Developmental Biology **207,** 322–333 (1999)
Article ID dbio.1998.9155, available online at http://www.idealibrary.com on IDEAL®

in Mouse Spermatogenesis

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Male infertility in *HR6B* knockout mice is associated with impairment of spermatogenesis. The *HR6B* gene is a mammalian, autosomal homolog of the *Saccharomyces cerevisiae* gene *Rad6* encoding a ubiquitin-conjugating enzyme. In addition, X-chromosomal *HR6A* has been identified, in human and mouse. RAD6 in yeast is required for a variety of cellular functions, including sporulation, DNA repair, and mutagenesis. Since RAD6 and its mammalian homologs can ubiquitinate histones *in vitro*, we have investigated the pattern of histone ubiquitination in mouse testis. By immunoblot and immunohistochemical analysis of wild-type mouse testis, a high amount of ubiquitinated H2A (uH2A) was detected in pachytene spermatocytes. This signal became undetectable in round spermatids, but then increased again during a relatively short developmental period, in elongating spermatids. No other ubiquitinated histones were observed. In the *HR6B* knockout mice, we failed to detect an overt defect in the overall pattern of histone ubiquitination. For somatic cell types, it has been shown that histone ubiquitination is associated with destabilization of nucleosomes, in relation to active gene transcription. Unexpectedly, the most intense uH2A signal in pachytene spermatocytes was detected in the sex body, an inactive nuclear structure that contains the heterochromatic X and Y chromosomes. The postmeiotic uH2A immunoexpression in elongating spermatids indicates that nucleosome destabilization induced by histone ubiquitination may play a facilitating role during histone-to-protamine replacement.

Key Words: **ubiquitin; RAD6; chromatin; protamine; testis; spermatogenesis; sex body; histone ubiquitination.**

INTRODUCTION

Homozygous mutation of *HR6B*, homolog of the *Saccharomyces cerevisiae* gene *Rad6* in the mouse, results in male, but not female, infertility (Roest *et al.*, 1996). In yeast, the RAD6 protein is required for a variety of cellular functions, including sporulation, DNA repair, and mutagenesis (reviewed by Lawrence, 1994). Two homologs of *Rad6* have been identified in mouse and man, X-chromosomal *HR6A* and autosomal *HR6B* (Koken *et al.*, 1991; Roest *et al.*, 1996). Both genes are expressed in many tissues, with the highest mRNA expression levels in brain, heart, and testis (Koken *et al.*, 1996). In different cell types, the amounts of HR6A and HR6B protein are similar, but in mouse and rat spermatids a relatively high level of HR6B protein is present (Koken *et al.*, 1996; and unpublished observations). The expression of X-chromosomal HR6A mRNA is strongly reduced in pachytene spermatocytes (Hendriksen *et al.*, 1995; Koken *et al.*, 1996). During this part of the meiotic prophase, the X and Y chromosomes are heterochromatic, forming the so-called sex body, which is transcriptionally inactive (Monesi, 1965). Following completion of the meiotic divisions, the X-chromosomal *HR6A* gene is reactivated. Despite this postmeiotic expression (Hendriksen *et al.*, 1995), there is a relative lack of HR6A protein in round and elongating spermatids (Koken *et al.*, 1996).

HR6A and HR6B are ubiquitin-conjugating enzymes. Ubiquitin is an ubiquitous protein of 76 amino acid residues, which can be covalently attached to cellular substrates through a multistep enzymatic process. Polyubiquitination often marks intracellular proteins for degradation, but it can for example also signal endocytosis of plasma membrane proteins or result in a functional (conformational) alteration of the target protein (reviewed by Varshavsky, 1997). The ubiquitination process involves activities of ubiquitin-activating (E1), -conjugating (E2), and -ligating enzymes (E3) and results in mono- or polyubiquitination of

0012-1606/99 $30.00

cellular substrates. The ubiquitin system is involved in a wide variety of fundamental cellular processes. The *HR6B* knockout mouse is the first mammalian animal model for a defect in any of the enzymes involved in the ubiquitin pathway. The finding of male-limited infertility as the only clear phenotypic defect in the *HR6B* knockout mouse may be explained by the high percentage of identity between mHR6A and mHR6B (96% identical amino acid residues), which implies that mHR6A can most likely functionally compensate for the lack of mHR6B in most tissues of the *HR6B* knockout mice (Roest *et al.*, 1996). Yet, the $HR6B^{-/-}$ phenotype points to a critical role for the ubiquitin system in spermatogenesis. In addition, several other observations indicate that male germ cell development and differentiation have a particular requirement for the ubiquitin system (see Baarends *et al.*, 1998). The level of ubiquitin is high in chicken (Agell and Mezquita, 1988) and mammalian (Lanneau and Loir, 1982) testis, and testis-specific mRNAs are produced through alternative splicing of a heat-shock-inducible polyubiquitin gene in chicken (Mezquita *et al.*, 1997).These findings might be related to the fact that, during spermatogenesis, a high turnover of proteins occurs, such as the turnover of nuclear proteins during the postmeiotic histone-to-protamine transition (Agell and Mezquita, 1988; Oliva and Dixon, 1991). This transition is one of the several reorganizing events, which occur as part of the extensive germ cell differentiation process. Such transitions probably require rapid and massive downregulation of the cellular concentration of a number of proteins and could pose a high demand on ubiquitin-dependent proteolysis, carried out by the 26S proteasome. This multisubunit structure is actively involved in the breakdown of (poly)ubiquitinated proteins (Varshavsky, 1997).

Derailment of spermatogenesis in the *HR6B* knockout mice becomes overt during the postmeiotic condensation of chromatin in spermatids. Based upon the relatively high expression of HR6B in spermatids, and its localization to the nucleus, the underlying mechanism might involve lack of ubiquitination of proteins in spermatids that are actual components of chromatin, or lack of ubiquitination of indirect regulators of chromatin structure. The postmeiotic nuclear elongation and nuclear condensation processes in spermatids require the replacement of nucleosomal histones by transition proteins and subsequently by protamines. Through association of protamines with DNA, linear side-by-side arrays of chromatin are formed. This results in the formation of a nucleus containing tightly compacted DNA, at least sixfold more highly condensed than the DNA in mitotic chromosomes (reviewed by Ward and Coffey, 1991).

It has been shown that the yeast and rabbit RAD6 proteins can ubiquitinate histone H2A and, to a lesser extent, H2B *in vitro* (Haas *et al.*, 1991). In somatic cells of many species, approximately 10% of H2A and 1–1.5% of H2B are ubiquitinated (West and Bonner, 1980). The function of histone ubiquitination is not fully clear. There are indications that histone ubiquitination results in destabilization of the H2A–H2B dimer, which may facilitate replacement by newly synthesized histones and/or potentiate DNA transcription (Li *et al.*, 1993).

Two types of histone modifications have been documented during spermatogenesis. In rat spermatids, occurrence of highly acetylated H4 is associated with histone displacement (Meistrich *et al.*, 1992). Furthermore, during chicken and trout spermatogenesis, ubiquitinated histones were detected during late stages, when nucleosomes are disassembled (Agell *et al.*, 1983; Nickel *et al.*, 1987). To investigate histone ubiquitination during mammalian spermatogenesis, and the possible function of HR6A and/or HR6B therein, we have performed a study to determine the pattern of histone ubiquitination in mouse testis.

