Although this chapter focuses on methods we have used to study the BRM and ISWI chromatin-remodeling factors, similar approaches can be used to study other chromatin-remodeling and modifying enzymes. Together with biochemical studies, these genetic and cytological methods should facilitate the investigation of the complex regulatory network of chromatin-modulating proteins.

## Acknowledgments

We would like to thank Mary Kay Phillips from Jackson ImmunoResearch Laboratories (West Grove, PA), for advice concerning the Fab fragment-based polytene staining protocol. We also would like to thank Dr. Renate Deuring and Vidhya Srinivasan for their useful comments on this manuscript. Most of the stocks described in this article can be obtained from the Bloomington Stock Center or our laboratory. Work in our laboratory is supported by a Grant from the NIH (GM49883) to J. W. T. J. A. A. has been supported by the Damon Runyon Cancer Research Fund (Fellowship DRG-1556) and D. F. V. C. has been supported by EMBO and HFSP postdoctoral fellowships.

# [5] Genetic Analysis of H1 Linker Histone Subtypes and Their Functions in Mice

*By* YUHONG FAN and ARTHUR I. SKOULTCHI

In most eukaryotic cells, the chromatin fiber consists of nearly one molecule of linker histone for each nucleosome core particle. Therefore, linker histones are expected to play a key role in the structure of the chromatin fiber. A large variety of *in vitro* experiments with chromatin, and other correlative findings, support this view. These studies indicate that two important functions of linker histones are to stabilize the DNA as it enters and exits the core particle and to facilitate the folding of nucleosome arrays into more compact structures. Linker histones also affect nucleosome core particle spacing and mobility *in vitro*.[1,2]

Surprisingly, however, elimination of the linker histone in Tetrahymena, yeast and fungi led to the conclusion that H1 is not essential in these unicellular eukaryotes.[3–7] But double stranded RNA-mediated interference

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(RNAi) of H1.1 in *C. elegans*[8] or antisense-mediated decrease of certain linker histone subtypes in tobacco[9] does lead to effects on germ line development. Delaying synthesis of somatic H1 in *Xenopus* embryos at the midblastula stage with specific ribozymes caused prolonged mesodermal competence.[10] These studies indicate that linker histones do play important roles in chromatin function during development in higher organisms.

In mice there are at least eight H1 subtypes, including the widely expressed somatic H1a through H1e, the testis-specific H1t, the oocyte-specific H1oo, and the replacement linker histone $H1^0$.[11,12] The genes for H1a–H1e and H1t are linked on mouse chromosome 13,[13] whereas the $H1^0$ gene is on mouse chromosome 15 and the H1oo gene is located on mouse chromosome 6. These H1 subtypes differ significantly in amino acid sequences; for example, $H1^0$ is only 30–38% identical to H1a–H1e and even amongst H1a–H1e sequence divergence approaches 40% between certain subtypes.[13] These subtypes also exhibit distinct patterns of expression during development.[11] Much of the differential regulation occurs at the transcription level. The accumulation of $H1^0$ is generally associated with terminal differentiation and terminal cell division.[14] The levels of certain subtypes during development can reach a major fraction of the linker histones present in specific cell types, for example $H1^0$ and H1e constitute 28 and 42%, respectively, of H1 in adult mouse hepatocytes[15] and H1t constitutes 40–50% of H1 in pachytene spermatocytes.[16,17] Many of these features are conserved between mice and humans, including genomic organization, amino acid sequence of corresponding subtypes, developmental

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regulation where studied, and even DNA sequences in regions of the mouse and human corresponding genes, such as the 5′ and 3′ untranslated regions and the promoters, that differ even more amongst the genes for the subtypes in one species.[18] This high level of conservation in mammalian evolution also suggests important roles for these subtypes in fine tuning chromatin structure.

The development of techniques for inactivating genes in murine embryonic stem (ES) cells and for producing mice from such modified ES cells provides an approach for undertaking a genetic analysis of H1 linker histones in mammals. In the following sections we first discuss methods for inactivating the genes for individual members of the H1 linker histone gene family and the procedures for analyzing the composition of chromatin from mice null for individual H1 subtypes. Surprisingly, these studies show that, despite the abundance of certain subtypes in specific tissues, none of the six individual H1 subtypes eliminated to date is essential for proper mouse development. Chromatin analyses from single H1 null mice show that compensation by upregulation of other H1 subtypes is a likely explanation for the absence of a phenotype in these mice. Nevertheless, by introducing into some of these single H1 null strains, a globin transgene, it is possible to show that specific H1 subtypes do have differential effects on gene expression. Finally we describe methods for producing compound H1 null mice. In certain compound null strains, the compensation among H1 family members is disrupted. These strains have been used to show that: (1) H1 linker histones are essential for mouse development; (2) marked reductions in stoichiometry of total H1 in chromatin are tolerated in mice.[19]

## Production of Mice Lacking a Single H1 Subtype

The general procedure for inactivating a gene in mice is gene targeting by homologous recombination in ES cells (see Figs. 1 and 2). First, genomic clones including portions of the gene of interest are isolated from a mouse genomic library. For a gene family consisting of highly related members, such as the H1 histone family, clones containing a specific family member(s) can be identified by hybridization with gene-specific flanking region probes or by gene-specific PCR. The use of a genomic library derived from a strain of mice congenic with the ES cell line used is recommended. The advantage of this practice is to increase the homologous recombination

[18] Z. F. Wang, A. M. Sirotkin, G. M. Buchold, A. I. Skoultchi, and W. F. Marzluff, *J. Mol. Biol.* **271,** 124 (1997).

[19] Y. Fan, T. Nikitina, E. M. Morin-Kensicki, J. Zhao, T. R. Magnuson, C. L. Woodcock, and A. I. Skoultchi, *Mol. Cell. Biol.* **23,** 4559 (2003).

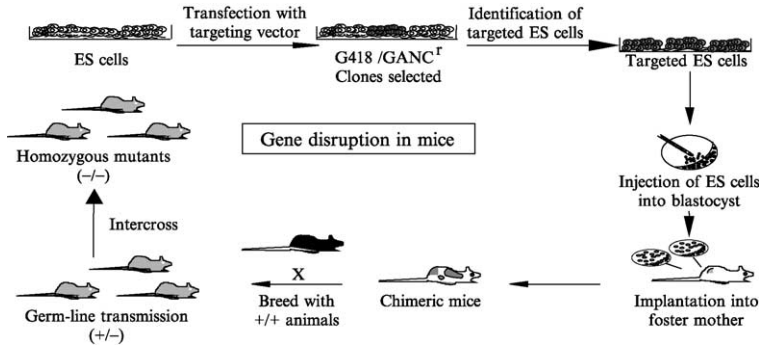


FIG. 1. Procedure of gene disruption in mice.

frequency during gene targeting. A variety of ES cell lines can be used. Many of these lines are derived from the 129J/sv mouse strain, for example, the E14-1 ES cell line,[20] or have a major portion of their genome derived from 129J/sv, for example, the WW6 ES cell line.[21] The use of genomic clones derived from the 129J/sv library increases the frequency of gene targeting by homologous recombination in these lines.[22] We have had particularly good success with the WW6 ES cell line which grows robustly, is easy to maintain, yields efficient germline transmission, and can be used for several rounds of sequenctial gene targetings with different selection markers (see later section). In addition, WW6 cells contain a 11-Mb $\beta$-globin transgene on chromosome 3 which can serve as an inert, nuclear-localized marker to identify donor ES-derived cells in chimeric mice using DNA in situ hybridization.[21]

Targeting vectors for gene inactivation are generally constructed containing both positive and negative selection marker genes for isolation of stably transfected ES cell clones.[22] Useful positive selection marker genes include those conferring resistance to G418 (Geneticin, Gibco-BRL), hygromycin (Boehringer Mannhem) or puromycin (Sigma). Transcription of these genes is usually driven by the strong, ubiquitously expressed phosphoglycerate kinase (PGK) promoter. The most widely used negative

[20] R. Fodde, W. Edelmann, K. Yang, C. van Leeuwen, C. Carlson, B. Renault, C. Breukel, E. Alt, M. Lipkin, P. M. Khan *et al.*, *Proc. Natl. Acad. Sci. USA* **91,** 8969 (1994).

