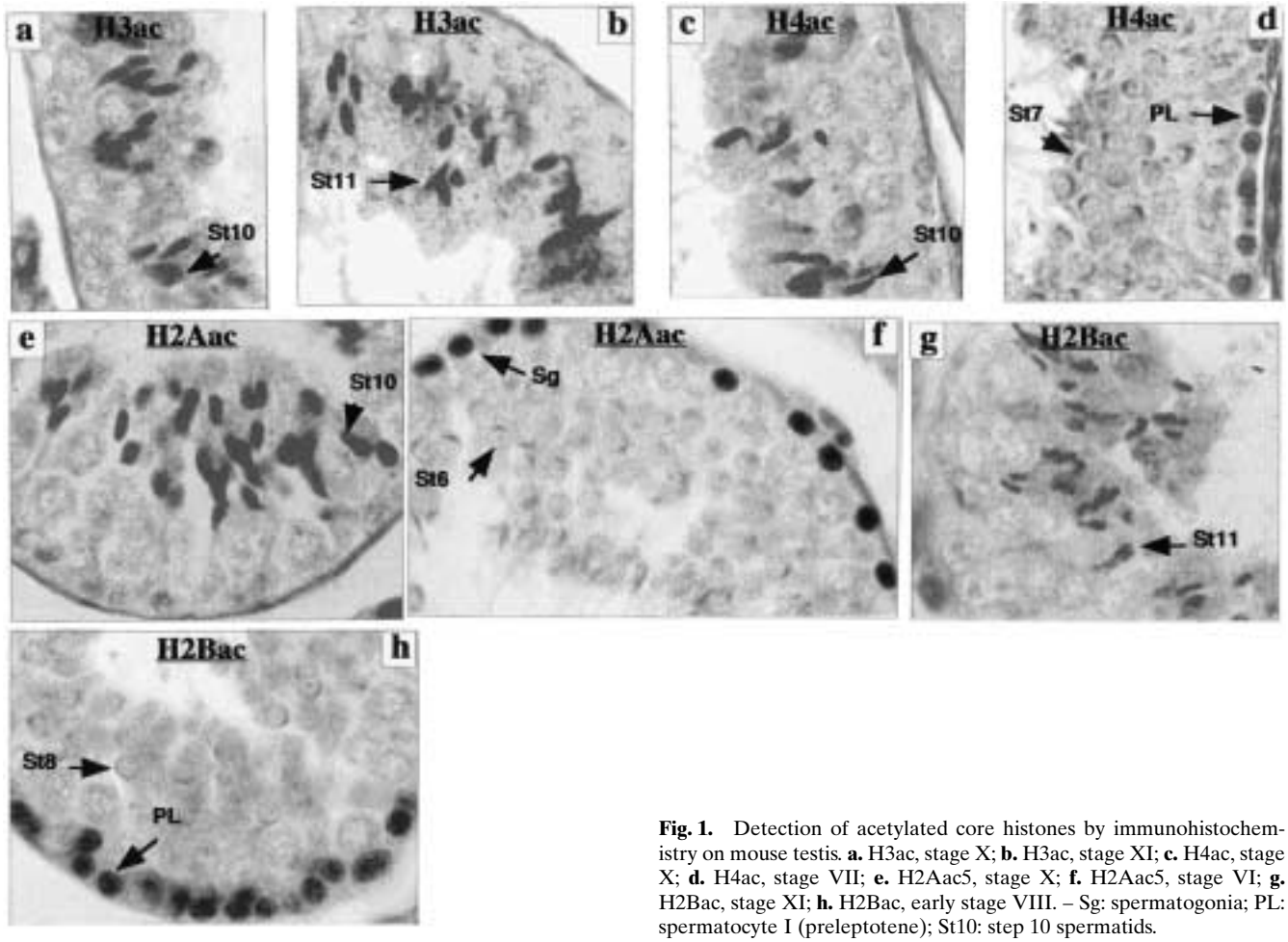

Fig. 1. Detection of acetylated core histones by immunohistochemistry on mouse testis. **a.** H3ac, stage X; **b.** H3ac, stage XI; **c.** H4ac, stage X; **d.** H4ac, stage VII; **e.** H2Aac5, stage X; **f.** H2Aac5, stage VI; **g.** H2Bac, stage XI; **h.** H2Bac, early stage VIII. – Sg: spermatogonia; PL: spermatocyte I (preleptotene); St10: step 10 spermatids.

it was conserved between species, we repeated the same immunohistochemical experiments on human testis. Similar patterns of acetylation were observed on human testis (our unpublished data).

Hence, histone acetylation during mouse and human spermatogenesis affects two stages: the spermatogonia/preleptotene spermatocytes and the spermatids. Between these two stages, at the pachytene stage, a specific histone deacetylation was observed. Histone hyperacetylation could be involved in two major events, which respectively affect these cell stages: premeiotic cell chromatin assembly/transcription in the spermatogonia/preleptotene spermatocyte, and histone replacement by transition proteins and protamines in the spermatid. In order to clarify the significance of histone acetylation observed in spermatogonia and preleptotene spermatocytes and to evaluate the link between acetylation and the proliferative capacities of these cells, we have looked for signs of DNA synthesis activity through spermatogenesis by detecting in situ PCNA protein. PCNA is known to be a part of the DNA polymerase complex and essential for DNA synthesis in both normal cell division and DNA repair.