MATERIALS AND METHODS

Isolation of Different Cell Types from Mouse Testis

Spermatocytes and round spermatids were purified from 30-day-old and adult mice using collagenase and trypsin treatment, followed by sedimentation at unit gravity (StaPut procedure) and density gradient centrifugation through Percoll (Grootegoed *et al.*, 1986). The purity of the cell fractions was >90% as observed by microscopic analysis of an aliquot of the purified cells fixed in Bouins' fixative on glass slides.

An alternative protocol for purification of spermatocytes involved inclusion of 10 mM iodoacetamide throughout the cell isolation procedure (iodoacetamide inhibits protein deubiquitination; Matsui *et al.*, 1982). To limit negative effects of iodoacetamide on cell viability, this shorter isolation procedure was carried out as follows. Decapsulated testes were shaken (90 cycles/min, amplitude 10 mm) at 32–34°C in 20 ml phosphate-buffered saline (PBS) with Ca^{2+} and Mg^{2+} (PBS + Ca/Mg: 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 1.1 mM $CaCl_2$, 0.5 mM $MgCl_2$), containing 1 mg/ml trypsin (40–110 U/mg; Boehringer Mannheim, Mannheim, Germany), 1 mg/ml collagenase (0.435 U/mg, Boehringer Mannheim), and 0.5 mg/ml hyaluronidase (1000 U/mg, Boehringer Mannheim) in a siliconized 100-ml Erlenmeyer flask for 25 min. The tubule fragments obtained by this enzyme treatment were shaken (120 cycles/min) at 32–34°C in 20 ml PBS without Ca^{2+} and Mg^{2+} (PBS − Ca/Mg: 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4) for 10 min. Large cell clumps were removed using a Pasteur pipette, and the cell suspension was filtered through a 60-μm nylon filter. The obtained cell preparation was centrifuged at 62*g* for 3 min.

Elongating spermatids were isolated from five adult mice that had been irradiated with 1 and 5 Gy on day 1 and day 2, respectively. On day 19, testes were isolated, and one testis was used for histological examination and ubiquitinated H2A (uH2A) immunohistochemistry. The histology demonstrated that the irradiation had resulted in complete lack of spermatocytes and the presence of only very few round spermatids. The uH2A immunoexpression pattern in the remaining germ cell types did not differ from the pattern observed in control testis. The other testes were used for isolation of elongating spermatids using the StaPut procedure, performed as described above, but at 7°C and in the presence of 10 mM iodoacetamide throughout the procedure. Microscopic analysis of the purified cells confirmed the absence of spermatocytes and somatic cells from the cell preparation, which consisted of a mixture of elongating and condensing spermatids.

Isolation of Acid-Soluble Nuclear Proteins

Testes or isolated cell preparations were homogenized in 10 ml of buffer A (100 mM KCl, 10 mM Tris, pH 7.5, 5 mM $MgCl_2$, 10 mM sodium butyrate, 1 mM phenylmethanesulfonyl fluoride, 0.1 μg/ml aprotinin, and 10 mM iodoacetamide) with 0.1% (v/v) NP-40, in an automated potter. Acid-soluble nuclear proteins were isolated according to Davie and Murphy (1994). Proteins were precipitated with 25% trichloroacetic acid and collected by centrifugation. After being washed with acetone/0.006 N HCl and subsequently with acetone, the pellet was dried and the proteins were dissolved in water.

Gel Electrophoresis and Detection of Ubiquitinated Histones

A total amount of 10μg acid-soluble proteins was run on 15% SDS–polyacrylamide gels and blotted on nitrocellulose (0.45 μm) using the Bio-Rad Mini-Protean II electrophoresis and blot cells (Bio-Rad, Hercules, CA). Blotting was performed for 1 h at room temperature in 16.5 mM Tris, 150 mM glycine, 20% (v/v) methanol (pH 8.3). First-dimension acetic acid–urea–Triton (AUT) gels were run according to Davie (1982) and contained 0.8 M acetic acid, 6 M urea, and 0.375% (v/v) Triton X-100. The AUT strips were placed on SDS–PAGE gels and run as described above for separation in the second dimension.

Ubiquitinated histones were detected with α-ubiquitin (α-ubi) (polyclonal; Sigma, St. Louis, MO) or α-uH2A (monoclonal; provided by Dr. Celis, Denmark) (Vassilev *et al.*, 1995). After nonspecific sites were blocked with 2.5% (w/v) nonfat milk in 20 mM Tris–HCl, pH 7.5, 0.15 M NaCl (buffer A), for 1 h at room temperature, α-ubi was diluted 1:500 in buffer A containing 0.2% nonfat milk and incubated with the immunoblot for 90 min. Washings were performed in buffer A with 0.05% (v/v) Tween 20. For the final washing step after the second antibody incubation (peroxidase-labeled goat anti-rabbit; Sigma), 10 mM Tris–HCl, pH 8.0, 0.15 M NaCl, 0.05% Tween 20 was used. For detection with α-uH2A, nonspecific sites were blocked with 1% (w/v) bovine serum albumin (BSA, Fraction V; Sigma) in PBS, 0.1% Tween 20. The α-uH2A antibody was diluted 1:100 in the blocking solution, and the washing steps were performed with PBS, 0.1% Tween 20. After the second antibody reaction (peroxidase-labeled goat anti-mouse and goat anti-rabbit; Sigma), antigen–antibody complexes were detected using a chemiluminescence kit (Du Pont de Nemours, NEN products, Bad Homburg, Germany) according to the instructions provided by the manufacturer.

Immunohistochemistry

Testes were isolated and fixed in phosphate-buffered formalin (30 mM NaH_2PO_4, 45 mM Na_2HPO_4, 4% (v/v) formaldehyde, pH 6.8) for 6–18 h at 4°C, dehydrated, and embedded in paraffin. Eight-micrometer sections were collected on 3-aminopropyltriethoxysilane-coated (Sigma) slides and dried overnight. After deparaffinization, endogenous peroxidase was blocked by incubation in 3% (v/v) H_2O_2 for 20 min, followed by a rinse with tap water. Then the slides were incubated for 20 min in a microwave oven in 0.01 M citric acid, pH 6.0. The slides were allowed to cool down and washed with distilled water and PBS. The slides were blocked for 20 min in PBS, 0.5% BSA, 0.5% nonfat milk, and incubated with α-uH2A (diluted 1:10 in 10% BSA in PBS) overnight at room temperature. Subsequently, the slides were washed in PBS and incubated for 1 h at room temperature with the second antibody (biotinylated goat anti-mouse; DAKO, Glostrup, Denmark) which was diluted 1:200 in PBS containing 2% normal goat serum. The antibody–antigen complexes were detected by incubation for 30 min with avidin–biotin complex reagent (DAKO) according to the protocol supplied by the manufacturer, followed by staining with 3,3′-diaminobenzidine tetrahydrochloride (DAB)/metal concentrate (Pierce, Rockford, IL), counterstaining with hematoxylin, and mounting.