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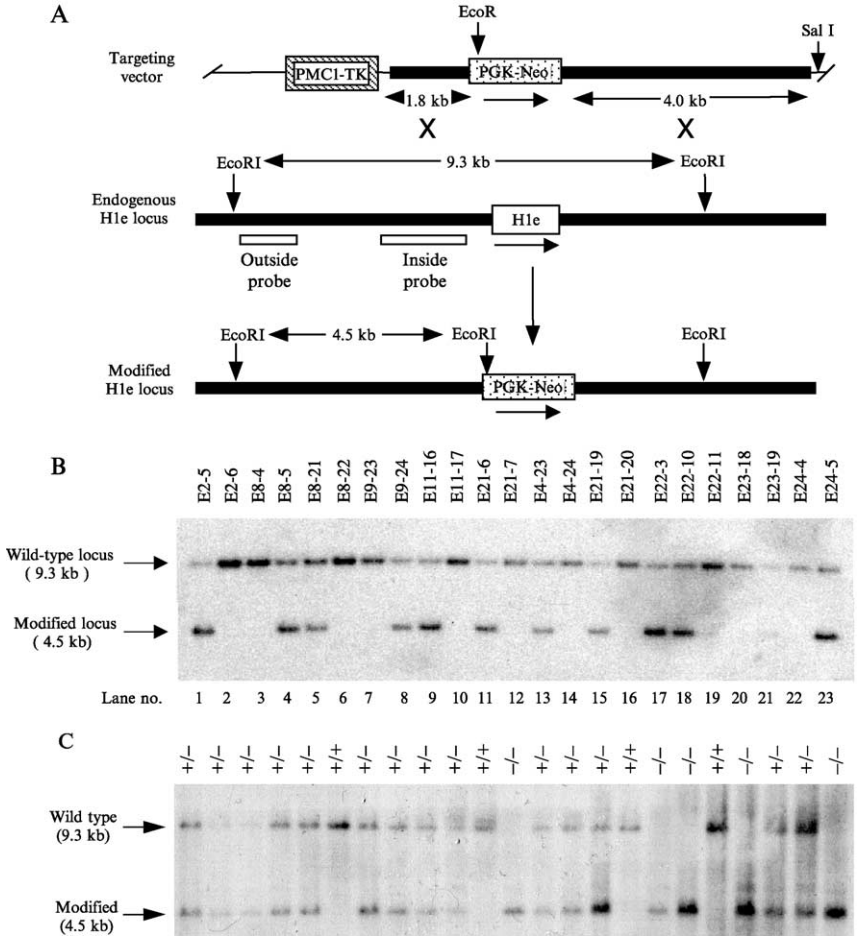



FIG. 2. Targeted disruption of the H1e gene in mouse ES cells and mice. (A) Homologous recombination strategy in ES cells. The H1e targeting vector (top) was constructed by replacing a 720 bp Msc I fragment containing the H1e coding region (open box) along with 49 bp of 5′ noncoding sequence and 11 bp of 3′ noncoding sequence with the PGK-Neo gene. A negative selection gene PMCI-TK was inserted 5′ of the 1.8 kb short arm of genomic DNA. A homologous recombination event (Xs) between the targeting vector and the endogenous H1e locus (middle) results in production of a modified H1e locus (bottom). (B) Identification and confirmation of ES cell clones containing the modified H1e allele. ES cell DNA (10 ug) was digested with EcoRI followed by Southern blot analysis using the 5′ outside probe shown in panel A. The expected position of the hybridizing fragments from the unmodified (wild-type) and modified H1e loci and their respective sizes are indicated. (C) Genotype analysis of offspring from parents heterozygous for the modified H1e allele. Siblings that were heterozygous for the modified H1e allele were bred and 15 ug of tail DNA from offspring were digested with EcoRI, blotted and hybridized with the 5′ inside probe. The deduced genotype of each animal is indicated above each lane. The wild-type and modified H1e loci and their corresponding sizes are indicated. Reproduced with permission from Fan *et al.*[23]

selection marker gene is Herpes Simplex thymidine kinase (HSV-*tk*) gene that, when expressed, causes cells to be sensitive to ganciclovir (Syntex). In the vector, the positive selectable marker gene is flanked by two arms of genomic DNA that are homologous to regions upstream and downstream of the gene to be inactivated. The two arms of genomic DNA in the vector may include none of the histone coding sequence, in which case, homologous recombination leads to complete deletion of the gene at the modified locus, or it may include a portion of the gene in which case an incomplete gene remains at the modified locus. Design of the targeting vectors for most H1 histone genes is facilitated by the fact that, except for the H1oo gene, none of the mouse H1 genes contain introns and their coding regions are small, ranging in size from 579 ($H1^0$) to 660 bp (H1d).[12,18] The negative selection marker gene (PMCI-TK in Fig. 2A) is placed either upstream or downstream of the regions of homology. Selection for loss of expression of this marker (with ganciclovir) increases the frequency of homologous recombinants among antibiotic resistant clones.[24]

Figure 2 shows an example of a gene targeting experiment at the H1e locus. To construct the H1e targeting vector, a positive selection marker gene, PGK-Neo, was flanked by two arms of genomic DNA that are homologous to the regions of the 5′ and 3′ region of H1e gene. The HSV-*tk* gene was inserted upstream of the 5′ homology region for negative selection. Homologous recombination between the targeting vector and the endogenous H1e locus will result in a modified locus in which a segment from 49 bp 5′ of the ATG codon to 11 bp 3′ of the stop codon in the H1e gene is replaced with the PGK-Neo gene (see Fig. 2A).

In the later sections we provide the protocol we have used to inactivate several H1 linker histone genes. Readers are referred to gene targeting manuals for even more detailed procedures.[22,24,25]

### *Methods for ES Cell Transfection and Selection*

1. ES cells are grown on mitotically inactive feeder layers of $G418^r$ SNL fibroblasts and cultured with ES cell medium supplemented with leukemia inhibitory factor (LIF) (ESGRO, Chemicon) at 1000 units/ml. (Note: In the sequential gene targeting experiments [see later], $G418^r$ $Hygro^r$ SNLH or $G418^r$ $Puro^r$ SNLP fibroblasts were used in subsequent targeting steps accordingly.)

[23] Y. Fan, A. Sirotkin, R. G. Russell, J. Ayala, and A. I. Skoultchi, *Mol. Cell. Biol.* **21,** 7933 (2001).
[24] R. Ramirez-Solis, A. C. Davis, and A. Bradley, *Methods Enzymol.* **225,** 855 (1993).
[25] E. J. Robertson, ''Teratocarcinomas and Embryonic Stem Cells.'' IRL, Oxford, 1987.