Immunodetection of PCNA and TP2

PCNA was detected by immunohistochemistry with an anti-PCNA antibody on mouse testis sections. It stained spermatogonia and spermatocytes (Fig. 4a – c) but did not stain the spermatids at any stage of development. PCNA was slightly positive in spermatogonia of stages II – III, IV and V (Fig. 4a). Intense staining appeared in spermatogonia at stage VI, and remained in preleptotene (Fig. 4b), leptotene (Fig. 4c), and zygotene spermatocytes until pachytene spermatocytes of stage I. Thereafter, PCNA staining was still positive but less intense in pachytene spermatocytes of stages II – III up to stage VII (Fig. 4a).

In order to place histone acetylation in the chronology of events leading to their replacement by transition proteins, we have also detected TP2. TP2 was found in elongating spermatids step 11 (stage XI) up to the condensing spermatids step 12 (stage XII) (Fig. 4d and 4e), as shown earlier by Kistler et al. (1996). Therefore, histone acetylation was shown to precede and overlap the presence of transition protein TP2.

3D detection and analysis of acetylated histones

Immunofluorescence (IF) is known to offer more topological resolution than immunohistochemistry (IH). Also, the cell preparation procedure used for IF included a fixation of the cells with formaldehyde, and was different from the fixation in Bouin and slide preparation for IH. It was used with anti-acetylated H4 antibody on testis cell preparations. In order to study the 3D distribution of acetylated histone H4 within the nuclei, cells were spread on the slides in a way that preserved

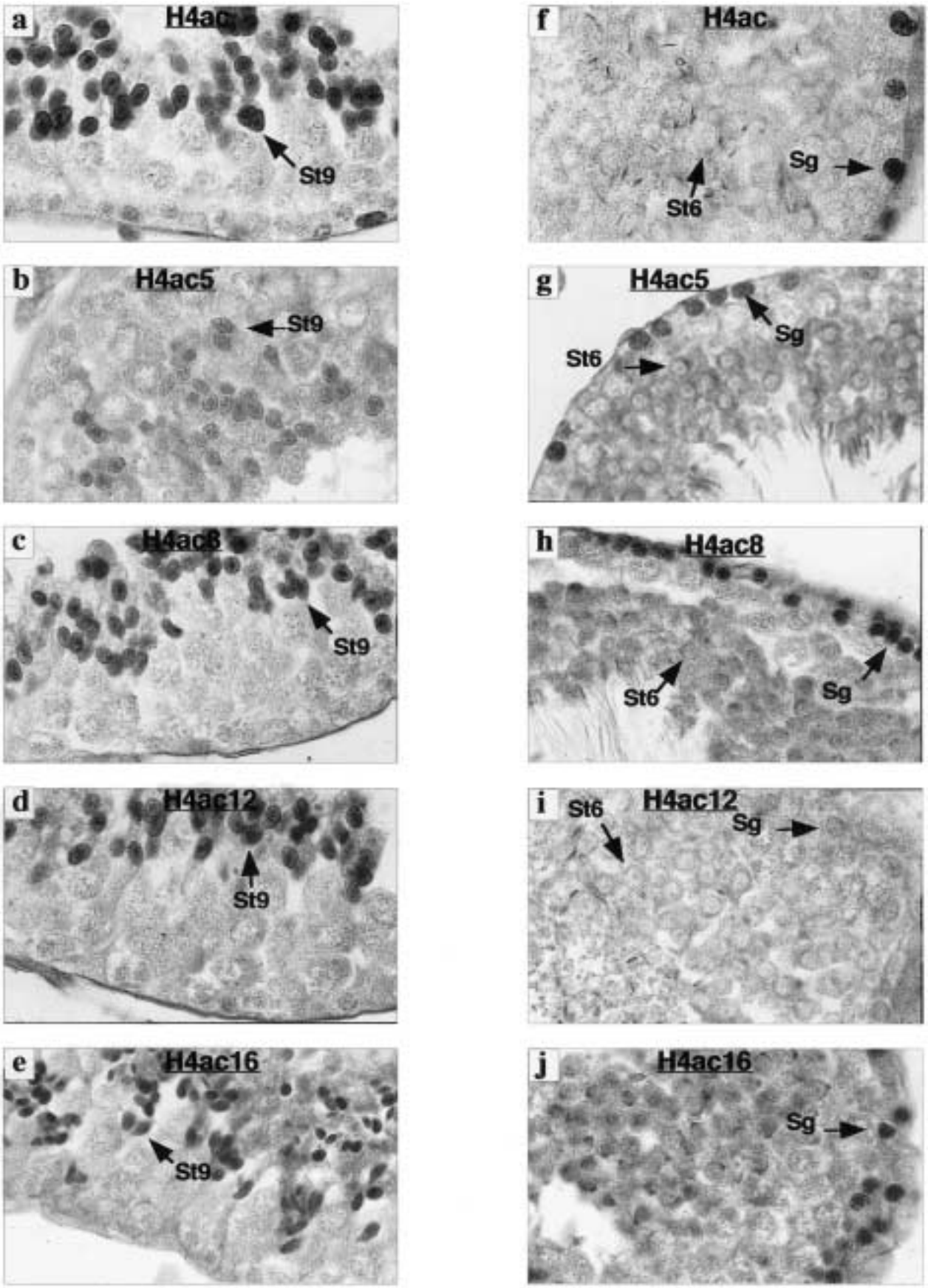

Fig. 2. Immunohistochemical detection of histones H4 acetylated at different lysine sites on mouse testis. **a – e.** stage IX; **f – j.** stage VI; **a, f.** H4ac; **b, g.** H4ac5; **c, h.** H4ac8; **d, i.** H4ac12; **e, j.** H4ac16. – Sg: spermatogonia; St9: step 9 spermatids.