Immunocytochemistry

Surface spreading of mouse meiotic germ cells was performed according to Peters *et al.* (1997). For the antibody reaction, slides were washed extensively in PBS containing 0.05% Triton X-100 and blocked in PBS with 0.05% Triton X-100 and 5% nonfat milk (blocking solution). α-uH2A was added (1:10, in blocking solution) and the incubation was continued overnight. After extensive washing in PBS containing 0.05% Triton X-100, the slides were incubated in PBS containing 5% nonfat milk and 10% normal goat serum for 20 min at room temperature. The same solution was used for second-antibody incubations (FITC-labeled goat anti-mouse; DAKO), and after three washes in PBS the slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA) containing 2.5 μg/ml propidium iodide.

RESULTS

Immunoblot Analysis of Ubiquitinated Histones in Mouse Testis

Basic nuclear proteins were isolated from total testis from 30-day-old mice and analyzed using SDS–PAGE. The amount of ubiquitinated histones was studied using a polyclonal antibody directed against ubiquitin (α-ubi), on immunoblots. The results of these experiments, presented in Fig. 1 (lane 1), show one major and one minor band of approximately 28 kDa, representing the bulk of ubiquitinated histones. The other indicated bands may represent polyubiquitinated histones, and the high-molecular-weight band most likely represents a variety of (poly)ubiquitinated nuclear substrates. A monoclonal antibody that specifically recognizes ubiquitinated H2A (α-uH2A) (Vassilev *et al.*, 1995), also specifically reacted with the major and minor band of approximately 28 kDa, identical to the bands which were detected with α-ubi (Fig. 1, lane 3). The appearance of uH2A as a doublet on SDS–PAGE gels has also been described by others (Davie and Murphy, 1990). Two-dimensional immunoblot analysis was performed, for acid-soluble nuclear proteins, separated on AUT gels in the first dimension and SDS–PAGE gels in the second dimension (Fig. 2A). The two 28-kDa spots, both recognized by α-ubi (Fig. 2B) and α-uH2A (not shown), migrated at the designated uH2A position in the AUT dimension. No other ubiquitinated histone species were detected.

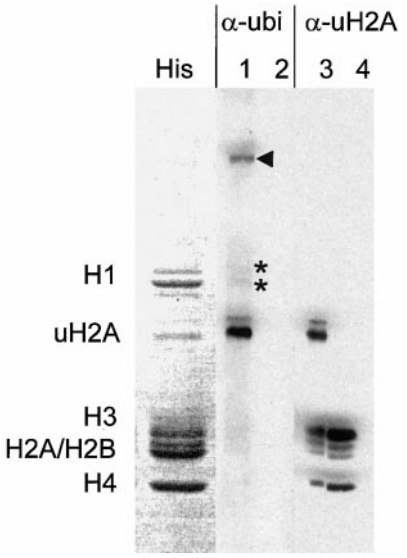

FIG. 1. Analysis of ubiquitinated histones in nuclear extracts from mouse testis. Basic nuclear proteins from total mouse testis were analyzed on 15% SDS–PAGE gels as described under Materials and Methods. His, Ponceau-stained representative lane of the immunoblot. Lanes 1, α-ubi; 2, control (no first antibody); 3, α-uH2A; 4, control (no first antibody). The positions of the major histones and uH2A are indicated. *Possible polyubiquitinated histone forms; arrowhead, polyubiquitinated basic nuclear proteins. Note that the peroxidase-conjugated anti-mouse antibody reacts aspecifically with a number of histone proteins (lane 4). In a different experiment, using another batch of second antibody, the results clearly showed that the first antibody does not react with the unmodified histones (not shown).

Immunohistochemical Analysis Reveals a Distinct Nuclear Localization of uH2A, Primarily in Pachytene Spermatocytes and Elongating Spermatids

Testicular uH2A was localized using the monoclonal α-uH2A on formaldehyde-fixed testis tissue sections. The control experiment revealed no aspecific staining, except in the interstitium (due to the second antibody), so no information concerning uH2A in Leydig cells and peritubular myoid cells could be obtained using this assay (Fig. 3A). Within the tubules, Sertoli cells and spermatogonia were frequently found to be positive (Fig. 3C). A marked uH2A signal was present during the prophase of meiosis I, in pachytene spermatocytes. In these cells, a distinct structural and temporal staining pattern was observed. First, during early pachytene, uH2A was most clearly present in a specific round subregion of the nucleus, located in the periphery. This region most likely is the sex body, a distinct nuclear structure containing the X and Y chromosomes (Fig. 3C). Subsequently, during midpachytene, the granular pattern of α-uH2A staining extended and covered the whole nucleus (Fig. 3C). Late pachytene spermatocytes lost most of the signal, finally also from the sex body (Fig. 3C). No uH2A staining was apparent during the diplotene stage and the subsequent meiotic divisions. Postmeiotically, uH2A was observed only in nuclei of steps 8–12 elongating spermatids (Figs. 3B and 3C).

Immunocytochemical Analysis of Pachytene Spermatocytes Confirms the Presence of uH2A in the Sex Body

Surface spreads of spermatocytes were used to study the exact intranuclear localization of uH2A (Fig. 4). Clear and granular uH2A staining was found in a rounded peripheral

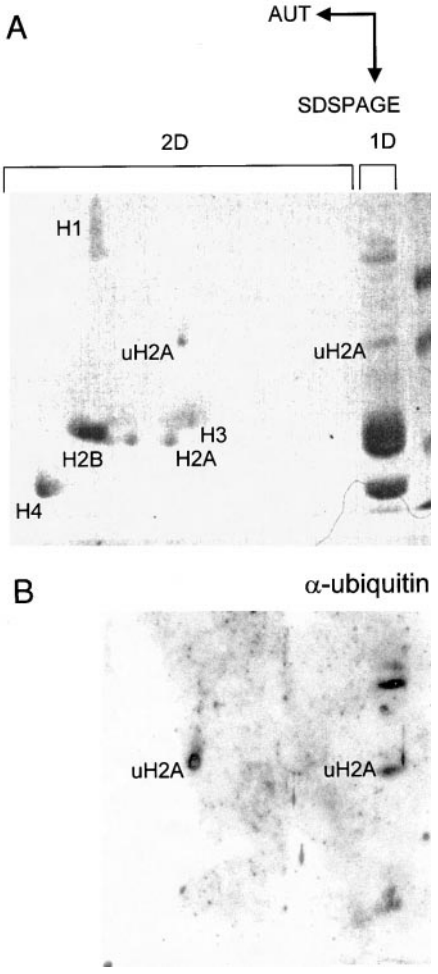

FIG. 2. Two-dimensional electrophoretic analysis of ubiquitinated histones in nuclear extracts from mouse testis. Basic nuclear proteins were analyzed on AUT SDS–PAGE two-dimensional gels (AUT, first dimension; SDS–PAGE, second dimension); one lane containing a one-dimensional SDS–PAGE analysis was included. (A) Ponceau-stained Western blot; the approximate localizations of H1, H2A, H2B, H3, and H4 spots are indicated. (B) Western blot reacted with α-ubi; the specifically recognized spots are indicated.