2. Feed ES cells with fresh ES cell medium 4 h before harvesting them for electroporation.

3. Twenty-five micrograms of SalI linearized targeting vector is electroporated into $2 \times 10^7$ ES cells at 400 V and 250 $\mu$F using a Bio-Rad Gene Pulser. The electroporated ES cells are subsequently plated onto five 10-cm plates that were coated with inactivated SNL cells.

4. Twenty-four hours after electroporation, change the ES cell medium to ES cell medium containing G418 (200 $\mu$g/ml). Forty-eight hours after application of G418, change the culture medium to ES medium containing both G418 (200 $\mu$g/ml) and ganciclovir (2 $\mu M$). Refeed cells when the medium begins to become acidic, usually daily for the first five days and every two days afterwards.

5. Ten days after transfection, ES cell colonies resistant to G418 and ganciclovir are picked, trypsinized and cultured in feeder cell coated 24-well plates. After three days, cells from each well are trypsinized and split into two 24-well plates (one plate is treated with gelatin, and the other plate is coated with inactivated feeder cells). The cells grown on the gelatin-coated plate can be grown to confluence and used for extraction of DNA. The absence of feeder cells allows for detection of targeting events in the ES cells without interference by feeder cell DNA. After 2–3 days, the feeder cell-coated plate is frozen by changing the medium to ES cell freezing medium (ES medium with 10% v/v DMSO). The plate is then wrapped with foil, placed in a styrofoam box and deposited into a $-80^\circ$ freezer. After determining which ES cell clones have undergone gene targeting, these clones can be recovered by thawing the plate and culturing cells from the appropriate wells.

*Preparation of ES Cell DNA for Screening Clones*

This method is modified from Laird *et al.*[26]

1. Allow the cells on the gelatinized plate to grow to confluence, which usually takes four days. During this period, change the medium daily with G418 selection only.

2. Aspirate the medium from the wells, rinse the well with PBS once, and fully aspirate the PBS at room temperature. There is no need to change tips during aspiration from one well to the other.

3. Add 500 $\mu$l of ES cell lysis buffer (100 m$M$ Tris pH 8.5, 5 m$M$ EDTA, 0.2% SDS, 200 m$M$ NaCl, 100 $\mu$g/ml Proteinase K). Incubate at $37^\circ$ for 3 h with agitation.

[26] P. W. Laird, A. Zijderveld, K. Linders, M. A. Rudnicki, R. Jaenisch, and A. Berns, *Nucleic Acids Res.* **19,** 4293 (1991).

4. Add one volume (500 $\mu$l) of isopropanol to each lysate.
5. Swirl a glass capillary sealed at one end in the well until the genomic DNA precipitates and wraps around the capillary. Dip the capillary in a beaker containing 75% ethanol and place it with the sealed end up in an Eppendorf tube to air dry.
6. After isolating DNA from one plate of clones, reverse the capillary so that now the end containing the DNA is inside the Eppendorf tube. Use a diamond-tipped pen to cut the capillary near the mouth of the tube, add 200 $\mu$l of 10 m*M* Tris pH 8.0 and allow the DNA to dissolve overnight with agitation at 55°.

In the gene targeting experiment described in Fig. 2, genomic DNA from 240 individual G418/ganciclovir-resistant colonies was prepared, pooled (2 colonies/pool), digested with EcoRI, and screened for homologous recombination events by Southern blot analysis (see Fig. 2B) with a 0.9 kb 5′ flanking region probe (Outside Probe—Fig. 2A). 11 clones gave rise to a 4.5-kb EcoRI hybridizing fragment expected from the modified allele (see Fig. 2B).

Four clones containing the modified H1e locus were injected into C57BL/6 recipient blastocysts, and the blastocysts were transferred to CD-1 pseudopregnant females to generate chimeric mice (these steps are usually carried out by highly trained personnel in a gene targeting core facility). Two cell lines generated chimeras ranging in chimerism between 70 and 100% based on coat color. Male chimeras from these two cell lines were mated with C57B1/6 mice. Both cell lines successfully transmitted the modified H1e allele through the germline. Male and female chimeras from each line were then mated with C57B1/6 mice. ES cells derived from the 129J/sv strain of mice contain the dominant agouti coat color gene, and the recipient C57B16 mice has a black coat color. Therefore, if the injected ES cells are able to contribute to the germline, the F1 progeny of the chimeric mice will have an agouti coat color. Generally, both agouti mice and black mice are present in the F1 progeny. The agouti mice among the F1 progeny were selected and their tail DNAs were extracted as described earlier.[22] Southern blot analyses were used to verify the transmission of the modified allele through the germline of chimeric mice produced from correctly targeted ES cell clones.

Mice heterozygous for H1e gene mutation were phenotypically normal.[23] These heterozygous mice were interbred to generate H1e homozygous mutant mice. Southern blot analyses (see Fig. 2C) or PCR assays were used to genotype F2 progeny using mouse tail DNA. Among 48 F2 progeny, 10 (21%) carried only the wild-type allele, 24 (50%) carried one copy of the modified allele and 14 (29%) carried two copies of the modified

allele. The ratio of the three classes of animals, is not significantly different from the expected values for Mendelian transmission of the two alleles.

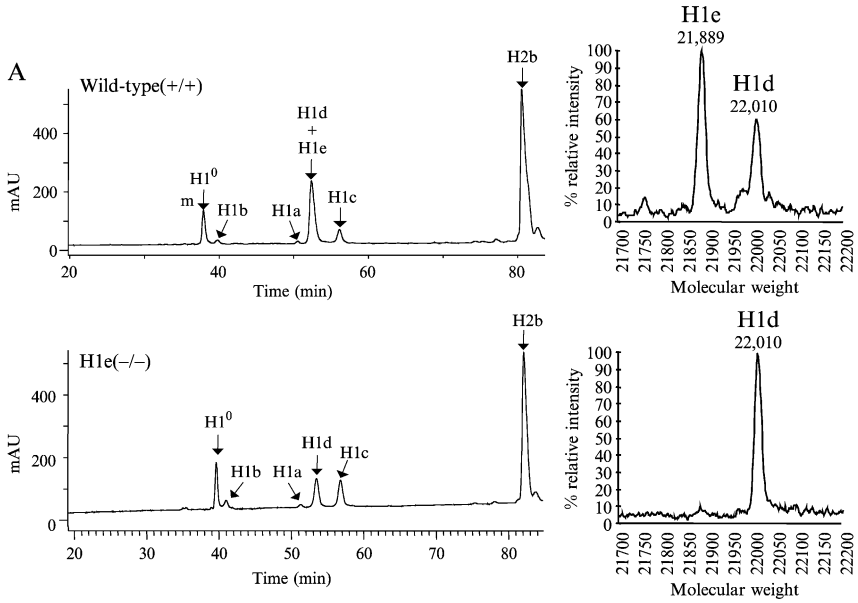
To confirm that the H1e gene targeting produced a null allele, total histone extracts from livers of F2 mice were analyzed by reverse-phase HPLC (see procedures later) (see Fig. 3A).[15,27–29] As shown in the figure, this method resolves $H1^0$ and the five somatic H1s. H1d and H1e elute as a single peak in the chromatogram, but by collecting this peak and subjecting it to TOF-MS analysis, these two subtypes also can be quantified separately. Whereas H1e was readily detected in extracts of wild-type animals, it was not detectable in extracts from homozygous mutant animals (see Fig. 3A).