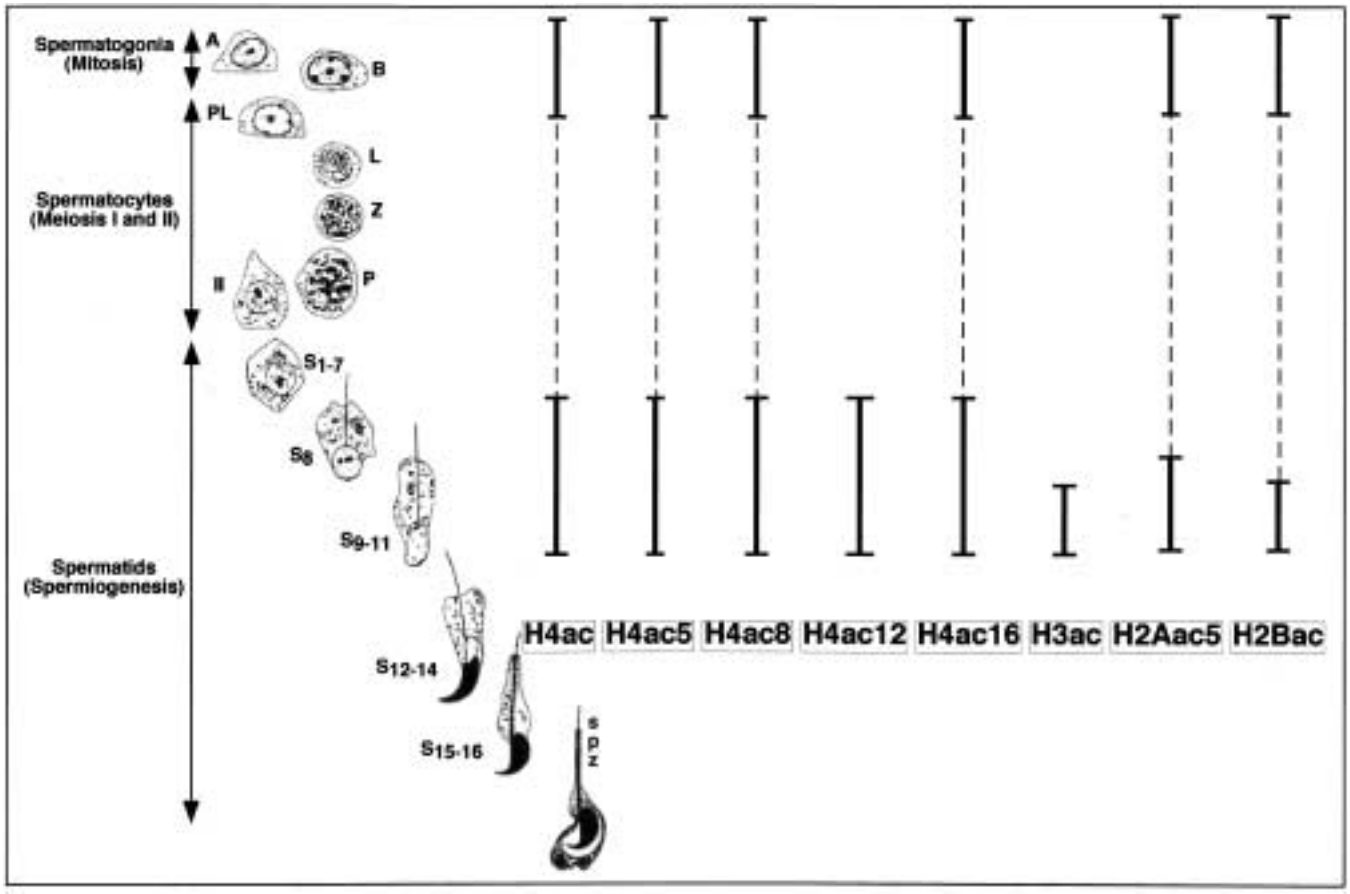

Fig. 3. Chronological appearance of acetylated histones along with mouse spermatogenesis. The depiction of germinal developmental stages was inspired by Russell et al. (1990).

their 3D structure, and immunofluorescence was followed by confocal microscopy. The results are shown in Table I and in Fig. 5.

