Reductions in Linker Histone Levels Are Tolerated in Developing Spermatocytes but Cause Changes in Specific Gene Expression*

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H1 linker histones are involved in packaging chromatin into 30-nm fibers and higher order structures. Most eukaryotic cells contain nearly one H1 molecule for each nucleosome core particle. Male germ cells in mammals contain large amounts of a germ cell-specific linker histone, HIST1H1T, herein denoted H1t, which is particularly abundant in pachytene spermatocytes. Despite its abundance in male germ cells and significant divergence in primary sequence from other H1 subtypes, inactivation of the H1t gene in mice showed that it is not required for spermatogenesis. Analysis of germ cell chromatin from H1t null mice showed that other H1 subtypes, especially the testis-enriched HIST1H1A, herein denoted as the H1a subtype, were able to compensate for the absence of H1t to maintain a normal total H1 to nucleosome core ratio. To disrupt the compensation, we generated H1t and H1a double null mice by two sequential gene-targeting steps in embryonic stem cells. Elimination of both H1t and H1a led to a 25% decrease in the ratio of H1 to nucleosome cores in double null germ cells. Surprisingly, the reduction in H1 did not perturb spermatogenesis or produce detectable defects in meiotic processes. Microarray analysis of gene expression showed that the reduced linker histone levels did not affect global gene expression, but it did cause changes in expression of specific genes. Our results indicate that a partial reduction in linker histone-nucleosome core particle stoichiometry is tolerated in developing male germ cells.

Histones are small, basic proteins that are unique to eukaryotes and found in all eukaryotic nuclei. Histones are involved in packaging DNA into a compact structure called chromatin. There are five major classes of histones, the core histones H2A, H2B, H3, and H4, and the linker histone H1. Two molecules of each of the core histones constitute the protein octamer of the nucleosome core particle. H1 histones bind to the linker DNA between nucleosome core particles and facilitate the folding of nucleosomes into the 30-nm chromatin fiber and higher order chromatin structures (1, 2). Therefore, they would be expected to have profound effects on many cellular processes that require access to the genome.

A general feature of histone proteins is that their amino acid sequences are conserved. However, the H1 family of histones is the most diverse class, with multiple subtypes in most organisms (3). In mice eight H1 subtypes have been described (4, 5), including the “somatic” subtypes H1a through H1e, the replacement subtype H1FOO (here H1o), the oocyte-specific H1FPOO, here denoted as H1o, and the testis-specific H1t. These subtypes are also present in humans, and the genomic organization of the genes encoding the H1 subtypes appears to be very similar in humans and mice (6, 7).

Although all of the mouse H1 subtypes have the basic tripartite structure of metazoan H1s, they do differ extensively in amino acid sequences, with H1o, H1o, and H1t being the most divergent subtypes. The different subtypes also exhibit distinct expression patterns during development, with the tissue-specific H1oo and H1t being the most extreme examples. Certain H1 subtypes can constitute a major portion of the linker histones in specific cell types. For example, H1t represents 40–50% of the H1 in pachytene spermatocytes (8). Because linker histones are nearly equimolar with nucleosome core particles in most higher eukaryotic cells, this implies that in some cell types a large fraction of the nucleosomes contains a particular H1 subtype, e.g. H1t in developing spermatocytes. Nevertheless, gene inactivation experiments in mice have shown that elimination of any one of several of these subtypes does not disrupt normal development, even in tissues in which the specific subtypes are highly abundant (9–14). For example, mice lacking H1t were fertile and showed no detectable defect in spermatogenesis (9, 13, 14). The most likely explanation for the absence of a phenotype in these single H1 null animals is that other H1 subtypes compensate for the lost subtype. Consistent with this view, analysis of chromatin from such mice showed that indeed the levels of other subtypes are increased so as to maintain a normal ratio of total H1 linker histones to nucleosome core particles (9).

Under these circumstances, one approach to investigating the in vivo roles of linker histones in mammals is to attempt to reduce the stoichiometry of H1 to nucleosome core particles by generating compound H1 null cells or mice. This is an arduous task for most H1 subtypes, because the genes for six of the eight subtypes are tightly linked on mouse chromosome 13 (7). We reported previously that in Hist1h1tmAis/Hist1h1tmAis (denoted H1t−/− or H1t-null) germ cells, the H1a subtype constitutes more than 50% of the remaining H1 (9). Therefore, we
generated H1t and H1a null mice by two sequential gene-targeting steps in mouse embryonic stem (ES) cells. The double null germ cells from such mice have a 25% decrease in ratio of H1 to nucleosomes. Remarkably, the reduction in H1-to-nucleosome stoichiometry did not perturb spermatogenesis or cause any apparent meiotic abnormalities, nor did it affect global gene expression. However, changes in expression of certain genes were found.

MATERIALS AND METHODS

Disruption of the H1a Gene in ES Cells and Generation of Chimeric Mice—The genomic DNA clones used in construction of the H1a targeting vector were isolated by screening a 129X1/SvJ (briefly, 129X1) genomic library as described previously (15). To generate an H1a targeting vector (Fig. 1A), a 1.2-kb SacII-TthIII fragment was inserted into the XbaI site of pTK-KS vector by blunt-end ligation as the short 5′ arm (Fig. 1A). The pTK-KS vector already contains a PGK-TK cassette. Then a 6.5-kb EcoRI fragment was inserted into the EcoRI site of the plasmid as the long 3′ arm. Finally, a PGK-Hygro cassette was blunt-end-inserted into the BamHI site of the plasmid in between these two arms. To generate ES cells with a targeted disruption at the H1a locus, 25–50 μg of NotI-linearized H1a targeting vector (Fig. 1A) were electroporated into 2–4 × 105 T3–12 ES cells containing a disrupted H1t allele, as described previously (9). The T3–12 ES cell line was shown previously to provide very good germ-line transmission of the disrupted H1t allele. ES cell clones resistant to 100 μg/ml G418, 150 μg/ml hygromycin, and gancyclovir were picked after 9–10 days and transferred to duplicate 48-well plates. Ten ES clones containing a modified H1a allele were identified among 192 triploid resistant clones analyzed by Southern blot hybridization (Fig. 1B). Four clones (D18, D32, E43, and E45) were injected into mice and analyzed by Northern blotting as described previously (26).