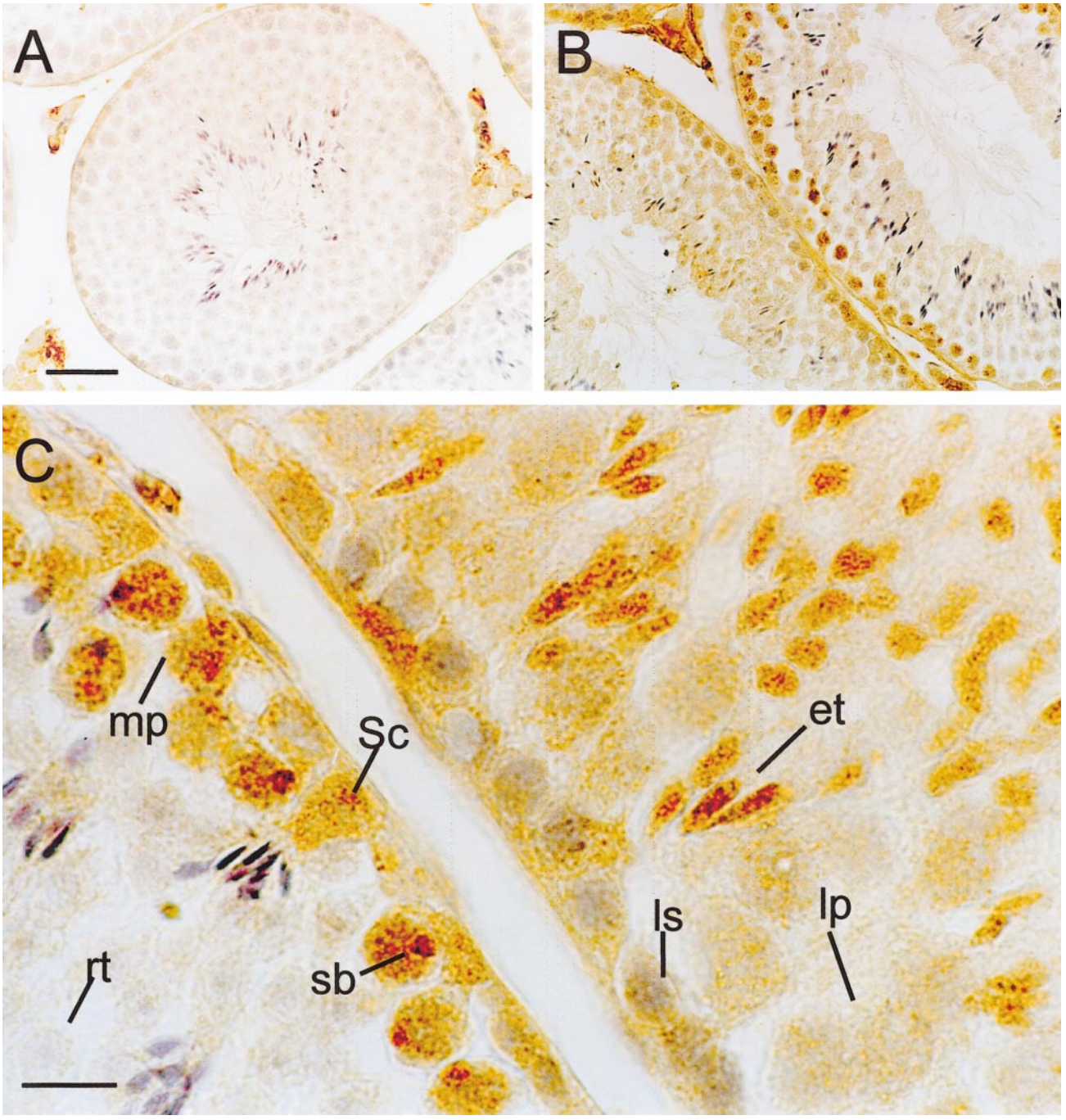

FIG. 3. uH2A immunoexpression in mouse testis. Formaldehyde-fixed mouse testis sections were incubated without (A) or with (B, C) the α-uH2A monoclonal antibody. After the second antibody reaction, the antigen–antibody complexes were detected through DAB staining (brown). (A) Negative control; some nonspecific staining covering the interstitial cells and the tubule lining can be observed. Scale bar represents 50 μm. (B) Representative uH2A immunostaining in wild-type testis viewed at the same magnification as in (A); note the clear uH2A immunoexpression in midpachytene spermatocytes in the right tubule and the very low signal in the early pachytene cells of the left tubule. Also, some staining is still found in a few condensing spermatids (step 13) of the left tubule. (C) Higher magnification of uH2A immunoexpression in midpachytene spermatocytes (mp), in particular in the sex body (sb), and in elongating spermatids (et) and Sertoli cells (Sc). A very low uH2A level is observed in late pachytene spermatocytes (lp), and no specific uH2A staining can be detected in round spermatids (rt) and leptotene spermatocytes (ls). Scale bar represents 10 μm.

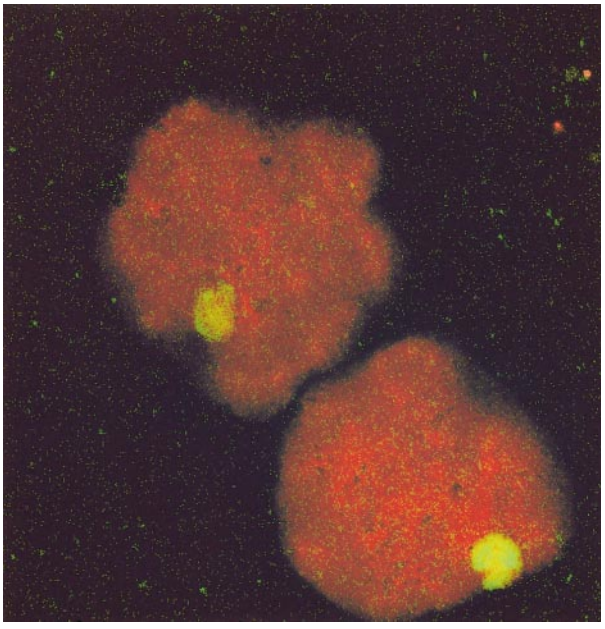

FIG. 4. Immunocytochemical detection of uH2A in the sex body of pachytene spermatocytes. Spermatocytes were spread on glass slides and uH2A was detected using α-uH2A antibodies in an immunofluorescence staining procedure (see Materials and Methods). The nuclear material is counterstained with propidium iodide (red) and the green staining reflects uH2A immunoexpression in the condensed X and Y chromosomes, forming the so-called sex body. Using this method, uH2A immunoexpression in the autosomes was low or undetectable.

region, one in each nucleus. Counterstaining of the DNA with propidium iodide showed that the uH2A signal covered the more densely stained sex chromosomal DNA.