Some cells were classified as round spermatids thanks to an emerging acrosome structure, which gave a little protuberance at the surface of their nuclei. Most of these round spermatids were negative for the analyzed acetylated histone (also see Fig. 6a). In most early elongating spermatids (steps 8 and early 9, stages VIII and IX), anti-acetylated H4 antibody showed homogeneous staining within the nucleus (Fig. 5a and 5b). In advanced step 9 spermatids, staining began to be slightly heterogeneous in several nuclei (Fig. 5c and 5d). The signal became even more heterogeneous in elongating step 10 spermatids (stage X) where it was mostly concentrated in a few limited regions inside each nucleus (Fig. 5e). Thereafter, the signals occupied a region between the caudal and the central part of the early step 11 spermatid nucleus (Fig. 5f). It was then restricted only to the caudal nuclear area in the late elongating step 11 spermatids (stage XI, Fig. 5g). Acetylated H4 was absent in the condensing spermatids (steps 12 and later). In all analysed spermatids, the DNA was homogeneously stained with propidium iodide (data not shown).

In addition, staining was also observed in round and ellipsoid cell nuclei which, according to their morphological aspect, were more likely to be spermatogonia or preleptotene spermatocytes (although this cannot be ascertained without specific markers). A cloudy signal was found in the volume of all thirty analyzed nuclei (data not shown).

Role of histone deacetylases during spermiogenesis

In order to evaluate the role of histone deacetylases, spermatogenic cells from an adult mouse testis were treated in vitro with TSA, a deacetylase inhibitor. The acetylated histone H4ac was then detected by IF and slides with TSA+(TSA-treated) and TSA- (untreated) spermatogenic cells were

Tab. I: Spermatid nuclei positive for acetylated H4 in immunofluorescence.

Spermatid steps	8	8–9	9	9–10	10	10–11	11
Number of analyzed nuclei (82 nuclei)	8	6	22	8	24	8	6
% of cells showing a non-homogeneous nuclear staining		14%	50%	87%	100%	100%	100%
Description of signals		(Fig. 5a, 5b)	(Fig. 5c)	(Fig. 5d)	(Fig. 5e)	(Fig. 5f)	(Fig. 5g)

Eighty-two positive spermatid nuclei were analyzed in this 3D study. In early elongating spermatids, 14% of the nuclei showed a very slight heterogeneity in their staining. The number of heterogeneously stained nuclei increased towards 50% among step 9 spermatid nuclei to reach 100% for steps 10 and 11 spermatid nuclei. Also, the heterogeneity of the staining pattern increased along with the elongation process.