Preparation and Analysis of Histones—Mice were sacrificed by cervical dislocation, and the testes were excised and immediately rinsed with Dulbecco’s modified Eagle medium. Seminiferous epithelial cells were dissociated and prepared as previously described (18). The germ cells were isolated from testes, chromatin was prepared from the nuclei of the isolated spermatogenic cells, and histone proteins were extracted from the chromatin with 0.2 N sulfuric acid and fractionated by high performance liquid chromatography (HPLC) as described previously (9). The effluent was monitored at 214 nm, and peak areas were determined as described (16).

Germ Cell Culture and Treatment—Isolated testicular germ cells were prepared as described previously (17) and washed and resuspended in HEPES-buffered minimal essential medium (Sigma) supplemented with 25 mM NaCl, 5% v/v fetal bovine serum, 20 mM sodium lactate, 50 μM penicillin, and 100 μg/ml streptomycin (19). The cells were plated at a concentration of 2.5 × 106 cells/ml in 4-well Nunc plates and cultured at 32 °C in a humidified atmosphere of 95% air/5% CO2. After a 2-h recovery period, the cells were treated with 5 μM okadaic acid (OA) for 4 h. An equal volume of the solvent, 100% EtOH, was added to the control wells.

Air-dried Chromosome Preparations—Chromosome condensation was assessed in air-dried chromosomes prepared by a modification of the Evans procedure (20). Briefly, 2.5 × 106 cultured cells were pooled and pelleted by centrifugation. After washing in 2.2% sodium citrate, the cells were allowed to swell in a 1% (hypotonic) sodium citrate solution. After swelling, the testicular germ cells were fixed in a 3:1 absolute methanol–acetic acid solution. The cells were dropped onto a prewarmed slide and allowed to air dry. The air-dried cell preparations were stained with Giemsa and scored for the presence of meiotic metaphase I figures or other degrees of chromosome condensation using brightfield optics with a 40× objective.

Surface-spread Chromatin Preparations—Cells were fixed for indirect immunofluorescence analysis in 2% paraformaldehyde, 0.03% SDS and processed as previously described (21). The antisera were used polyclonal anti-SYP-C3 (22), anti-phospho-H2AX (Upstate Biotechnology), anti-histone H1t (23), and anti-phospho-histone H3 (Upstate Biotechnology). Secondary antibodies, conjugated with either rhodamine or fluorescein, were purchased from Pierce (Rockford, IL). Coverslips were mounted with Prolong Antifade (Molecular Probes, Eugene, OR) containing 0.1 mg/ml 4′,6-diamidino-2-phenylindole to visualize the DNA. Localization was detected with a 100× objective on an Olympus epifluorescence microscope. Images were captured to Adobe Photoshop with a Hamamatsu C5810 color chilled charge-coupled device camera.

Histological Analysis of Mouse Testes—Testes were fixed and stained with hematoxylin and eosin and examined as described previously (9). Total normal sperm numbers were determined by counting with a hemocytometer as described previously (9).

Microarray Procedures—A description of the microarray hardware and procedures used in this study are available from www.aecom.y-u.edu/home/molgen/facilities.html. cDNA fluorescent probe preparations, prehybridization, and hybridization were performed as described (24). Briefly, for cDNA microarray analysis, RNA was extracted from germ cells of wild-type and H1a, H1t null littersmate male mice using TRizol reagent (Invitrogen). 100 μg of total RNA pooled from the germ cells of three males were used in each experiment. Total RNA was reverse-transcribed in the presence of Cy3-dUTP or Cy5-dUTP with Superscript II RT. The cDNA products from wild-type and mutant cells were combined and purified on a Micron YM 50 column and hybridized to a 9000 gene mouse cDNA microarray overnight at 50 °C. Hybridized cDNA microarrays were washed and scanned on an Axon GenePix 4000, and signal intensities were extracted. Four replicate array hybridization experiments were carried out, with the dyes were exchanged between the samples in the experiments.

Microarray Quality Control and Data Analysis—The spot intensity was extracted from the array image using GenePix Pro 3.0 as described (24), and the intensity data file was imported into R statistical packages for data analysis. To ensure the quality of spots used in data analysis, the statistical t test between spot intensity and surrounding background intensity was carried out for each spot of each channel. The significance p < 0.001 for both Cy3 and Cy5 channels were eliminated from further analysis. The intensities of the remaining spots were adjusted by subtracting the surrounding background intensity from the spot intensity. The ratio of the experimental sample over the control sample was calculated and further log transformed with base 2 (denoted as log2 R/G, where R and G are the intensities of the experimental sample and the control sample, respectively), because the log-ratios form a more normal distribution than the ratios. To avoid systematic errors such as unequal loading of cDNA samples and bias of dye labeling and incorporation, intensity-dependent normalization within the array was performed to balance the intensities of both channels by applying a Lowess (locally weighted scatterplot smoothing) regression to the distribution of log-ratio (log2 R/G) and log-intensity (1/2 log R+G) (25). The correlation coefficient of normalized log-ratios for all spots between two replicates was calculated to determine the good reproducibility of the replicates, and a cutoff for the correlation coefficient of 0.5 was used. To identify differentially regulated genes, the filtered data was imported into an Access database and queried for genes with the median of log2 of ratios ≥ 0.58 or ≤ –0.58 considered to be up-regulated (1.5-fold or more) or down-regulated (1.5-fold or more), respectively, and a p value < 0.05 of statistical t test on the repeated measurements. A cutoff of 0.584 for the median of log-ratios was determined by data from a number of arrays generated by the same array platform and experimental procedure as performed in this study.