The Amount of uH2A Varies during Postnatal Testis Development and Differs between Specific Isolated Germ Cell Types

Using immunoblot analysis, it was observed that the testicular uH2A level was higher in 7-day-old mice, compared to mice of 3 to 4 weeks of age (Fig. 5A). This indicates that during progression of the first wave of spermatogenesis, a relatively low level of uH2A in certain testicular cell types results in a decrease of the total uH2A amount/mg protein. To investigate this in more detail, and to confirm the uH2A immunolocalization pattern, we expected to be able to study the uH2A level in basic nuclear extracts from different isolated testicular germ cell types on immunoblots. However, we found that the amount of ubiquitinated histones in a total germ cell preparation clearly decreased during incubation in PBS at room temperature for 1 h or longer (not shown), apparently as a result of protein deubiquitination. Hence, the StaPut purification procedure was carried out at 7°C and in the presence of iodoacetamide which inhibits deubiquitination (Fig. 5B) through inactivation of deubiquitination enzymes which require free sulfhydryl groups (Matsui *et al.*, 1982). Unfortunately, it was found that, under these conditions, spermatids did not survive the Percoll gradient centrifugation step that follows the StaPut procedure and is performed to further purify the cells. Thus, highly purified cell preparations of spermatocytes and round spermatids can be obtained with a combined StaPut and Percoll purification procedure, but only without the addition of iodoacetamide. When isolated in the absence of iodoacetamide, the pachytene spermatocytes still contained a relatively large amount of ubiquitinated histones, but no detectable amount of ubiquitinated histones was found in nuclear extracts from isolated round spermatids (Fig. 5C). In view of possible deubiquitination that occurs during cell isolation, these results do not exclude a low level of ubiquitinated histones in round spermatids. On the other hand, the immunoblot results are in agreement with the immunohistochemical observations, which also show a lack of uH2A in round spermatids. Furthermore, the observed developmental decrease of the testicular uH2A level/mg protein during the initiation of spermatogenesis (Fig. 5A) can be explained by the first appearance of an uH2A-negative spermatid fraction, which constitutes an increasing proportion of the testicular cell population from 14 to 28 days. However, developmental changes in the uH2A level of somatic cell types of the testis cannot be excluded. Elongating and early condensing spermatids (steps 8–12), which did show histological uH2A immunoexpression, represent only a small portion of the total germ cell population, and these cells are difficult to purify with StaPut and Percoll methods due to their broad sedimentation pattern, which overlaps with the sedimentation of round spermatids and also partially with that of spermatocytes (Meistrich *et al.*, 1973). In order to obtain a preparation of these cells, we used an approach that involved *in vivo* elimination of all spermatocytes and most of the round spermatids, through γ-irradiation of mice (see Materials and Methods). Histological analysis of a representative γ-irradiated testis confirmed the absence of spermatocytes and most of the round spermatids, with the elongating and condensing spermatids still being present. A highly purified fraction of elongating and condensing spermatids was isolated from these γ-irradiated mouse testes, in the presence of iodoacetamide at 7°C using the StaPut procedure. Ubiquitinated histones were detected in this fraction, albeit at a lower level than in a spermatocyte-enriched germ cell preparation that was isolated in parallel from testes of nonirradiated mice (Fig. 5D).

Preparations enriched for different somatic cell types of the testis, in particular Leydig cells, peritubular myoid cells, and Sertoli cells, all contained ubiquitinated H2A (not shown).

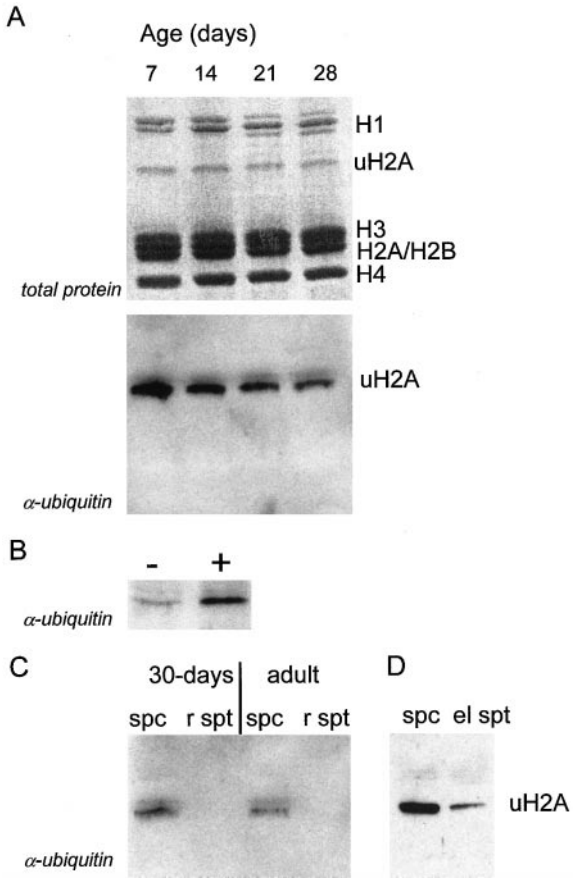

FIG. 5. Changes in uH2A level during mouse testis and germ cell development. (A, top) Basic nuclear proteins from testes of 7-, 14-, 21-, and 28-day-old mice, showing equal amounts of protein present in each lane; (bottom) immunoblot reacted with α-ubi antibodies. (B) α-Ubi immunostaining of uH2A present in basic nuclear proteins from an enriched spermatocyte preparation. The spermatocytes were isolated in the absence (−) or presence (+) of iodoacetamide. (C) α-Ubi immunostaining of uH2A present in basic nuclear proteins from spermatocytes (spc) and round spermatids (r spt), isolated as described under Materials and Methods from immature (30 days) and mature (adult) mice. (D) α-Ubi immunostaining of uH2A present in basic nuclear proteins from an enriched pachytene spermatocyte preparation (isolated in the presence of iodoacetamide) (spc) and elongating spermatids (el spt). The elongating spermatids were isolated from testis of γ-irradiated mice using the StaPut procedure (at 7°C, with 10 mM iodoacetamide) (see Materials and Methods and Results). An equal amount of protein was present per lane in gels from B–D, results from the Ponceau-stained Western blot are not shown.

Comparison between Wild-Type and HR6B Knockout Mouse Testes Reveals No Overt Differences in the Amount and Localization of uH2A

Acid-soluble nuclear proteins were obtained from StaPut/Percoll preparations of pachytene spermatocytes and round spermatids, isolated in the absence of iodoacetamide, from 30-day-old wild-type and *HR6B* knockout mice. Animals of this age were used because the first wave of spermatogenesis in *HR6B* knockout mice starts apparently normally, permitting isolation of populations of pachytene spermatocytes and round spermatids. The impairment of spermatogenesis starts to occur with the appearance of elongating and condensing spermatids, and such late spermatids cannot be isolated in sufficient quantity, neither from immature nor from adult *HR6B* knockout mice. For wild-type mouse testis, no difference was found in the amount of ubiquitinated histones in germ cell preparations isolated from 30-day-old and adult mice (Fig. 5C). Then, when we analyzed wild-type versus knockout, immunoblot analysis revealed that there is no overt difference in the amounts of uH2A between spermatocytes of wild-type and knockout mouse testes. As expected on the basis of the results described above, no signal was observed for the isolated round spermatids (Fig. 6).