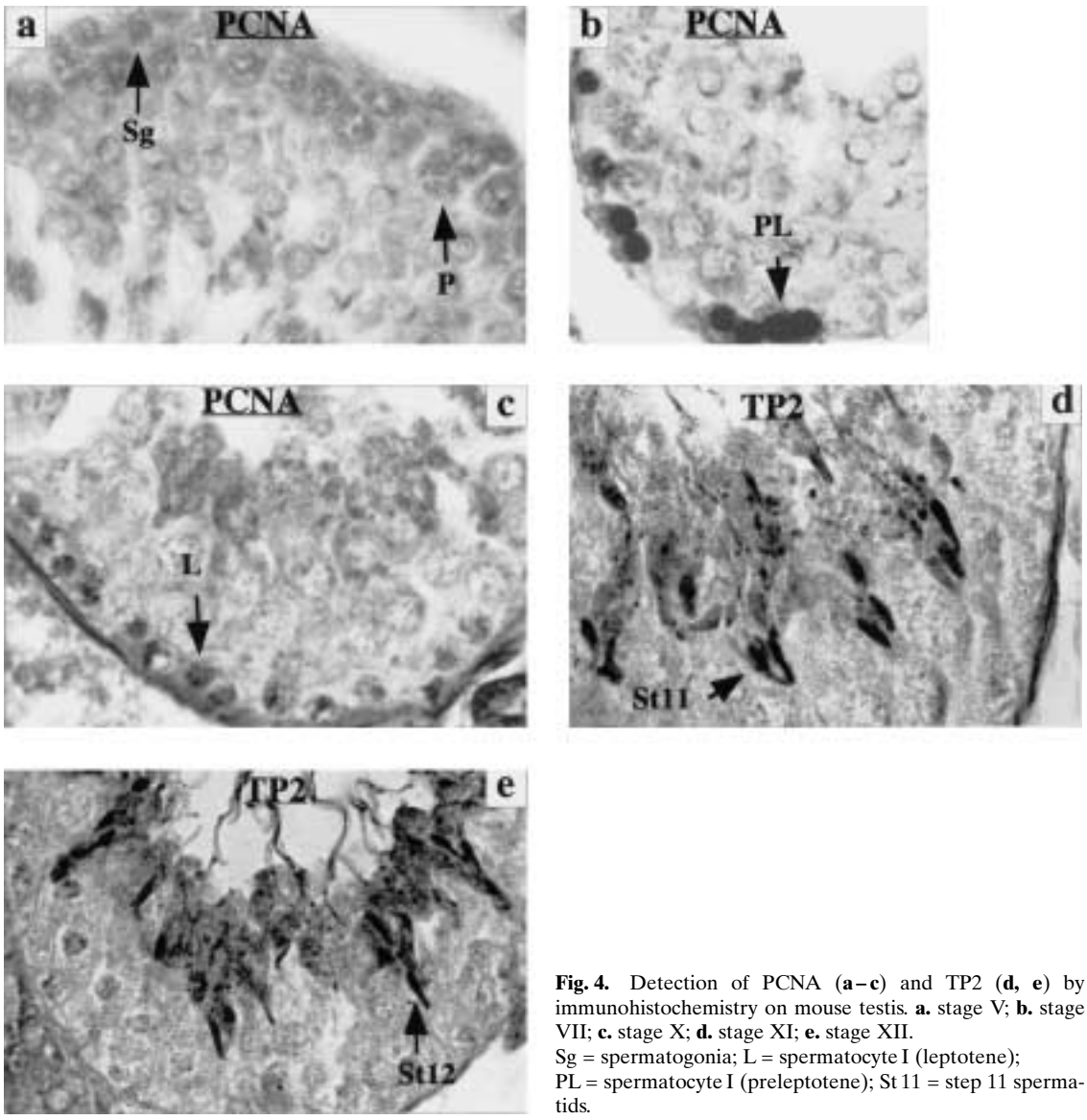

Fig. 4. Detection of PCNA (**a–c**) and TP2 (**d, e**) by immunohistochemistry on mouse testis. **a.** stage V; **b.** stage VII; **c.** stage X; **d.** stage XI; **e.** stage XII.
Sg = spermatogonia; L = spermatocyte I (leptotene); PL = spermatocyte I (preleptotene); St 11 = step 11 spermatids.

compared. Under TSA treatment, a dramatic increase of H4 acetylation was observed in round spermatids (Fig. 6). Both the number of labeled round spermatids and the intensity of the H4ac signal were increased in TSA+cells. In round spermatids, the signals were homogeneous, covering the whole area of the nucleus. This observation suggests that a deacetylase activity is necessary for the maintenance of a histone hypoacetylation in round spermatids. On the contrary, there was no evidence for variations in the content of acetylated histones in elongating and condensing spermatids. Condensed spermatids remained negative with H4ac antibody, event after TSA treatment. This observation suggests that the histones would be displaced/removed in their acetylated state (and not deacetylated prior to their replacement).

The presence of two histone deacetylases, HDAC1 (a member of class I deacetylases) and HDAC6 (a member of class II deacetylases, shown to be over-expressed in the testis, Verdel and Khochbin, 1999) was detected on immunoblots from spermatogenic cells at different stages of maturation, along with the transition protein TP2. Both HDAC1 and HDAC6 were found in the spermatogenic cells from 6 days old mice (G), as well as in the pachytene spermatocytes (P) and in the round and elongating spermatids (RES). Amounts of both deacetylases decreased in condensing spermatids (CS), a stage at which TP2 became positive (Fig. 7).

Discussion

Hyperacetylation had been previously reported for histone H4 in the testis of various species, and was also correlated with spermatid elongation and replacement of histones by protamines (Wolf et al., 1993; Christensen and Dixon, 1982; Christensen et al., 1984; Oliva and Mezquita, 1982; Grimes and Henderson, 1984a, b; Meistrich et al., 1992). In this work, the core histones H2A, H2B, H3 and H4 were shown to be acetylated in mouse spermatogonia and preleptotene spermatocytes, deacetylated throughout meiosis and in round spermatids, and then re-acetylated in elongating spermatids.