Northern Blot Analysis—Total RNA was isolated from male germ cells and analyzed by Northern blotting as described previously (26).

RESULTS

Generation of ES Cells with Disrupted Alleles of Both H1a and H1t—To generate ES cells with disrupted H1a and H1t genes, we utilized a previously described ES cell clone (T3–12) in which one allele of the H1t gene had been inactivated and replaced with a PGK-Neo cassette (9). The mouse H1a gene does not contain introns, and it encodes a protein of 213 amino acids. The single short exon of the H1a gene enabled us to replace the entire H1a coding sequence with a selection marker. The H1a targeting vector (Fig. 1A) contained a PGK-Hygro cassette that allows positive selection for transfectants with hygromycin and a PGK-TK cassette that allows negative selection for hygromycin-resistant cells.
FIG. 1. Targeted disruption of the \( H1a \) gene in mouse ES cells and mice. A, homologous recombination strategy in \( H1t(+/−) \) ES cells. The \( H1a \) targeting vector (top) was constructed by inserting a 1.3-kb SacII-ThIII fragment just upstream of the \( H1a \) start codon into the XbaI site of the pKS-TK vector, which contains a PGK-TK cassette in the pSK-Bluescript vector. Next a 6.5-kb EcoRI fragment beginning about 0.5 kb downstream of the stop codon was inserted into the EcoRI site of the vector. Finally, a blunt-ended 1.8-kb PGK-Hygro cassette was inserted into the blunted BamHI site between the two previously inserted fragments. A homologous recombination event (Xs) between the targeting vector and the endogenous \( H1a \) locus (middle) results in production of a modified \( H1a \) locus (bottom), in which a 1.2-kb fragment, including the entire \( H1a \) coding sequence along with 0.5-kb 3′-non-coding sequence is removed. A 0.45-kb Smal-EcoRV fragment (5′-probe) lying outside of the targeting construct was used as a DNA probe for Southern blotting analysis of DNA from ES cells and mice. B, identification of ES cell clones containing the modified \( H1a \) allele. DNA (10 μg) was digested with EcoRI and hybridized with the 5′-probe shown in A. The expected positions of the hybridizing fragments from the wild-type and the modified \( H1a \) loci and their respective sizes are indicated. The 4.4-kb hybridizing band (globin transgene locus) is due to hybridization between contaminating plasmid sequences in the 5′-probe and multiple copies of an inactive globin transgene present in the parental WW6 ES cells (47). C, PCR genotype analysis of agouti offspring from chimeras produced with double targeted ES cells. Male chimeras produced from each of the four ES cell clones D18, D32, E43, and E45 were mated with C57BL/6 females. Genomic DNA was prepared from the agouti offspring and analyzed by PCR for the presence of either the disrupted \( H1t \) gene (\( H1t \)-Neo) or the disrupted \( H1a \) gene (\( H1a \)-Hygro). All agouti progeny derived from ES cell lines D18 and E43 were shown to be heterozygous for either the \( H1a \)-modified allele or the \( H1t \)-modified allele. All agouti progeny derived from ES cell lines D32 and E45 were shown to be wild-type for both the \( H1a \)-modified allele and the \( H1t \)-modified allele. D, genotype analysis of offspring from parents heterozygous for either the modified \( H1a \) allele alone (\( H1a \) knockout) or both the modified \( H1a \) and \( H1t \) alleles (\( H1a \) and \( H1t \) double knockout). 10 μg of tail DNA from offspring was digested with EcoRI, and Southern blots were hybridized with either the \( H1a \) 5′-probe (upper panel D) shown in panel A or the \( H1t \) 5′-probe (lower panel D) (9). Other details are as described for panel B. The deduced genotype of each mouse is indicated above each lane.
selection with gancyclovir. Homologous recombination between the targeting construct and the endogenous H1a locus resulted in the generation of a modified H1a allele in which the entire H1a coding sequence was replaced by the PGK-Hygro cassette (Fig. 1A). The targeting vector was linearized by NotI digestion, electroporated into T3–12 ES cells, and cells resistant to G418, hygromycin, and gancyclovir were selected. The gancyclovir-negative selection resulted in a 4-fold enrichment of G418 and hygromycin-resistant clones. DNA from a total of 192-resistant clones was screened by both recombination-specific PCR and Southern blot hybridization with a 5′ probe lying outside of the H1a targeting construct (Fig. 1A). Ten positive clones in which a homologous recombination event had occurred at the H1a locus were identified (examples shown in Fig. 1B). Southern blot analyses with a H1t gene-specific 5′ probe (9) confirmed that all ten clones were H1t (+/−), indicating that the disrupted H1t allele was not lost during ES cell propagation and passage (data not shown). The targeting of the H1a allele can occur in cis or in trans to the previously inactivated H1t allele. The former type of event can be used to generate H1a, H1t double mutant mice, whereas the latter type of event can be used to generate H1a mutant animals.