*Method for Preparation of Total Histones*

All operations are done at 4°, except as indicated.

1. Dissect tissue, rinse in PBS, mince into small pieces with a razor blade, suspend in sucrose buffer (0.3 *M* Sucrose, 15 m*M* NaCl, 10 m*M* HEPES [pH 7.9], 2 m*M* EDTA, 0.5 m*M* PMSF [added fresh]) at 1 g tissue per 10 ml sucrose buffer.
2. Homogenize in a Dounce homogenizer (B pestle), 10–15 strokes.
3. Transfer the homogenated tissue to 15 ml conical tubes, centrifuge at 500 rpm for 30 s. (Centrifuge model: Eppendorf 5810R)
4. Carefully transfer the supernatant to a fresh tube (discard the pellet) and centrifuge at 2000 rpm, 5 min.
5. Discard the supernatant; resuspend the pellet in 10 ml sucrose buffer + 0.5% NP-40 and homogenize with a B pestle, 10 strokes.
6. Centrifuge 2000 rpm for 5 min and discard supernatant.
7. Resuspend pellet together in high salt buffer at 3 ml per 1 g tissue, and homogenize in a small Dounce homogenizer, 10 strokes [high salt buffer: 0.35 *M* KCl, 10 m*M* Tris (7.2), 5 m*M* $MgCl_2$, 0.5 m*M* PMSF (added fresh)].
8. Transfer to a 1.5 ml microcentrifuge tube and let stand on ice for 20 min.
9. Centrifuge at 14,000 rpm for 10 min and discard supernatant.
10. Resuspend each pellet in 0.8 ml 0.2 N $H_2SO_4$ by using an Eppendorf tube pestle to grind the pellet well, and let stand at 4° for overnight.

[27] D. T. Brown and D. B. Sittman, *J. Biol. Chem.* **268,** 713 (1993).
[28] W. Helliger, H. Lindner, O. Grubl-Knosp, and B. Puschendorf, *Biochem. J.* **288,** 747 (1992).
[29] H. Lindner, W. Helliger, A. Dirschlmayer, M. Jaquemar, and B. Puschendorf, *Biochem. J.* **283,** 467 (1992).




B

| | % of total H1 | | | | | | Total H1 per nucleosome |
|---|---|---|---|---|---|---|---|
| | H1$^0$ | H1a | H1b | H1c | H1d | H1e | Ratio |
| WT (+/+) | 27.4 | 0.9 | 1.9 | 11.6 | 16.1 | 42.1 | 0.73 |
| H1e (–/–) | 38.49 | 1.47 | 4.46 | 24.25 | 31.34 | 0 | 0.75 |

FIG. 3. Analysis of chromatin from H1e knockout mice. (A) Analysis of histones extracted from livers of wild-type and H1e homozygous mutant mice. Panels on the left show reverse phase HPLC analyses of approximately 100 $\mu$g of total liver histone extracts from a 20-week-old wild-type (upper panel) and a H1e homozygous mutant (lower panel). The abscissa represents the elution time and the ordinate represents absorbency at 214 Å. The identity of the histone subtype(s) in each peak is indicated. Panels on the right show TOF-MS analysis of a fraction eluting between 52 and 54 min (corresponding to the peak marked H1d + H1e). The identity of the H1d and H1e subtypes detected in this analysis was shown earlier.[18] (B) H1 subtype composition of liver chromatin from 5-month-old wild-type and H1e mutant mice. Data were calculated from HPLC analyses of wild-type and H1e mutant strains like that shown in (B). The percentage of total H1 was determined by the ratio of the $\overline{A}_{214}$ of the indicated H1 peak to the total $\overline{A}_{214}$ of all H1 peaks. Total H1 per nucleosome was determined by the ratio of the total $\overline{A}_{214}$ of all H1 peaks to half of the $\overline{A}_{214}$ of the H2b peak. The $\overline{A}_{214}$ values of the individual H1 peaks and the H2b peak were adjusted to account for the differences in the number of peptide bonds in each H1 subtype and H2b. (Fig. 3A is adapted with permission from Fan *et al.*[23])

11. Centrifuge at 14,000 rpm for 10 min, transfer the supernatant to two 1.5 ml microcentrifuge tubes (400 $\mu$l each).

12. Add 2.5 volumes of ice cold ethanol directly to each tube and leave at $-20^{\circ}$ overnight.

13. Centrifuge at 14,000 rpm for 10 min and wash the pellet 3 times with 70% EtOH. Air dry about 20–30 min or longer as necessary or dry for 3–5 min in a speed-vac centrifuge. The dried protein can be stored at $-20^{\circ}$ for up to 12 months.

*Methods for HPLC Analysis of Histone Proteins*

1. 50–100 $\mu$g of dried total histone preparations are resuspended in 1 ml $H_2O$/0.1% TFA and injected into a Vydac 300A, 5 mm C18 reverse phase column (0.46 cm $\times$ 25 cm) on a Hewlett-Packard 1090 HPLC system.

2. Total histone proteins are fractionated by an increasing acetonitrile gradient as follows:

| Time (min) | $H_2O$/0.1%TFA (%) | Acetonitrile/0.1%TFA (%) |
|---|---|---|
| 0.01 | 100.0 | 0.0 |
| 1.00 | 95.0 | 5.0 |
| 11.0 | 75.0 | 25.0 |
| 26.0 | 70.0 | 30.0 |
| 45.0 | 65.0 | 35.0 |
| 66.0 | 60.0 | 40.0 |
| 75.0 | 57.0 | 43.0 |
| 126.0 | 45.0 | 55.0 |
| 131.0 | 10.0 | 90.0 |
| 136.0 | 95.0 | 5.0 |
| 151.0 | 100.0 | 0.0 |

3. The effluent from the HPLC column is monitored at 214 nm and the peaks are recorded using the Hewlett-Packard 1090 system. Peak areas are determined with a Hewlett-Packard peak integrator program.

## Compensation Among Linker Histone Subtypes in Single H1 Null Mice

Using the procedures described in the preceding section, strains of mice that are null for each of six individual H1 subtypes were generated, including the somatic H1s, H1a, H1c, H1d, and H1e, the testis-specific H1t, and the highly variant, replacement linker histone $H1^0$.[15,23,30,31] Each of the six

types of single H1 null mice were found to be indistinguishable from their heterozygous and wild-type littermates. Homozygous null mice were fertile and they produced litters of normal size and their progeny appeared normal. Examination of hematoxylin-and-eosin-stained sections from each null strain did not show any pathology or histologic abnormality. No differences were found even in testis or liver in which particular subtypes are especially abundant (H1t 40–50% in pachytene spermatocytes; $H1^0$ and H1e 27% and 42% in liver).

In the absence of an obvious phenotype in knockout mice, it is often assumed that other proteins with similar functions compensate for the protein that has been eliminated. This hypothesis may be examined by generating compound mutants (see a later section). For H1 histones, another approach to investigating this possibility is to examine the content and stoichiometry of the remaining H1 subtypes by reverse phase HPLC. As shown in Fig. 3, HPLC, in combination with TOF-MS, allows quantitation of the relative amounts of $H1^0$ and each of the somatic H1 subtypes, H1a through H1e. H1t, which migrates at about 64 min in the chromatogram also can be quantitated.[30] Furthermore, the method also separates the H1s from the nucleosomal core histones. This allows one to estimate the ratio of linker histones to nucleosome core particles by measuring the total H1 amount compared to one of the core histones, for example, H2b.