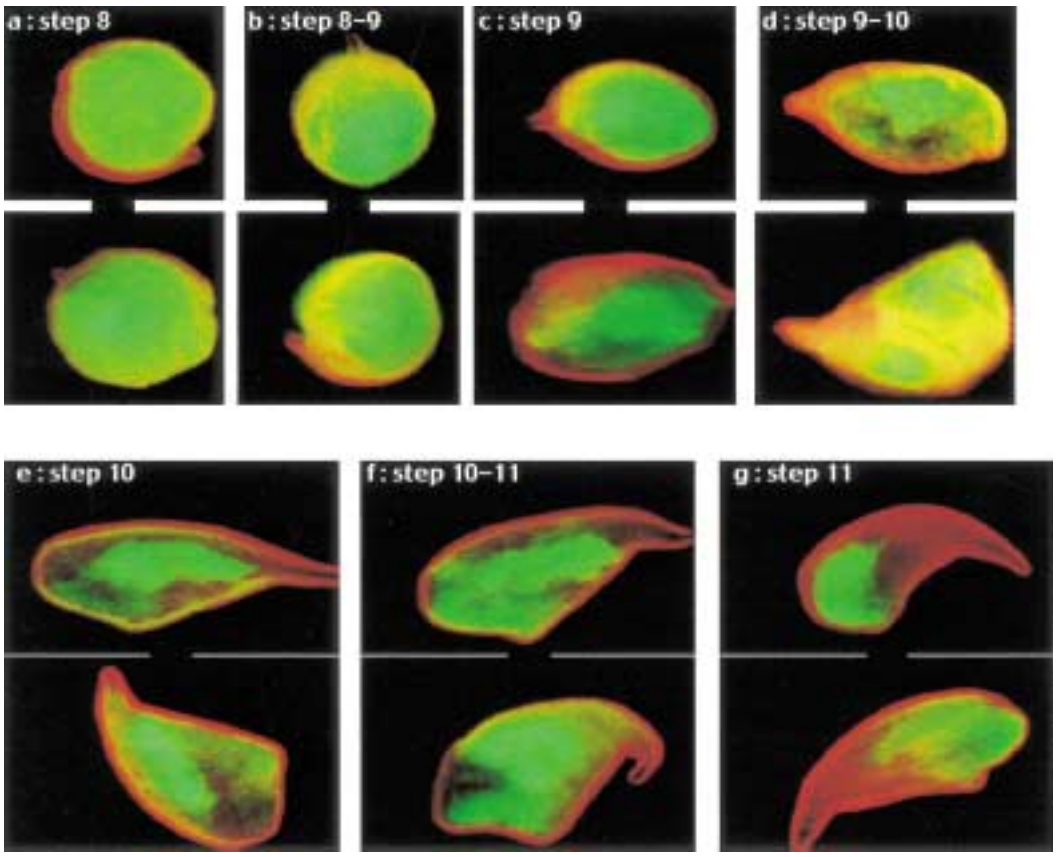

Fig. 5. Sections of spermatid nuclei at different stages of maturation after IF with anti-acetylated H4 and 3D reconstruction: spatial dynamic sequence of acetylated H4 in spermatids during elongation phase of spermiogenesis. Two different nuclei are shown for each of the seven identified spermatid steps. (**a**) step 8 spermatid, (**b**) step 8 – 9 spermatid, (**c**) step 9 spermatid, (**d**) step 9 – 10 spermatid, (**e**) step 10 spermatid, (**f**) step 10 – 11 spermatid, (**g**) step 11 spermatid (for more details, see the description of signals in Table I).

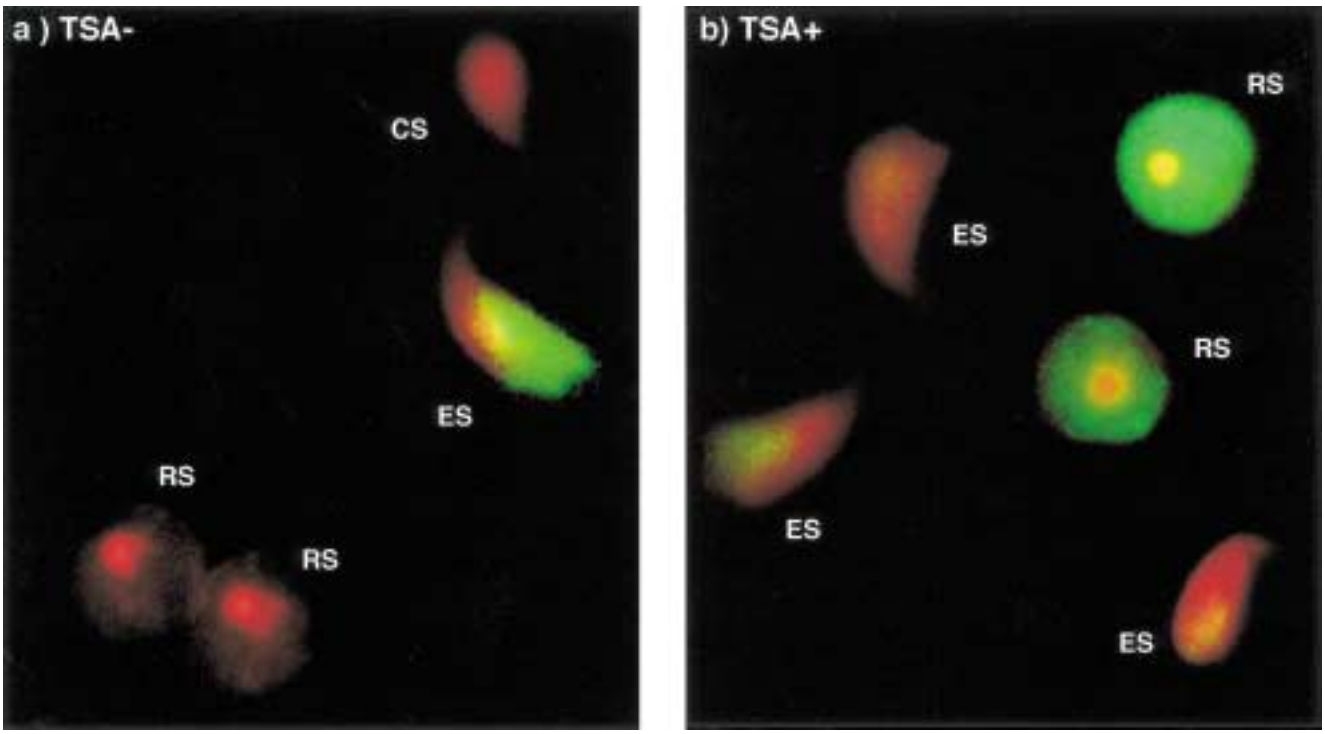

Fig. 6. Effect of TSA treatment on the presence of H4ac in spermatogenic cells detected by IF and analyzed with a CCD camera. Histone H4ac, detected by Alexa 488, is shown in *green* and the nuclei are shown in *red*. **a.** Untreated spermatogenic cells: the two round spermatids (RS) are negative for H4ac, the elongating spermatid (ES) is positive with H4ac located on the caudal part of the nucleus, and the condensing spermatid (CS) is negative. **b.** Spermatogenic cells after a 3-hours treatment with TSA: H4 is acetylated in the two round spermatids (RS), and all three elongating spermatids (ES) contain H4ac in their caudal part.