Generation of H1a-null Mice and H1a- and H1t-double-null Mice—To generate both H1a homozygous mutant mice and mice homozygous mutant for both H1a and H1t, four ES clones (D18, D32, E43, and E45) containing disrupted H1a and H1t alleles (Fig. 1B) were injected into C57BL/6 blastocysts to produce chimeric mice. All four cell lines gave rise to chimeras ranging in chimerism from 50% to 99% as judged by coat color. Two to four male chimeras from each line were mated with C57BL/6 females. All males mated produced agouti progeny, indicating that all four ES cell lines contributed to the germ line. Some agouti progeny from all four lines were shown to contain the disrupted H1a allele by PCR (Fig. 1C). These results were confirmed by Southern blot analysis (data not shown). By analyzing the genotypes of the agouti animals at both the H1a and H1t loci it is possible to determine whether the targeting of the H1a gene occurred in cis or trans to the inactivated H1t allele. All agouti progeny from D18 and E43 double-targeted ES cells were either heterozygous at H1a or heterozygous at H1t (Fig. 1C, left panel), indicating that the mutated H1a allele and the disrupted H1t allele were in trans in these two lines. On the other hand, all agouti progeny from the D32 and E45 cell lines were either wild-type at H1a and H1t loci or heterozygous at both loci (Fig. 1C, right panel), suggesting that the mutated H1a and H1t alleles were located on the same chromosome in these two lines.

Mice heterozygous for either the H1a mutation or both the H1a and H1t mutations were phenotypically normal. To determine the possible effects of the H1a mutation alone, mice derived from the D18 and E43 cell lines and heterozygous for the modified H1a allele were interbred, and tail DNA from the progeny was analyzed by PCR or Southern blot hybridization (Fig. 1D, left panel). Of 197 animals analyzed by either PCR or Southern blot hybridization, 43 (22%) carried only wild-type H1a alleles, 111 (56%) had one copy of the H1a modified allele, and 43 (22%) carried two copies of the H1a-mutated allele. The ratio of the three classes of mice is consistent with Mendelian transmission of the two alleles. H1a homozygous mice were normal in size and body weight (Table 1), and they were indistinguishable from heterozygous and wild-type littermates. Male H1a−/− mice also did not exhibit any abnormalities in spermatogenesis by several criteria. They were fertile, producing litters of normal size, and both their testis weight and epididymal sperm numbers were normal (Table 1). No abnormalities were detected in testis histology. These results are consistent with an earlier report (12) indicating that mutation of the mouse H1a gene does not impair normal development or spermatogenesis.

Because the entire H1a coding region was deleted in the modified H1a allele, H1a−/− mice would be expected to lack H1a protein. To demonstrate that homozygous mutant animals do not have H1a protein, total histone extracts of germ cell chromatin were analyzed by reverse-phase HPLC. The HPLC chromatogram of germ cell histones from wild-type animals had a peak eluting at −50 min (Fig. 2A), which is absent in germ cell histone extracts from H1a−/− animals (Fig. 2B). To prove that this peak is indeed H1a, we collected the material eluting in this position and subjected it to time-of-flight mass spectrometry. The mass spectrophotograph showed a single component with a molecular mass of 21,705 Da (data not shown), consistent with the expected size of H1a protein (21,790 Da) predicted from the H1a gene sequence after accounting for cleavage and acetylation of the resulting N-terminal serine. Because this peak is missing from germ cells extracts of H1a−/− mice, we conclude that the modified H1a allele is indeed a null mutation. The H1a peak was also not present in extracts of liver and spleen from the H1a−/− mice (data not shown).

Reduced H1 Content in Germ Cells of Mice Null for Both H1a and H1t—To generate animals null for both H1a and H1t, H1a+/−, H1t+/− mice derived from cell lines D32 and E45 (in which the mutant H1a and H1t alleles were linked) were intercrossed and tail DNA from the F2 progeny was analyzed by PCR or Southern blot hybridization (Fig. 1D, right panel). Of 171 animals analyzed by these methods, 39 (23%) carried only wild-type alleles, 88 (51%) had one copy of the mutated H1a and H1t alleles, and 44 (26%) carried two copies of the chromosome containing both the H1a and H1t null alleles. The ratio of the three classes of mice is consistent with Mendelian transmission of the doubly mutated chromosome. H1a, H1t double homozygous mutant mice were normal in weight and size, and they were indistinguishable from heterozygous and wild-type littermates (Table 1). These results indicate that the presence of both the modified H1a allele and the disrupted H1t allele does not impair normal development.

Because both the H1a and H1t disruptions are null mutations, it is expected that H1a−/−, H1t−/− mice would be completely lacking both H1a and H1t proteins. This expectation was confirmed by HPLC analysis of total histone extracts of male germ cell chromatin from such mice (Fig. 2C).

To determine the effect of eliminating both H1a and H1t on the H1 subtype composition and H1-to-nucleosome stoichiometry, we analyzed these parameters by HPLC of extracts from germ cell chromatin of wild-type and homozygous double mutant male littermates. As shown in Fig. 2A, this method resolves the H1 histones in male germ cells into six peaks; H1d and H1e migrate as a single peak and therefore their amounts

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**Table I**

| Genotype | Body wt. | Testis wt. | Sperm no. |
|----------|----------|------------|-----------|
|          | g        | mg         | ×10⁷      |
| H1a (+/+)| 31.0 ± 1.8 | 97.8 ± 8.2 | 3.4 ± 1.1 |
| H1a (+/-)| 30.1 ± 1.9 | 101.5 ± 13.6 | 2.8 ± 0.7 |
| H1a (-/-)| 34.5 ± 1.7 | 95.7 ± 7.2 | 4.5 ± 1.2 |
| H1a/H1t(+/+, +/+) | 35.1 ± 3.5 | 105.2 ± 10.4 | 3.7 ± 1.2 |
| H1a/H1t(-/-, +/+) | 34.9 ± 6.1 | 101.2 ± 10.1 | 3.0 ± 1.1 |
| H1a/H1t(-/-, -/-) | 34.4 ± 5.2 | 90.0 ± 12.2 | 2.7 ± 1.0 |