The results of this type of analysis carried out with each of the six strains of single H1 null mice showed that the stoichiometry of total linker histones compared to nucleosomes is not altered in the knockout mice. For example, in H1e null mice, despite the fact that H1e accounts for 42% of total H1 in adult liver, the elimination of H1e did not result in a decrease in the H1/nucleosome ratio (see Fig. 3B). Instead, other H1 subtypes, especially $H1^0$, H1c, and H1d, are upregulated in chromatin so as to maintain the ratio in the null mice at the same level as in wild-type mice. This is also true in mice null for highly specialized H1 subtypes, such as the differentiation specific $H1^0$ which account for 27% of H1 in adult liver and the testis specific H1t which is 28% of H1 in testis.[15,23,30]

The mechanism(s) that serve to maintain a normal linker histone to nucleosome stoichiometry in single H1 null mice is not fully understood. One possibility is that excess linker histone molecules are synthesized normally and when the gene for one subtype is inactivated, some of the excess of the remaining subtypes is deposited in chromatin in place of the missing subtype. This explanation is supported by the finding that in several types of single H1 null mice, the relative amounts of the remaining subtypes found

[30] Q. Lin, A. Sirotkin, and A. I. Skoultchi, *Mol. Cell. Biol.* **20,** 2122 (2000).
[31] Q. Lin, Ph.D. thesis: Albert Einstein College of Medicine, Bronx, New York (1998).

in chromatin are nearly the same as their relative amounts in wild-type mice. However, observations made with compound H1 null mice, generated as described later, suggest that increased mRNA synthesis of specific H1 subtypes may also be involved in the feedback or cross-regulation of transcription within the H1 family. Interestingly, this cross-regulation may include the highly variant $H1^0$ gene (YF and AS unpublished observations) which is not located at the histone locus on mouse chromosome 13 and which, unlike most H1 genes, produces polyA + mRNA.

## Deletion of Specific H1 Linker Histone Subtypes Affects Expression of Transgenes in Mice

Although compensation within the H1 gene family apparently leads to the absence of a phenotype in single H1 null mice, these strains of mice can be used to examine the effects of specific H1 subtypes on gene regulation *in vivo*. Many studies have shown that expression of transgenes in mice (and other eukaryotes) are subject to position effects. Although the basis for position effects on transgene expression are mostly not understood, they are thought to reflect aspects of chromatin structure. Transgenes are thought to be very sensitive indicators of perturbations in local chromatin structure. To study the effect of H1 subtypes on transgene expression, in collaboration with Dr. Eric Bouhassira and colleagues (Albert Einstein College of Medicine), we bred several different single H1 null mice with a transgenic strain carrying a human $\beta$-globin transgene that is subject to age-dependent silencing (see Fig. 4A, B). The 4.4 kb human $\beta$-globin transgene contains the entire coding sequence of the $\beta$-globin gene plus 2.1 kb of upstream flanking sequences and 0.8 kb of downstream coding sequences. Analysis of transgene expression at the single cell level with flow cytometry using a FITC-labelled monoclonal anti-human $\beta$-globin antibody[32] showed that the transgene is silenced due to a gradual decrease in the proportion of expressing cells as the transgenic mice age (see Fig. 4B). This transgene is therefore subject to an age-dependent variegating position effect.

To determine if the silencing of the transgene is modulated by linker histones, we produced by breeding mice hemizygous for the globin transgene and homozygous or heterozygous for deletion of the H1(0), H1a, H1c, H1d, or H1e genes. We then periodically monitored the fraction of red blood cells expressing the transgene and found that the absence of linker histones H1e or H1d strongly attenuated the silencing (see Fig. 4C). Loss of one allele of the H1d gene has less of an effect on attenuating

[32] R. Alami, J. M. Greally, K. Tanimoto, S. Hwang, Y. Q. Feng, J. D. Engel, S. Fiering, and E. E. Bouhassira, *Hum. Mol. Genet.* **9,** 631 (2000).

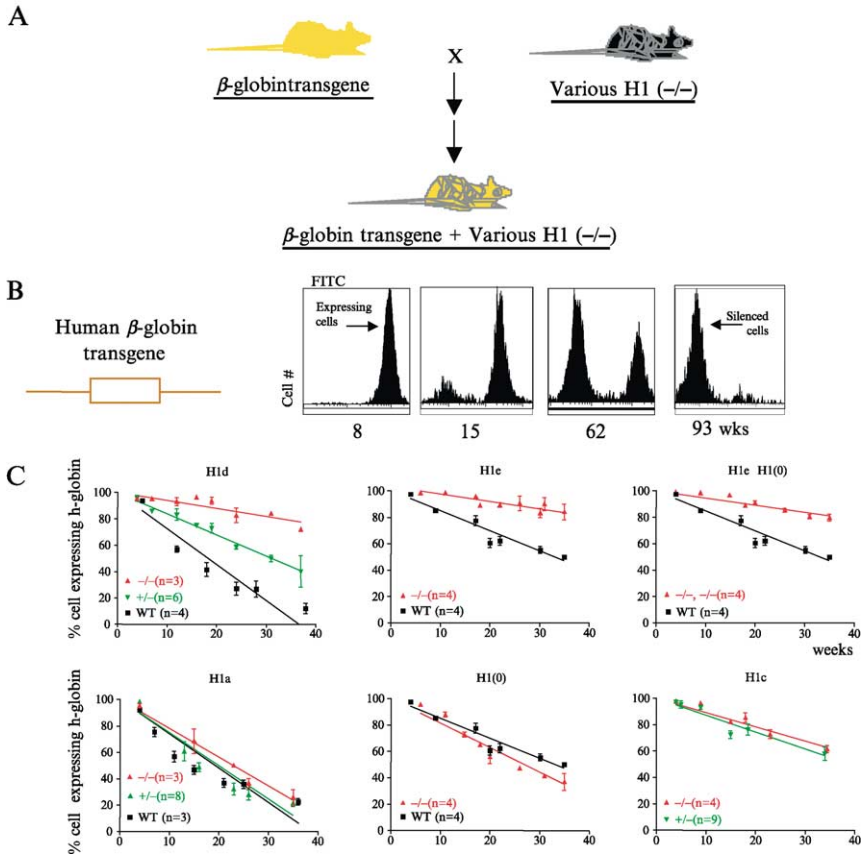



FIG. 4. Age-dependent silencing is modulated by specific linker histone deletions. (A) Scheme of experimental strategy for generating mice containing the human $\beta$-globin transgene and deletion of a single H1 subtype. (B) The 4.4 kb human $\beta$-globin gene transgene is subject to an age-dependent variegating position effect. Permeabilized RBCs were stained with FITC-labelled anti human-$\beta$-globin antibodies and periodically analysed by flow cytometry. The proportion of RBCs expressing the human transgene decreases with age. (C) Linker histone subtypes differentially affect transgene expression. Silencing of the human-$\beta$-globin transgene in mice homozygous or heterozygous for deletions of linker histones was monitored over time by flow cytometry and compared to controls. Deletion of H1d, or H1e or (H1e and $H1^0$) dramatically attenuates the rate of silencing. Deletions of H1a, H1c, and $H1^0$ has no effect on the rate of silencing. *n* is the number of mice analyzed. (Fig. 4B, C are reproduced from Alami *et al.*[33] Copyright National Academy of Sciences, USA.)