Immunolocalization of uH2A in 6-week-old *HR6B* knockout mouse testis showed the presence of uH2A in spermatocytes, in a pattern very similar to that of wild-type mouse testis (Figs. 7B and 7D). The nuclear uH2A staining in elongating spermatids of *HR6B* knockout mice was different from the staining observed for wild-type testis, due to the aberrant shape of the spermatid nuclei of the knockout testis and the overall disorganization of the spermatogenic epithelium containing the late spermatids. However, the remaining elongating spermatids in *HR6B* knockout mice still seem to contain a considerable amount of uH2A. It is certain that subtle quantitative differences in the amount of ubiquitinated histones present in nuclei of germ cell types from wild-type and knockout mouse testes cannot be detected using these immunoblotting and immunohistochemical methods.

DISCUSSION

Two-dimensional gel electrophoresis and immunoblot analysis of basic nuclear proteins isolated from total mouse testis revealed that the major ubiquitinated histone in mouse testis is H2A. Ubiquitination of other histones was not detected. This result was confirmed by the finding that a polyclonal anti-ubiquitin antibody and a monoclonal anti-uH2A antibody both recognized two bands around 28 kDa on SDS–PAGE gels. The variation in the amount of uH2A during spermatogenesis in the mouse was studied using immunohistochemistry and also by means of immunoblot analysis of basic nuclear proteins isolated from purified germ cell preparations. The immunoblot results confirm the immunohistochemical data, as schematically summarized in Fig. 8. The amount of uH2A was maximal during the pachytene stage of the meiotic prophase. The uH2A immunoexpression in pachytene nuclei was first limited mainly to the sex body (early pachytene), then it

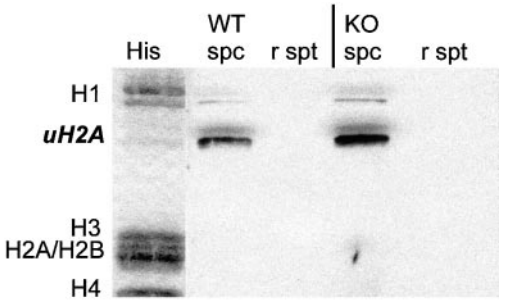

FIG. 6. Level of uH2A in isolated spermatocytes and round spermatids from wild-type and *HR6B* knockout mice. Spermatocytes (spc) and round spermatids (r spt) were isolated from wild-type (WT) and *HR6B* knockout (KO) mouse testes, using the StaPut/Percoll procedure as described under Materials and Methods. uH2A was detected with α-ubi. His, Ponceau-stained lane of the Western blot containing basic nuclear proteins from wild-type spermatocytes; this amount of protein was present in all lanes (not shown).

covered the whole nucleus (midpachytene) and was subsequently again limited to the sex body of late pachytene spermatocytes.

Thus, we found the highest uH2A level in the condensed and transcriptionally inactive XY chromatin of the sex body (Monesi, 1965; Oud *et al.*, 1979). During midpachytene, uH2A staining on the transcriptionally active autosomal chromatin becomes clearly visible, but the sex body still remains the most densely stained structure, even when the sex body chromatin partially decondenses, during late pachytene. Although we cannot formally exclude the possibility that the monoclonal anti-uH2A antibody recognizes an epitope in the sex body chromatin that is not part of uH2A, the finding that this antibody recognizes only uH2A and no other basic nuclear testicular protein on immunoblots, combined with the observation that anti-uH2A also specifically binds in an evenly distributed manner to the sex body chromatin of spread spermatocyte nuclei, strongly supports the conclusion that uH2A is enriched in the sex body chromatin. This is a surprising finding, in light of the fact that deubiquitination is currently viewed as a general aspect of chromosome condensation (Matsui *et al.*, 1979). Although the exact function of histone ubiquitination is not known, it has been shown that this nuclear protein modification alters nucleosome stability and thereby may facilitate processes that require access to DNA (Li *et al.*, 1993). It could be hypothesized that histone ubiquitination during pachytene facilitates replacement of somatic histones by the testis-specific isotypes that are synthesized in pachytene spermatocytes, such as testis-specific H1t (Fig. 8) (Drabent *et al.*, 1996).

There are some data that point to a direct functional link of ubiquitination of H2B, and to a lesser extent that of H2A, with gene transcription (Nickel *et al.*, 1989; Davie and Murphy, 1990, 1994; Davie *et al.*, 1991). However, it is uncertain whether this role of histone ubiquitination is a general phenomenon. Certainly, the present results on the X and Y chromosomes of the sex body indicate that uH2A can also localize to inactive, relatively condensed chromatin. There is one report that describes a similar correlation between inactive chromatin and ubiquitin immunolocalization, for polytene chromosomes of *Drosophila* (Izquierdo, 1994). Also, it is of interest to note that there is genetic evidence for yeast (Moazed and Johnson, 1996; Huang *et al.*, 1997) and *Drosophila* (Henchoz *et al.*, 1996) that ubiquitination of unknown substrates stimulates silencing of certain genes.

The observed lack of histone ubiquitination in round spermatids, both in isolated cell preparations and in testis tissue sections, is a very unexpected finding. A different result was recently reported by Chen *et al.* (1998), who found a moderate uH2A level in round spermatid nuclei isolated from rats. The difference with our finding might very well be explained by the fact that the round spermatid preparation as isolated by Chen *et al.* (1998) still contained a considerable amount of spermatocytes (11%) and somatic cells (11%). Furthermore, species differences might play a role, although this seems less likely, since we have found similar uH2A immunoexpression patterns in mouse and rat tissue sections (unpublished observations).

Thus far no mammalian cell type without ubiquitinated histones has been described, apart from cell-cycle dependent deubiquitination and reubiquitination, at metaphase and anaphase, respectively (Matsui *et al.*, 1979; Wu *et al.*, 1981). Although we cannot exclude that a very small amount of ubiquitinated histones is present in round spermatids, but is lost during the isolation procedure, it is clear that in comparison with pachytene spermatocytes the amount of uH2A has dramatically decreased after completion of the meiotic divisions. Possibly, there is increased deubiquitinating activity, just prior to the first meiotic division, which causes the loss of uH2A from diplotene spermatocytes. This would be comparable to histone deubiquitination during, or just prior to, mitotic metaphase. Throughout the second meiotic division and the first seven steps of spermiogenesis, deubiquitination rather than ubiquitination remains the prevailing steady-state situation. In steps 8–12 spermatids, ubiquitinated histones reappear, as observed both through immunohistochemistry and through immunoblot analysis.