Values are means ± S.D. of individual determinations made for six 3-month-old mice. Sperm numbers are the numbers of sperm obtained from two epididymides.
cannot be estimated separately unless the peak containing them is collected and subjected to mass spectrometry (27). As discussed previously (11), the HPLC analysis also allows an estimate of the amount of linker histones in cells relative to nucleosomes, by measuring the total amount of all of the H1 subtypes relative to a nucleosomal histone such as H2B. Quantitative measurements from analyses like that shown in Fig. 2, performed on germ cell chromatin from five 3-month-old wild-type and H1a, H1t null mice (B), and a double homozygous H1a and H1t mutant mouse (C) were fractionated by reverse-phase HPLC as described under “Materials and Methods.” The identity of the histone subtype(s) in each peak is indicated.

Characterization of Germ Cells Null for Both H1a and H1t—Having determined that elimination of both H1a and H1t leads to a reduction in the molar ratio of total H1 to nucleosomes in germ cells, we sought to examine the effects of the H1 deficiency on the properties of the cells and the properties of their chromosomes during meiosis. To determine whether H1a−/−, H1t−/− male mice are fertile, they were bred to both wild-type and corresponding null mutant females. Litter sizes from these matings were indistinguishable from those of wild-type matings, and their progeny all appeared normal. However, mice can be fertile with less than 10% of the normal number of mature sperm (28). Therefore, it was important to measure the actual number of mature sperm in the double mutants and to examine the testes for any abnormalities. No significant differ-
were adjusted to account for the differences in the number of peptide bonds in each H1 subtype and H2b.

The

embryonic sections of testes from 3-month-old wild-type and 

gens, or homozygous double mutant mice (Table I). To compare 

dated testicular sperm. Therefore, using these methods no 

round spermatids, and condensed spermatids, and the lumen 

eter of the seminiferous tubules, the thickness of the seminif- 

somes was observed (Fig. 3).

SYCP3 (Fig. 3) and SYCP1 (data not shown) no defects in 

first meiotic division phase (29). As assessed by localization of 

plexes visible. SYCP3 persists on the centromeres through the 

chromosome pairing in mutant spermatocytes, localization of 

cytes on the unpaired homologous chromosomes. As the sper-

3). SYCP3 is a component of the lateral elements of the synap-

the synaptonemal complex protein SYCP3 was assessed (Fig. 

chromosome involved in synaptonemal complex formation, recombina-

ces were detected in testis weights of wild-type, heterozy-

uous, or homozygous double mutant mice (Table I). To compare 

the number of mature sperm produced by wild-type and mu-

tant mice, the sperm from the cauda epididymis were isolated 

and counted. No significant differences were detected in sperm 

number and sperm motility among the three classes of mice (Table I). Examination of hematoxylin-eosin-stained paraffin-

embedded sections of testes from 3-month-old wild-type and 

H1a/–, H1t/– littersmates did not reveal any abnormal histol-

ogical features in the mutant mice (data not shown). The 

testes of H1a/–, H1t/– mice contained closely packed semi-

inferous tubules and limited interstitial space, and the diam-

eter of the seminiferous tubules, the thickness of the seminif-

erous epithelium, and the size of the lumen appeared normal. 

The seminiferous epithelium of H1a/– or H1a/–, H1t/–/– testes contained Sertoli cells, spermatagonia, and multiple lay-

ers of spermatocytes, including pachytene spermatocytes, 

round spermatids, and condensed spermatids, and the lumen 

contained testicular sperm. Therefore, using these methods no 

abnormalities in the process of spermatogenesis could be 

detected in the testes of H1a/–, H1t/–/– mice.

H1a/–, H1t/– spermatocytes were further analyzed for defects in meiotic processes by using antibodies against pro-

teins involved in synaptonemal complex formation, recombina-

ation, and chromosome condensation. To monitor homologous 

chromosome pairing in mutant spermatocytes, localization of 

the synaptonemal complex protein SYCP3 was assessed (Fig. 

3). SYCP3 is a component of the lateral elements of the synap-

onemal complex that is first detected in leptotene spermatocytes 

on the unpaired homologous chromosomes. As the sper-

matocytes progress through prophase I, the localization of 

SYCP3 changes, allowing for the identification of specific cell 

types. At pachytene, SYCP3 is localized to the synapsed homol-

ogous chromosome pairs with 20 distinct synaptonemal com-

plexes visible. SYCP3 persists on the centromeres through the 

first meiotic division phase (29). As assessed by localization of 

SYCP3 (Fig. 3) and SYCP1 (data not shown) no defects in 

homologous chromosome pairing were detected in mutant sper-

matocytes and complete synopsis of the homologous chromo-

somes was observed (Fig. 3).

A hallmark of meiosis is the appearance of double strands 

breaks that occur as a result of recombination. The phospho-

rylation of the histone H2A variant, γ-H2AX, has been shown 

to be a marker for the presence of double strand breaks that 

occur as a result of meiotic recombination (30). Leptotene and 

zygotene spermatocytes from H1a/–, H1t/–/– mice showed 

intense localization of γ-H2AX over the entire nuclear area, 

and the same localization pattern was seen in the controls (Fig. 

3, E and F). By pachynema the localization of γ-H2AX showed 

the expected restriction to the sex body in both mutant and 

control pachytene spermatocytes (Fig. 3, G and H). The absence 

of staining over the autosomal chromosomes in mutant 

pachytene spermatocytes suggests that repair of the double-

strand breaks was not impaired. The localization of RAD51, a 

protein thought to participate in the repair of double strand 

breaks (31, 32), was also the same in both mutant and wild-

type controls (data not shown).