[33] R. Alami, Y. Fan, S. Pack, T. M. Sonbuchner, A. Besse, Q. Lin, J. M. Greally, A. I. Skoultchi, and E. E. Bouhassira, *Proc. Natl. Acad. Sci. USA* **28,** 28 (2003).

transgene silencing than homozygous H1d gene deletion, suggesting that even partial reduction in H1d amount can affect the rate of silencing. Absence of H1a, H1c, or $H1^0$ had no effect on the rate of silencing (see Fig. 4C). Mice lacking both H1e and $H1^0$ silenced at the same rate as mice lacking only H1e, confirming the results obtained with the H1e −/− and $H1^0$ −/− mice.[34]

The results of these studies show that, whereas mice null for individual H1 subtypes do not have apparent phenotypic changes, the absence of specific subtypes in such mice changes the properties of chromatin in subtle ways that lead to quantitative effects on expression of certain genes. These studies illustrate the utility of such H1 null mice for analysing the functions of specific H1 subtypes. In the future, single H1 null mice should be useful to study the role of specific H1s in regulation of other genes and other cellular processes that may be affected or controlled by specific subtypes.

## Generation of Compound H1 Knockout Mice

As discussed in the earlier sections, despite the extensive sequence divergence within the H1 family and despite the abundance of certain subtypes in specific tissues, elimination of any one of six different H1 subtypes by gene inactivation does not appear to affect mouse development. The absence of an effect on development is most likely due to compensation by other H1 subtypes. HPLC analysis showed that the chromatin of mice lacking any one of the H1 subtypes has a normal ratio of total H1 linker histones to core histones, and that increased amounts of the remaining H1 subtypes were deposited in chromatin and led to maintainence of a normal linker-to-core histone stoichiometry. When such compensation is present in knockout mice, one way to proceed is to generate compound null mice by combining two or more null mutations in a single strain. As described below, we have used this approach successfully for the H1 gene family.

Histone genes are often located in clusters at specific chromosomal loci. For example, the genes for H1a through H1e and H1t are tightly linked on mouse chromosome 13 interspersed with core histone genes.[18] On the other hand, the solitary $H1^0$ gene is located on mouse chromosome 15 and the H1oo gene is located on mouse chromosome 6.

There are three approaches to making compound knockout mice in which two or more genes have been inactivated. One approach is simply to breed single gene knockout mice and subsequently to intercross the double heterozygous mutants to generate double homozygous mutants.

[34] A. Puech, B. Saint-Jore, S. Merscher, R. G. Russell, D. Cherif, H. Sirotkin, H. Xu, S. Factor, R. Kucherlapati, and A. I. Skoultchi, *Proc. Natl. Acad. Sci. USA* **97,** 10090 (2000).

This approach is suitable for generating compound mutant mice for genes that are not linked, such as for the $H1^0$ gene in combination with any of the other H1 genes. However, we found that this approach is not practical for most of the H1 genes on chromosome 13 because they are too tightly linked and thus the recombination frequency is very low. In these cases, a second, but more arduous approach of sequential gene targeting has been successful, as described later. A third approach, that we and others have used at nonhistone loci, is to generate a chromosomal deletion encompassing multiple genes.[34,35] Such deletions can be produced in ES cells or mice by engineering through gene targeting a chromosomal region flanked by loxP sites. The intervening sequences between loxP sites can then be removed by Cre-mediated recombination. Application of this approach to the H1 locus on mouse chromosome 13 is limited, however, because many core histone genes and possibly other genes[13,36] are interspersed among the six H1 genes at the locus. Nevertheless, the deletion approach has been used to attempt to address linker histone functions in chicken DT40 cells.[37–39]

Taking advantage of the fact that $H1^0$ is not located on chromosome 13 where the somatic H1 genes reside, we have used the first approach (breeding strategy) to generate three types of compound H1 mutant mice lacking $H1^0$ and either H1c or H1d or H1e. First, doubly heterozygous mutant mice were produced by breeding $H1^0$−/− mice with each of strains null for one of the three somatic subtypes. Double mutant heterozygotes of each specific type were then interbred to produce double null mice, namely $H1^0$H1c, $H1^0$H1d, and $H1^0$H1e homozygous mutants. All three types of double H1 null mice are fertile and appear to be normal.[23] Just as in the analysis of single H1 null mice, here also the chromatin from tissues of these mice had a normal linker histone to nucleosome ratio.[23] This is the case even in the livers of $H1^0$H1e null mice in which 70% of the H1 normally present has been eliminated. Thus, it seems that there is sufficient excess capacity within the H1 gene family to maintain normal total H1 amounts even when the genes for two abundant subtypes are inactivated. In this circumstance, it is necessary to produce mice with three (or even more) H1 genes inactivated.

[35] B. Zheng, M. Sage, E. A. Sheppeard, V. Jurecic, and A. Bradley, *Mol. Cell. Biol.* **20,** 648 (2000).
[36] W. Albig, B. Drabent, N. Burmester, C. Bode, and D. Doenecke, *J. Cell. Biochem.* **69,** 117 (1998).
[37] Y. Takami and T. Nakayama, *Genes Cells* **2,** 711 (1997).
[38] Y. Takami and T. Nakayama, *Biochim. Biophys. Acta* **1354,** 105 (1997).
[39] Y. Takami, S. Takeda, and T. Nakayama, *J. Mol. Biol.* **265,** 394 (1997).

As mentioned earlier, to inactivate two or more of the six H1 genes on chromosome 13, the second approach of sequential gene targeting strategy is needed (see Fig. 5). The first step in the sequential strategy was the generation of ES cells in which one allele of the H1c gene is replaced by a PGK-Hygro cassette. The second step in the sequential gene inactivation strategy was the inactivation of the H1e gene in H1c +/− ES cells by replacing the H1e coding region with a PGK-Neo cassette (see Fig. 5).

To generate compound knockout mice by sequential gene targeting, it is essential to obtain ES cell clones in which the targeting events have