Histone deubiquitination in somatic cells and also in spermatogenic cells is most likely catalyzed by one of the members of the large family of deubiquitinating enzymes (DUBs). The specific enzyme involved in the mitotic cell-cycle-dependent mechanism has not been identified (Wilkinson, 1997). One of the DUB enzymes, PGP9.5, is selectively expressed in brain and testis of rat (Kajimoto *et al.*, 1992). Furthermore, human and mouse homologues of the *Drosophila Fat facets* gene, which encodes a DUB involved in eye development and oogenesis, are located on the X and Y chromosomes (Jones *et al.*, 1996; Brown *et al.*, 1998). The X-chromosomal gene (*DFFRX/Dffrx*) is ubiquitously expressed, but with enhanced expression in both

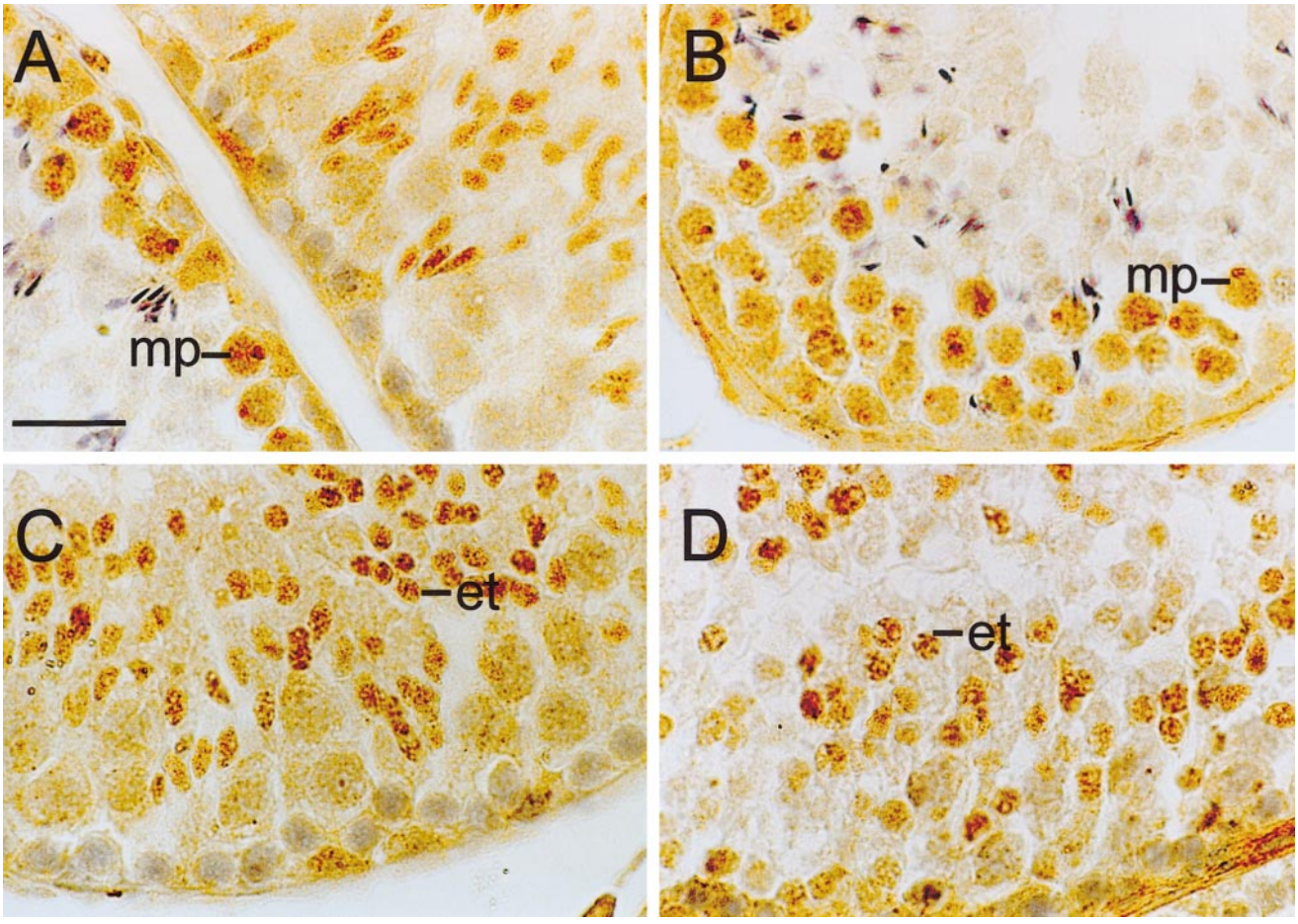

FIG. 7. uH2A immunoexpression in testes from wild-type and *HR6B* knockout mice. (A and C) Wild-type testis sections reacted with α-uH2A. (B and D) *HR6B* knockout testis sections reacted with α-uH2A. No detectable change in uH2A staining of pachytene spermatocytes (compare A to B) and early elongating (steps 8–9) spermatids (compare C to D) was observed in testes from *HR6B* knockout mice. mp, pachytene spermatocyte; et, elongating spermatid. Scale bar represents 30 μm.

human and mouse testis. The Y-chromosomal gene (*DFFRY/Dffry*, 89% identical base pairs with the X-chromosomal gene) is ubiquitously expressed in the human and expressed in a testis-specific manner in the mouse. This latter gene maps to the infertility-associated regions AZFa (human) and Sxr^b (mouse) (Brown *et al.*, 1998). It will be of much interest to determine whether any of the genes encoding DUB enzymes show enhanced expression in round spermatids.

When the experiments described herein were started, our main aim was to test the hypothesis that histone ubiquitination occurs during the histone-to-protamine transition, and that this process is impaired through loss-of-function of one of the two mammalian homologs of yeast *RAD6*, in *HR6B* knockout mice. This hypothesis was based on three observations concerning RAD6 function in yeast. First, RAD6 is able to ubiquitinate histones *in vitro* (Jentsch *et al.*, 1987). Second, this ubiquitination requires an intact polyacidic C-terminal tail of RAD6, and this tail is also essential for sporulation (reviewed by Lawrence, 1994). Third, although histone ubiquitination *in vivo* in yeast has not been detected, and mutation of the ubiquitination site in the H2A protein does not reduce yeast cell viability, this mutation exerts a small negative effect upon sporulation efficiency (Swerdlow *et al.*, 1990).

The presence of uH2A in elongating spermatids, with the relatively high immunoexpression of uH2A in the nuclei of these cells, immediately precedes the replacement of histones by transition proteins. This may point to a functional role for histone ubiquitination in the histone-to-protamine replacement. There is overall agreement with the findings of increased histone ubiquitination during late phases of spermatogenesis in chicken and trout (Agell *et al.*, 1983; Nickel *et al.*, 1987). Very recently, when the experiments described herein were completed, ubiquitinated H3 was detected in elongating spermatids from rat (Chen *et al.*,