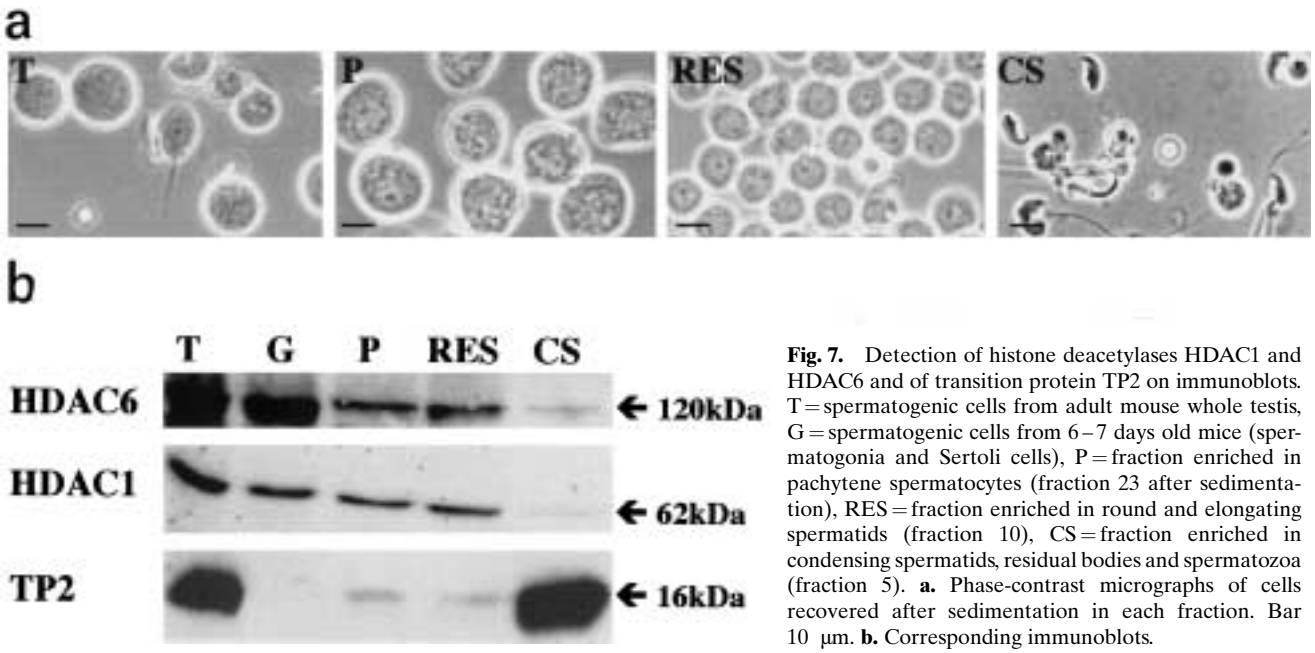

Fig. 7. Detection of histone deacetylases HDAC1 and HDAC6 and of transition protein TP2 on immunoblots. T = spermatogenic cells from adult mouse whole testis, G = spermatogenic cells from 6–7 days old mice (spermatogonia and Sertoli cells), P = fraction enriched in pachytene spermatocytes (fraction 23 after sedimentation), RES = fraction enriched in round and elongating spermatids (fraction 10), CS = fraction enriched in condensing spermatids, residual bodies and spermatozoa (fraction 5). **a.** Phase-contrast micrographs of cells recovered after sedimentation in each fraction. Bar 10 μm. **b.** Corresponding immunoblots.

Each antibody labeled nuclei at specific stages of spermatogenesis giving a specific staining pattern. This could be explained by differences in antibody specificity or in antibody accessibility. However, all antibody staining patterns were restricted to some nuclei and did not stain other cells, where chromatin is known to be more de-condensed and could be therefore more accessible to antibodies. In addition, immunohistochemical staining pattern of acetylated H4 on Bouin-fixed paraffin sections was compatible with the results of immunofluorescence on PFA-fixed cell suspensions. Spermatogenic cells prepared in two different ways gave the same results (Fig. 2 and Fig. 5). Moreover these results were confirmed by Western blot experiments (data not shown). Therefore, the differences in staining patterns are likely to reflect differences in antigen compositions.

Studies have shown that acetylation of specific lysines is likely to be linked to specific functions: for example, in *Drosophila* H4 acetylation at residue 12 is preferentially associated with heterochromatin, whereas H4 acetylation at residue 16 is mostly found in male hyperactive X chromosome (Turner et al., 1992; Bone et al., 1994; Turner, 1998). In the present work, the study of H4 acetylated at specific lysine sites showed that H4 acetylated at lysine 5, lysine 8, lysine 12 and lysine 16 were all detected in elongating spermatids. Therefore, the nuclei of these cells contain histone H4 in its hyperacetylated form. However, H4ac12 could not be detected in spermatogonia or in preleptotene spermatocytes, suggesting that, if H4 acetylation on lysine 12 is involved in heterochromatinisation, this process does not appear to be a major one in these cells.

Spermatogonia are cycling cells, which divide by mitosis, and preleptotene spermatocytes are cells undergoing DNA replication before meiosis. In both these stages, acetylated H4 as well as acetylated H2A and H2B could have a role in histone deposition/displacement in preparation for DNA replication as described at early S phase in *Physarum* by Loidl and Grobner (1987) and during nucleosome assembly (Perry et al., 1993). Similarly, during nucleosome assembly, acetylated chromatin was shown to be the preferential target for H2A/H2B exchange (Perry et al., 1993).