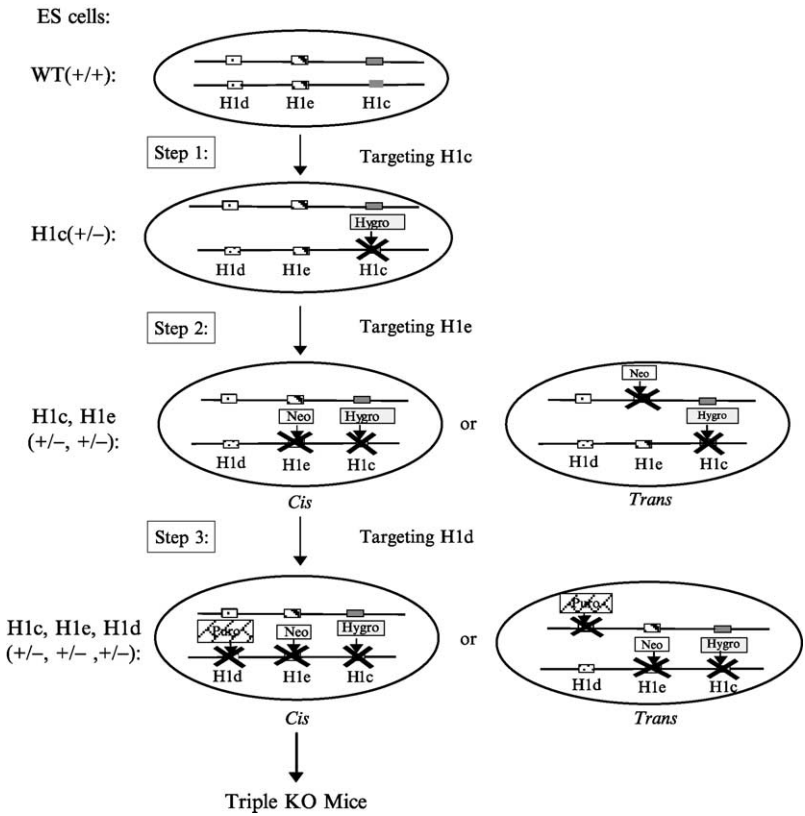



FIG. 5. Strategy for sequential inactivation of three H1 histone genes in mouse ES cells. The figure depicts the chromosome homologues containing the three, linked H1 genes to be targeted and the three steps used for targeting the genes, each with a different selectable marker gene. The figure also depicts the *cis* and *trans* configurations of gene targetings that can occur in Steps 2 and 3. Reproduced from Fan *et al.*[19] with permission from the American Society of Biology.

occurred in *cis* (see Fig. 5). There are two approaches for determining whether the two homologous recombination events occurred in the *cis* or the *trans* configuration. One approach is to use traditional genetic linkage analysis. For example, in the experiment shown in Fig. 5, several doubly targeted (H1c +/−, H1e +/−) ES cell clones were isolated, injected into blastocysts and chimeric mice were obtained from each cell line. The chimeric mice were mated with C57B16 mice and agouti progeny were genotyped by PCR analysis or Southern blot analysis of tail DNA to determine whether the modified H1c and H1e alleles cosegregated. Cosegregation of the H1c and H1e modified alleles indicates that the two targeting events occurred in *cis*, whereas independent segregation of the two modified alleles indicates that they occurred in *trans*.

An alternative approach to genetic linkage analysis is a new application of Expression FISH that we developed to distinguish between *cis* and *trans* gene targeting events in ES cells. The method is based on detection of transcripts from commonly used selectable marker genes inserted during homologous recombination. Nascent transcripts at the sites of transcription of these genes (e.g., PGKHygro and PGKNeo at Step 2 in Fig. 5) are detected in interphase nuclei making the preparation of mitotic cells unnecessary. The nascent transcripts are detected by *in situ* hybridization with specific, fluorescent oligonucleotides. In this way, one can determine the *cis* versus *trans* configuration of the two marker genes inserted during homologous recombination by examining whether or not the two signals colocalize. Compared to genetic linkage testing in chimeric mice, this method can greatly shorten the time required for determining the configuration of two gene targeting events in ES cells. Moreover, by providing such information about the modified ES cells prior to their injection into mouse blastocysts, the method allows for preselecting ES cell clones with a desired configuration. Readers who are interested in this approach are referred to the published procedure.[40]

Using the foregoing procedures, we developed mice null for the H1c and H1e subtypes. Surprisingly, these mice are also apparently normal. Once again, HPLC analysis showed that this strain had a normal H1 to nucleosome ratio in adult liver, indicating that other H1s (mainly $H1^0$ and H1d in liver) could compensate for loss of H1c and H1e. Accordingly, we carried out a third step of gene inactivation in *cis*-doubly targeted H1c, H1e (+, +/−, −) ES cells with a H1d targeting vector in which a PGK-Puro gene replaced the H1d coding region (see Fig. 5). Mice heterozygous for the three, linked mutant alleles were derived from several independent ES cell clones. These mice were intercrossed and their progeny were genotyped by

[40] Y. Fan, S. A. Braut, Q. Lin, R. H. Singer, and A. I. Skoultchi, *Genomics* **91,** 66 (2001).

PCR assays. From of a total of 638 F2 progeny analyzed, no triple, homozygous mutant animals were found.[19] The absence of triple homozygous mutants in litters is not due solely to H1d deficiency, because H1d null mice obtained from three H1d-*trans* targeted ES cell lines develop normally.[23] These results imply that H1 histones are required for proper mouse development. Thus, unlike lower eukaryotes, H1 is essential in mammals.

Genotype analysis of embryos (see Fig. 6A) at various stages of gestation revealed that no homozygous mutants were recovered after E11.5. The homozygous mutant embryos recovered from E8.5 to E11.5 were increasingly underrepresented in litters. They exhibited a wide spectrum of abnormalities, ranging from slight growth retardation to severe growth retardation with additional disruptions in development.[19]

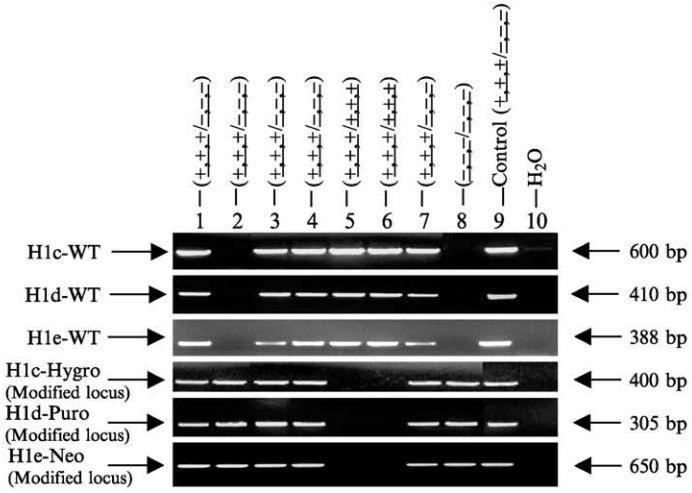
HPLC analysis of relatively normal appearing E10.5 triple mutant embryos showed that the H1-to-core histone ratio in these embryos is nearly 50% of that in wild-type embryos (see Fig. 6B, C). Thus inactivation of the three H1 genes indeed disrupts the compensation observed in single and double mutants.

It is interesting that reduction of total H1 levels in the embryos to about 50% of normal leads to lethality. Perhaps 50% is a critical amount for H1 protein, as it is for other proteins involved in haploinsufficiency syndromes. To explore this question further, we generated two different strains of compound H1 null mice. Mice null for H1c and H1e, but with one wild-type allele of H1d, (H1cH1dH1e [−, +, −/−, −, −], were produced by breeding H1cH1e double null animals with H1cH1dH1e triple heterozygous mutants. Mice null for $H1^0$ and H1c and H1e [$H1^0$ (−/−)H1cH1e(−, −/−, −]) were produced by intercrossing $H1^0$(+/−)H1cH1e(+, +/−, −) animals. Both types of mutants were very significantly underrepresented in litters, and in both cases, surviving mutants were much smaller shortly after birth and as adults. HPLC analyses of chromatin extracts from these mutants showed that their tissues have 20–50% reductions in the total H1 to nucleosome core ratio (see Table I). In both strains, we found that total H1 stoichiometry is most severely depressed in the thymus, in which the H1 to nucleosome ratio approaches 50% of wild-type levels (see Table I). Interestingly, both strains also have significantly smaller thymi than their wild-type littermates, again suggesting that having more than 50% normal total H1 levels is critical.