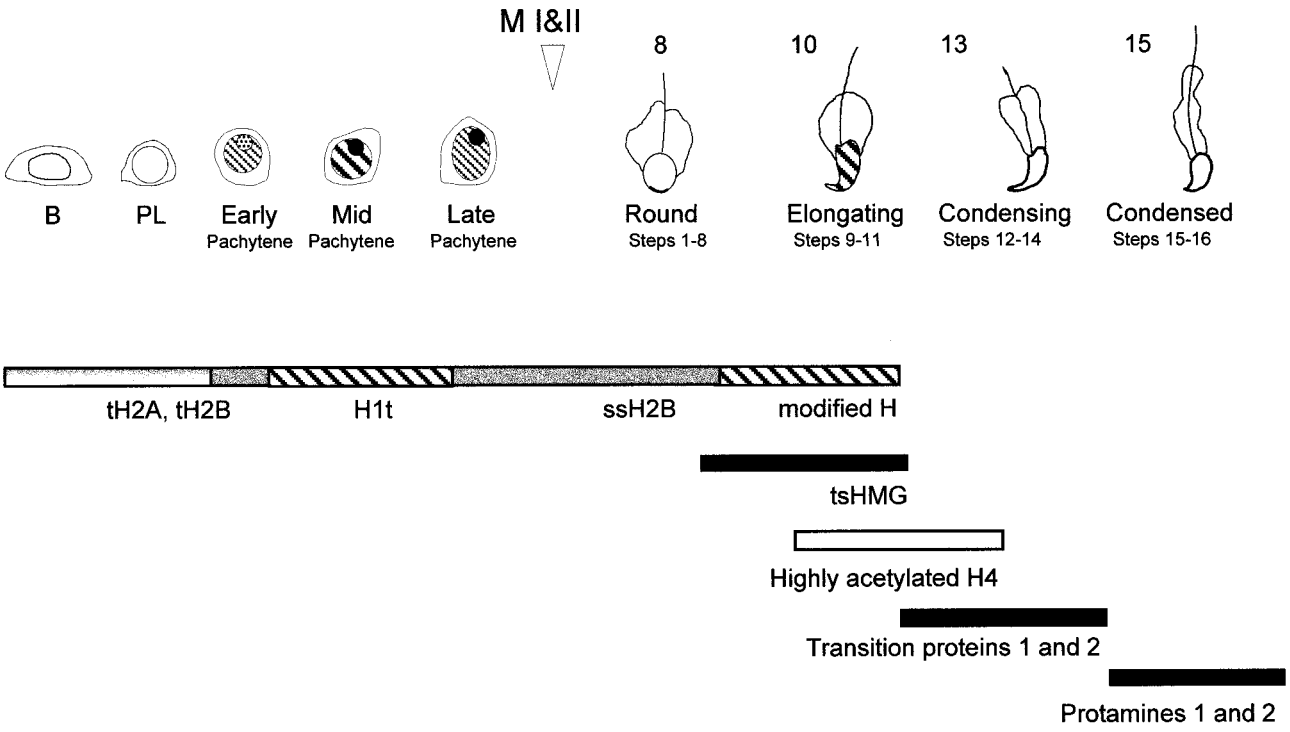

FIG. 8. Immunoexpression of uH2A during histone-to-protamine transition of mouse spermatogenesis. From left to right, the scheme represents the last step of entry into meiosis (B to Pl) and the subsequent meiotic and postmeiotic development. The bars indicate the presence or absence of specific proteins involved in chromatin structure. The upper bar represents the presence of histones, packaged in nucleosomes during spermatogenesis. The onset of expression of various testis-specific histones is shown. Histones remain present until spermatid nuclei have elongated. Then they are removed and replaced by transition proteins and subsequently by protamines (also indicated by bars). uH2A formation as detected on immunoblots of isolated pachytene spermatocytes and elongating spermatids is indicated by the striped region of the histone bar. The removal of histones is preceded by a marked increase in the detection of uH2A (this report) and testis-specific H2B (tH2B) (Unni *et al.,* 1995) (indicated by modified H). The testis-specific high-mobility group DNA-binding protein (tsHMG) may contribute to changes in chromatin structure through modulation of topoisomerase I activity (Boissonneault and Lau, 1993; Alami-Ouahabi *et al.,* 1996). Histone H4 hyperacetylation during nuclear elongation is indicated by an open bar, to indicate that this has been published for rat germ cells (Meistrich *et al.,* 1992); for the mouse, this has not been reported. Ubiquitinated H2A was detected also in pachytene spermatocytes, associated with the sex body and the autosomes, and in steps 8–12 spermatids, as is schematically indicated by different fill patterns of the nuclei. B, spermatogonium B; PL, preleptotene spermatocyte; MI&II, meiotic divisions I and II; tH2A, testis-specific H2A; H1t, testis-specific H1.

1998). No other cell type has ever been reported to contain ubiquitinated H3, and this may further point to a specific role for histone ubiquitination during postmeiotic chromatin reorganization. However, in mouse testis, we and others (M. L. Meistrich, personal communication) could not detect ubiquitinated H3.

A simple straightforward hypothesis is that ubiquitination of histones in elongating spermatids results in an alteration of chromatin structure, which allows other factors, such as the transition proteins, access to the DNA. Evidently, there is no future role for histones in spermatids, so it is likely that the released histones will be degraded, possibly through activity of the 26S proteasome. In contrast, in somatic cells undergoing mitotic proliferation, histones will take part in a ubiquitination/deubiquitination cycle (Matsui *et al.,* 1979; Wu *et al.,* 1981).

In the *HR6B* knockout male mice, progression of the development of elongating spermatids is severely impaired. However, the aberrant elongating spermatids that are still being formed do show uH2A immunoexpression. This indicates that HR6B is not necessary for H2A ubiquitination per se and that, at least in meiotic male germ cells, the absence of HR6B does not result in a decreased amount of uH2A. Although it cannot be excluded that a small but functionally significant reduction in the capacity to ubiquitinate H2A in elongating spermatids in the *HR6B* knockout mice causes part of the observed phenotype, we feel that dysregulation of ubiquitination of other HR6B substrates plays a role in causing the male infertility phenotype. Certainly, E2 enzymes other than HR6B can maintain H2A ubiquitination in testis of *HR6B* knockout mice. HR6A is the first obvious candidate, but most likely there are several

more, since a number of ubiquitin-conjugating enzymes show increased or exclusive expression in testis, such as rat $E2_{17kb}$ and its isoform 8A (Wing and Jain, 1995; Wing *et al.*, 1996), a human homolog of yeast UBC9 (Kovalenko *et al.*, 1996; Yasugi and Howley, 1996), and also UbcH-ben, which is encoded by a human homolog of the *Drosophila* gene *bendless* (Yamaguchi *et al.*, 1996). Partial functional redundancy, between HR6A and HR6B, complicates the interpretation of the *HR6B* knockout phenotype, and the subsequent identification of critical HR6A/B substrates that are involved. Apart from histones, no other candidate substrates are known. We have planned to try to identify candidate substrates, through approaches such as two-hybrid selection. However, in the *HR6B* knockout mice, the absence of HR6B may become critical at variable time points during meiotic and postmeiotic germ cell development, depending on the amount of HR6A that is still present and the compensatory potential of other E2 enzymes. Thus, a clear-cut difference between wild-type and *HR6B* knockout testes in the amount, or ubiquitination level, of certain critical substrates may not be easily found.

In conclusion, we have shown that ubiquitination of histones occurs during the postmeiotic histone-to-protamine transition. In addition, histone ubiquitination in pachytene spermatocytes is increased, in particular for the X and Y chromosomes in the sex body. As discussed, this latter observation is remarkable, in view of the fact that this points to a situation in which histone ubiquitination is correlated with transcriptionally inactivated chromatin.

ACKNOWLEDGMENTS

We thank Dr. Celis (Institute of Medical Biochemistry and Danish Center for Human Genome Research University of Aarhus, Aarhus, Denmark) for providing us with the α-uH2A antibody (mAb E6C5), Dr. P. de Boer (Department of Genetics, Agricultural University Wageningen, The Netherlands) for his advice on immunocytochemical methods, and Dr. W. A. van Cappellen for his assistance with the image analysis. This work was supported by the Dutch Science Foundation (NWO) through GB-MW (Medical Sciences), and by the Dutch Cancer Society (IKR 92-118).

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Received for publication June 16, 1998
Revised November 18, 1998
Accepted November 23, 1998