Because it is possible that spermatocyte histones regulate 

chromosome condensation at the time of the meiotic division, 

we assessed both localization of phosphorylated histone H3 (a 

marker of division-phase chromosome condensation) and the 

ability of chromosomes in cultured mutant spermatocytes to 

respond to precocious induction of the division phase. The 

localization of the phosphorylated form of histone H3 did not 

differ in pattern or timing between the double mutant sper-

matocytes and their wild-type littermate controls (Fig. 4). To 

monitor further the competence of the histone-depleted mutant 

chromosomes to condense at division phase, spermatocytes 

were cultured in the presence of the type I and IIa phosphatase 

inhibitor okadaic acid (OA), which induces a precocious met-

aphase-like state characterized by the condensation of chromo-

somes (19). In this experimental model, the double null sper-

matocytes were able to condense their chromosomes in a meiotic metaphase I configuration with no detectable differ-

ences from their wild-type littermate controls in the degree of 

condensation (Fig. 4).

A TUNEL assay was then performed on the sections to 

determine if the double null mice have increased levels of 

apoptosis. No increase in cell death was detected compared 

with wild-type littermate controls (data not shown).

Reducing H1 Content in Germ Cells Leads to Changes in 

Expression of Specific Genes—Because reducing the H1 content
of germ cells did not appear to affect their development or meiotic chromosome properties, we sought to determine whether the loss of H1a and H1t affected gene expression. We compared the gene expression profiles of wild-type and H1a<sup>−/−</sup>, H1t<sup>−/−</sup> germ cells by reverse-transcribing total RNA from each source in the presence of Cy3 or Cy5 dUTP and hybridizing a mixture of the two products to microarrays containing PCR products of about 9000 cDNA clones. After processing the data according to our criteria for spot intensity relative to background and reproducibility (see "Materials and Methods"), we found that expression of only 17 genes differed between the two types of samples by more than 2-fold. Interestingly, all 17 genes were expressed at lower levels in the mutant germ cells as compared with wild-type germ cells. By lowering the cut-off to 1.5-fold changes, we found 85 more genes that differed between the two samples. Once again, genes down-regulated in the mutant cells predominated, with 67 of the 85 genes expressed at lower levels in the mutant cells. The known genes with altered expression fell into many functional groups (Table III). The changes in expression detected with the microarrays were confirmed for several genes by Northern blot hybridization with germ cell RNA from three wild-type and mutant littermate pairs (Fig. 5). One of the up-regulated genes is a putative spermatogenic-specific transcription factor (AA064110), but most of the affected genes are not enriched in germ cells. These results indicate that loss of H1a and H1t and the resulting reduction in H1 to nucleosome stoichiometry does not affect global gene expression; instead expression of specific genes is affected.

**DISCUSSION**

Our previous work (9) and that of other investigators (13, 14) showed that the testis-specific linker histone H1t is not essential for spermatogenesis in mice. As discussed above, one possible explanation is that other H1 subtypes can compensate for loss of H1t. This explanation is supported by analysis of chro-
TABLE III
Functional categories of genes whose expression level are affected by knock-out of H1t and H1a

The functional categories refer to the terms in biological process category of gene ontology.