PCNA, which is a part of the DNA polymerase complex, was detected in spermatogonia and several prophase spermatocytes I stages but not in spermatids, as already described for mouse, bovine and human spermatogenesis (Wrobel et al., 1996; Kamel et al., 1997; Steger et al., 1998). In the present study, PCNA gave a weak signal in early spermatogonia (stages II to V) and was intensely positive in late spermatogonia (stage VI onwards), preleptotene, leptotene and zygotene spermatocytes. This is in contrast with the acetylated H4, H2A and H2B, which were positive in all spermatogonia until preleptotene spermatocytes. This difference in staining patterns of the acetylated histones and PCNA antibodies suggests that histone acetylation in these cells is not exclusively related to their proliferative capacity. The presence of PCNA in post replicative stages has been observed in the testes of other species. According to Wrobel et al. (1996), it cannot be entirely explained by the long half-life of PCNA nor by the finding that the S phase of the primary spermatocytes extends into the beginning of the leptotene phase. PCNA might be involved in other processes than DNA replication, such as DNA excision repair.

As the spermatocytes enter meiosis a deacetylation of the core histones occurs and all core histones remain unacetylated throughout the long prophase period, including pachytene stage and in the early stages of post-meiotic maturation in the round spermatids. This is somewhat surprising because spermatocytes as well as round spermatids are known to be actively transcribing cells (Nayernia et al., 1996; Cook, 1997) and transcription was shown to be correlated with histone acetylation (Kuo et al., 1996). However, core histones hyperacetylation could be restricted to regions containing promoters of actively transcribed genes and remain below the detection limits of our methods. Alternatively, transcription happening at this period of spermatogenesis could involve very specific mechanisms, different to those observed in somatic cells.

During post-meiotic spermatid maturation, no replication is known to take place and transcription is thought to stop at the beginning of the elongation phase (Soderstrom and Parvinen, 1976; Kierszenbaum and Tres, 1975, 1978; Nayernia et al.,

1996). Therefore acetylation of histones in elongating spermatids is probably dissociated from these two processes. It has been suggested that histone hyperacetylation is related to their displacement by transition proteins and protamines. Here, transition protein TP2 was detected in mouse step 11 spermatids, where acetylated histones were still present and remained until step 12 condensing spermatids, where acetylated histones were undetectable, showing that acetylation of histones immediately precedes and overlaps the process of their replacement by transition proteins. Other studies in several fish species have suggested that H4 hyperacetylation was a critical factor involved in histones displacement from nuclei by protamines (Oliva et al., 1987). In the rainbow trout, Christensen et al. (1984) observed that hyperacetylated H4 was clustered in certain regions (domains) of late stage testis chromatin and that, within these domains, the chromatin presented a highly relaxed structure. The authors suggested that these domains could represent an initial structural transition that is necessary for the histone removal and protamine replacement processes to take place. Similarly, our 3D data provide evidence for a specific nuclear 3D distribution of acetylated histones during spermatid elongation. Indeed, in the early elongating spermatid (step 8), the acetylated histone H4 presented a homogeneous distribution in the whole volume of the nucleus. It then moved to a more restricted location in a few limited regions within the nucleus of the elongating spermatid (steps 9 and 10) and finally to a caudal position in the late elongating spermatid (step 11), before its disappearance in condensing step 12 spermatids. This antero-caudal disappearance of H4ac seemed to precede the condensation of the nucleus, which also follows an antero-caudal movement, suggesting a tight link between histone replacement and nuclear condensation. These movements could be part of a process, which would regulate the histones/protamines replacement and chromatin remodeling. In the mouse sperm pronucleus after fertilization, Adenot et al. (1997), detecting hyperacetylated H4 by immunofluorescence, suggested a role of histone acetylation in the reverse process of protamines/histones exchange in the zygote.

In order to evaluate the contribution of histone deacetylases to the pattern of histone acetylation during spermatogenesis, spermatogenic cells were treated in vitro with trichostatin A (TSA), a deacetylase inhibitor. A dramatic increase of acetylated H4 was detected by immunofluorescence in the TSA-treated round spermatids, suggesting that a deacetylase activity is necessary to maintain histone hypoacetylation at this stage. It is therefore possible that a specific inhibition of this histone deacetylase activity is responsible for the hyperacetylation of the core histones, in the elongating spermatids. On the contrary, no acetylated H4 was detected in condensed spermatids, even after TSA treatment, suggesting that a deacetylase activity is not necessary for the histone removal, and that the core histones could be displaced in their acetylated state. Factors involved in histone displacement and/or chromatin remodeling could be directly or indirectly recruited by this acetylated chromatin.

In conclusion, H2A, H2B, H3 and H4 were found to be specifically acetylated in spermatogonia/preleptotene spermatocytes and elongating spermatids, whereas a specific histone deacetylation occurs during meiosis from the leptotene/pachytene stage until meiosis is completed, in the round spermatid. In the early elongating spermatids, histone hyperacetylation could be triggered by a specific inhibition of a histone deacetylase activity. Our data also suggest that histone acetylation in the nucleus of the elongating spermatid is associated with histones displacement and also tightly linked to nuclear condensation. In order to characterize more precisely the relationship between histone acetylation, chromatin remodeling and nuclear function, the next step will be the identification of remodeling factors involved in spermatogenesis.

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