| Accession | Symbol | Name | Expression (KO/WT) |
|-----------|--------|------|-------------------|
| **Cell cycle/communication/growth/maintenance** | | | |
| AA023523 | Parva | Parvin, alpha | 0.36 |
| AA016759 | Mcm6 | Minichromosome maintenance deficient 6 (MIS5 homolog, *S. pombe*) (*S. cerevisiae*) | 0.47 |
| AA388790 | Tsc1 | Tuberous sclerosis 1 | 0.54 |
| W47753 | Arpc5 | Actin-related protein 2/3 complex, subunit 5 | 0.58 |
| AA002910 | Fos | FBJ osteosarcoma oncogene | 0.61 |
| AA245312 | Thap1 | THAP domain-containing, apoptosis-associated protein 1 | 0.62 |
| AA240272 | Bcar1 | Breast cancer anti-estrogen resistance 1 | 1.56 |
| AA048426 | Cdc6 | Cell division cycle 6 homolog (*S. cerevisiae*) | 1.75 |
| AA030603 | Igf2 | Insulin-like growth factor 2 | 1.79 |
| **Metabolism** | | | |
| AA24457 | Mmp15 | Matrix metalloproteinase 15 | 0.44 |
| AA239282 | Phlda1 | Pleckstrin homology-like domain, family A, member 1 | 0.54 |
| AA06231 | Mgs2b1 | Mannosidase 2, alpha B1 | 0.55 |
| W89239 | Sf3a1 | Splicing factor 3a, subunit 1 | 0.55 |
| AA080231 | Man2b1 | Mannosidase 2, alpha B1 | 0.55 |
| AA269845 | Gpm6a | Glycoprotein m6a | 0.55 |
| AA466323 | Shapy-pending | Ca²⁺/H¹0011-dependent endoplasmic reticulum nucleoside diphosphatase | 0.56 |
| AA016923 | Mars | Methionine-tRNA synthetase | 0.56 |
| AA008747 | Ppt2 | Palmitoyl-protein thioesterase 2 | 0.57 |
| AA500330 | Fdps | Farnesyl diphosphate synthetase | 0.57 |
| AA200473 | Manb | Mannosidase, beta A, lysosomal | 0.59 |
| **Signal transduction** | | | |
| AA261662 | Dusp16 | Dual specificity phosphatase 16 | 0.45 |
| AA273310 | Digk | Discs, large homolog 4 (*Drosophila*) | 0.46 |
| AA239487 | Bcap37 | Beta-cell receptor-associated protein 37 | 0.54 |
| AA254769 | Cops7b | COP9 (constitutive photomorphogenic) homolog, subunit 7b (*Arabidopsis thaliana*) | 0.54 |
| W89195 | Prkcq | Protein kinase C, theta | 0.58 |
| AA016824 | Cck | Cholecystokinin | 0.60 |
| AA002708 | Sast-pending | Syntrophin-associated serine/threonine kinase | 0.64 |
| **Transcriptional regulation** | | | |
| AA286166 | Usf2 | Upstream transcription factor 2 | 0.45 |
| AA240305 | Hand1 | Heart and neural crest derivatives expressed transcript 1 | 0.47 |
| AA125197 | Smerel | SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily e, member 1 | 0.56 |
| AA002910 | Fos | FBJ osteosarcoma oncogene | 0.61 |
| AA518833 | Mrp38 | Mitochondrial ribosomal protein L38 | 0.61 |
| AA266002 | Bcl3 | B-cell leukemia/lymphoma 3 | 0.63 |
| AA118392 | Zfpl43 | Zinc finger protein 143 | 0.65 |
| **Transport** | | | |
| AA122470 | Dao1 | D-amino acid oxidase | 0.42 |
| AA002937 | Abcg4 | ATP-binding cassette, sub-family G (WHITE), member 4 | 0.55 |
| AA208297 | Psy2 | FXYD domain-containing ion transport regulator 2 | 0.56 |
| AA242805 | Pced1 | Pleckstrin homology, Sec7, and coiled-coil domains 1 | 0.58 |
| W30612 | Clic3 | Chloride intracellular channel 3 | 0.59 |
| AA458178 | Cd36 | CD36 antigen | 0.66 |
| W70943 | EST | RIKEN cDNA 1110030C19 gene | 1.60 |
| **Others** | | | |
| AA064326 | Hspa4 | Heat shock protein 4 | 0.44 |
| AA255141 | Agrp1a | Angiotensin receptor 1a | 0.46 |
| AA239753 | Pdr1 | Phosducin-like | 0.55 |
| AA062075 | Icos1 | Icos ligand | 0.56 |
| AA014404 | Ki121b | Kinesin family member 21B | 0.60 |
| AA254511 | EST | RIKEN cDNA 2310020H19 gene | 0.60 |
| AA061123 | Sfjp1 | Surfactant-associated protein B | 0.62 |
| AA276336 | C81 | CD 81 antigen | 0.62 |
| AA170590 | Gphn | Gephyrin | 0.63 |
| AA000073 | Rrm1 | Ribonucleotide reductase M1 | 0.63 |
| AA109951 | B2m | Beta-2 microglobulin | 0.65 |
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TABLE III—continued

| Accession | Symbol | Name | Expression(KO/WT) |
|-----------|--------|------|------------------|
| AA541888 | 2210068A03Rik | RIKEN cDNA 2210068A03 gene | 0.39 |
| W3413 | 2410127E18Rik | RIKEN cDNA 2410127E18 gene | 0.40 |
| AA60979 | Mus musculus, clone IMAGE. 106712, mRNA | 0.41 |
| AA27551 | M. musculus similar to RIKEN cDNA 6030426L16 (LOC385177), mRNA | 0.45 |
| AA49482 | | | 0.46 |
| AA64149 | | | 0.48 |
| W73539 | M. musculus similar to PTK9 protein-tyrosine kinase 9 (LOC233637), mRNA | 0.49 |
| AA46719 | E130103E02Rik | RIKEN cDNA E130103E02 gene | 0.54 |
| W6344 | 2900002H16Rik | RIKEN cDNA 2900002H16 gene | 0.54 |
| AA61338 | Phe3 | Polyhomeotic-like 3 (Drosophila) | 0.55 |
| W1821 | 2310057J16Rik | RIKEN cDNA 2310057J16 gene | 0.56 |
| AA266367 | | | 0.58 |
| AA78613 | A481100 | Expressed sequence A481100 | 0.57 |
| AA004178 | Cbfa23h | Core-binding factor, runt domain, alpha subunit 2; translocated to 3 homolog (human) | 0.57 |
| AA030925 | Farp1 | FERM, RhoGEF (Arhgef) and pleckstrin domain protein 1 (chondrocyte-derived) | 0.59 |
| AA067119 | M. musculus, similar to hypothetical protein FLJ21610, clone: IMAGE:5150298, mRNA, partial cds | 0.60 |
| AA061397 | | | 0.60 |
| AA498328 | Scamp4 | Secretory carrier membrane protein 4 | 0.60 |
| AA201871 | | | 0.61 |
| AA542183 | | | 0.61 |
| AA013802 | AW907654 | Expressed sequence AW907654 | 0.61 |
| W89816 | BC025462 | cDNA sequence BC025462 | 0.61 |
| W63092 | 5730494N06Rik | RIKEN cDNA 5730494N06 gene | 0.62 |
| AA161815 | 5730445M16Rik | RIKEN cDNA 5730445M16 gene | 0.62 |
| AA200478 | MGC38453 | Hypothetical protein LOC381045 | 0.62 |
| AA547884 | | | 0.63 |
| AA475992 | Sbf1 | SET binding factor 1 | 0.63 |
| AA007974 | Tde1 | Tumor differentially expressed 1 | 0.63 |
| W89835 | | | 0.64 |
| AA04906 | 1110038M16Rik | RIKEN cDNA 1110038M16 gene | 1.54 |
| A09171 | B130036O03 | Hypothetical protein B130036O03 | 1.55 |
| AA03663 | Pcbp4 | poly(rC) binding protein 4 | 1.56 |
| W189003 | Tex2 | Testis-expressed gene 2 | 1.56 |
| AA119797 | M. musculus LOC381690 (LOC381690), mRNA | 1.57 |
| AA060891 | 2810432L12Rik | RIKEN cDNA 2810432L12 gene | 1.58 |
| AA12957 | Zfp363 | Zinc finger protein 363 | 1.59 |
| AA12887 | mKIAA1064 | mKIAA1064 protein | 1.65 |
| AA272167 | BC024814 | cDNA sequence BC024814 | 1.70 |
| AA268786 | 1700023O11Rik | RIKEN cDNA 1700023O11 gene | 1.78 |

matin in H1t-null germ cells, which showed that other H1 subtypes are indeed increased in these cells so as to maintain a normal H1t-to-nucleosome core stoichiometry (9). The H1a subtype, in particular, appeared to be involved in compensation, as it represents more than 50% of the remaining linker histone in H1t-null germ cells. The present work was undertaken with the goal of creating an actual linker histone deficiency in murine germ cells. This goal was achieved by generating two linked null alleles for H1a and H1t through a two-step, sequential gene inactivation strategy in ES cells, and then producing mice that are homozygous null for both genes.

The nucleosome core particle is the fundamental organizational component of eukaryotic chromatin, and most of the DNA in cells is associated with core particles. Most eukaryotic cells also contain nearly one H1 linker histone molecule for each nucleosome core particle (1). It might be expected that alterations in the levels of these abundant cellular constituents would not be tolerated. Reductions in the cellular levels of core histones are lethal in the yeast *Saccharomyces cerevisiae* (33, 34). Surprisingly, however, the linker histone is not essential for viability in yeast (35, 36), although the normal level of this histone relative to nucleosomes appears to be significantly lower in yeast than in other eukaryotes (37). H1 also is not essential in several other unicellular eukaryotes (35, 36, 38–40). However, we recently reported that linker histones are essential in mice (41). Elimination of the somatic linker histones subtypes, H1c, H1d, and H1e, led to mid-gestational embryonic lethality. The null embryos exhibited a 50% reduction in the level of total H1 and a broad range of phenotypic defects.

The role of linker histones in mammalian spermatogenic cells is also of interest for several reasons. These cells contain H1t, one of the two tissue-specific linker histones in mammals. During the process of spermatogenesis, there are numerous and complex changes in chromosome structure, in meiotic and post-meiotic stages. There also are marked changes in chromatin protein composition, including, ultimately, complete replacement of histones by other basic proteins (8, 42). It might be expected that such cells and processes would be exquisitely sensitive to alterations in the balance of linker histones and nucleosomal histones. On the other hand, because germ cells are not essential for viability of mice, they are ideal for studying the effects of histone changes on chromatin stoichiometry.

By inactivating the genes encoding both H1a and H1t, we eliminated two H1 subtypes that normally constitute more than 65% of the total H1 histones in germ cells. However, this did not result in a 65% reduction in linker histone content in the cells. As we have observed previously, the synthetic capac-
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A.

B.

C.

GAPDH

FIG. 5. Northern blot analysis of two genes found to change expression in H1α, H1t null germ cells by cDNA microarray analysis. Panels show northern blots of total germ cell RNA from three littermate pairs of the indicated genotypes. The animals used for these experiments were different from the animals used for microarray analysis. Probes used were: A, spermatogenic-specific zic finger transcription factor (AA064110); B, propionyl coenzyme A carboxylase homolog (W35841); and C, glyceraldehyde-3-phosphate dehydrogenase. The arrows in the figure indicate the position of 18 S ribosomal RNA in the gels before transfer (not shown).

ity of the remaining H1 genes is sufficient to partially compensate for loss of the two H1 genes. In this case, H1c, H1d, and H1e constitute most of the H1 present in the double null germ cells, with H1c representing nearly 50% of the remaining H1. Nevertheless, the double gene inactivation did lead to a 25% reduction in the H11-to-nucleosome core stoichiometry (Table II). Furthermore, we found that the actual reduction of H1 in pachytene spermatocytes may be considerably higher than 25%, because H1t represents 40–50% of the linker histone in pachytene spermatocytes (8), whereas it is only about 30% of the H1 in germ cells, which we used for our measurements.

Based on our previous studies of compound and mutant embryos null for the somatic H1α (41), one might have expected that spermatocytes with a 25% or more reduction in total H1 would have obvious defects. But we did not detect any differences in spermatogenesis between wild-type and H1α, H1t double null mice, including differences in testis weight or histology or number of mature sperm. We also did not detect any abnormalities in meiotic processes, using antibodies against various proteins involved in homologous chromosome pairing, recombination, and chromosome condensation. Interestingly, there were no observable morphological differences in meiotic chromosome synapsis or condensation in the mutant spermatocytes, implying that normal stoichiometry of H1 histones is not critical for these dynamic chromatin-remodeling processes. Our analyses also suggest that other meiotic processes, such as formation of recombination-related DNA double strand breaks and establishment of chiasma as a result of recombination, are unaffected. Perhaps a greater reduction in H1 amount is needed to perturb these processes. The H1c gene would be an appropriate target for inactivation in the H1α, H1t doubly targeted ES cells, because H1c represents nearly 50% of the H1 remaining in double null germ cells (Table IIb).

One possible explanation for the absence of phenotypic abnormalities in the H1α, H1t null germ cells may lie in our finding that expression of relatively few genes is affected in the cells. Among about 9,000 genes assayed, we found only 17 genes that showed greater than 2-fold differences in expression between wild-type and double null germ cells. It is quite strik-

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