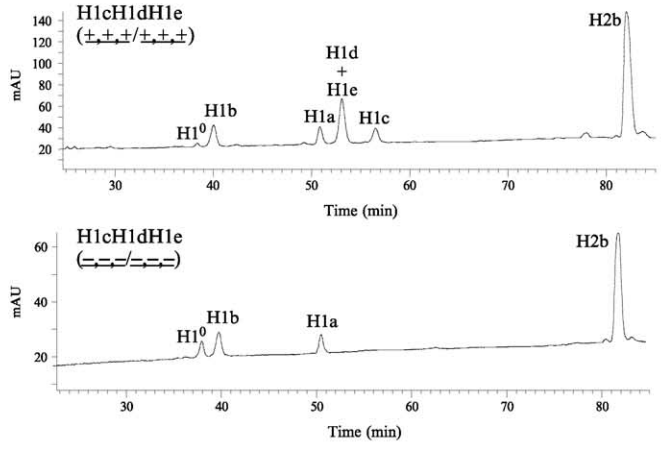
## Future Prospects

The procedures described above have allowed the generation of both single H1 null mice which have a normal H1 to nucleosome ratio as well as compound H1 mutant mice and embryos, some of which have reduced

A




B




C

| | % of total H1 | | | | | Total H1 |
|---|---|---|---|---|---|---|
| | $H1^0$ | H1a | H1b | H1c | H1d + H1e | per nucleosome |
| +,+,+/+,+,+ | 2.8 ± 0.1 | 14.4 ± 0.3 | 20.6 ± 0.3 | 15.9 ± 0.6 | 46.3 ± 0.2 | 0.74 ± 0.03 |
| −,−,−/−,−,− | 23.2 ± 4.4 | 28.4 ± 1.6 | 48.4 ± 2.8 | 0 | 0 | 0.40 ± 0.08 |

Individual H1 per nucleosome

| | $H1^0$ | H1a | H1b | H1c | H1d + H1e |
|---|---|---|---|---|---|
| +,+,+/+,+,+ | 0.02 ± 0.00 | 0.11 ± 0.00 | 0.15 ± 0.01 | 0.12 ± 0.01 | 0.34 ± 0.01 |
| −,−,−/−,−,− | 0.10 ± 0.04 | 0.11 ± 0.02 | 0.19 ± 0.04 | 0 | 0 |

total amounts of linker histones in chromatin, in some cases to levels approaching 50% of normal. It is possible to prepare various types of cultured cells from such animals, for example, ES cells, mouse embryonic fibroblasts, etc., that also can be used to study the role of H1 in processes that are more readily assayed in cell culture. The mice and cells lacking one of the H1 genes should be very useful to further understand how specific subtypes affect specific gene regulation. On the other hand, the compound mutants and cells derived from them can further our understanding of how H1 stoichiometry in general affects gene expression. The first step in this direction will be to use genome wide assays, such as microarray technology, to analyze the gene expression profile changes associated with the loss of H1 or specific H1 variants.

The compound mutants with reduced amounts of H1 also are useful for studying how H1 stoichiometry affects various aspects of global chromosome structure and functions. Much of our knowledge about the role of H1 in the structure of the chromatin fiber is derived from studies in which chromatin has been reconstituted *in vitro* with or without H1. Some of these studies have shown that other basic molecules (e.g., polyamines) can substitute for certain H1 functions.[41] It will be interesting to determine whether or not this is true *in vivo*. Analysis of nucleosome repeat lengths in some of the compound H1 null mice, carried out in collaboration with Dr. Christopher Woodcock (University of Massachusetts, Amherst), shows that reduced H1 content does lead to shortened nucleosome spacing, in agreement with *in vitro* findings.[19]

[41] T. A. Blank and P. B. Becker, *J. Mol. Biol.* **252,** 305 (1995).

FIG. 6. Analysis of chromatin from H1c, H1d, H1e triple null mouse embryos. (A) PCR genotype analysis of E7.5 embryos from intercrosses of H1cH1dH1e (+, +, +/−, −, −) mice. Embryo DNA was prepared and analyzed by PCR assays for H1c, H1d, and H1e wild-type and modified loci.[19] The deduced genotype of each embryo is indicated above each lane. The position of the PCR products from the wild-type and modified alleles are indicated. Control reactions contained tail DNA from a H1c, H1d, H1e triple heterozygous mutant mouse. (B) Reverse-phase HPLC analysis of histones in extracts from E10.5 wild-type and homozygous H1c, H1d, H1e mutant embryos. Approximately 20 ug of total histone extract of chromatin from wild-type (upper panel) and homozygous triple H1c,H1d,H1e mutant (lower panel) 10.5 d.p.c embryos were fractionated by reverse phase HPLC. Other details are as in the legend to Fig. 2B. (C) H1 subtype composition of chromatin from wild-type and H1cH1dH1e (−, −, −/−, −, −) 10.5 dpc embryos. Data were calculated from HPLC analyses of wild-type and H1c, H1d, H1e triple mutant embryos like that shown in (B). Reproduced from Fan *et al.*[19] with permission from the American Society of Biology.

TABLE I

H1 Subtype Composition and Total H1 Stoichiometry per Nucleosome in Wild-type and H1 Compound Knockout Mice[@]

| Tissue | Genotype | % of Total H1[*] | | | | | | | | | | | | Total H1 per Nucleosome[#] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $H1^0$ | | H1a | | H1b | | H1c | | H1d | | H1e | | | |
| Thymus | Wild-type (+/+) | 1.9 | 1.6 | 10.7 | 1.3 | 16.5 | 0.9 | 26.7 | 3.5 | 35.4 | 2.7 | 9.6 | 1.5 | **0.83** | **0.10, $n = 12$** |
| | $H1^0$(−/−)H1cH1e (−, −/−, −) | 0 | | 17.0 | 3.5 | 28.3 | 3.0 | 0 | | 54.7 | 5.5 | 0 | | **0.47** | **0.26, $n = 8$** |
| | H1cH1dH1e (−, +, −/ (−, −, −) | 5.9 | 4.6 | 25.0 | 3.0 | 35.4 | 3.1 | 0 | | 31.7 | 2.6 | 0 | | **0.41** | **0.16, $n = 6$** |
| Liver | Adult Wild-type (+/+) | 29.0 | 0.8 | 1.4 | 0.6 | 15.8 | 3.5 | 12.5 | 1.0 | 14.3 | 0.6 | 39.9 | 2.5 | **0.79** | **0.07, $n = 5$** |
| | Liver $H1^0$(−/ −)H1cH1e (−, −/ −, −) | 0 | | 2.2 | 0.3 | 6.3 | 1.3 | 0 | | 91.5 | 1.6 | 0 | | **0.64** | **0.01, $n = 4$** |
| | Neonatal Wild-type (+/+) | 9.5 | 4.9 | 6.1 | 2.4 | 14.2 | 1.7 | 20.9 | 4.5 | 30.3 | 2.3 | 19.0 | 1.5 | **0.76** | **0.07, $n = 5$** |
| | Liver H1cH1dH1e (−, +, −/ −, −, −) | 26.7 | 6.5 | 9.9 | 2.2 | 29.3 | 4.6 | 0 | | 32.7 | 5.2 | 0 | | **0.50** | **0.15, $n = 6$** |

Reproduced from Y. Fan, T. Nikitina, E. M. Morin-Kensicki, J. Zhao, T. R. Magnuson, C. L. Woodcock, and A. I. Skoultchi, *Mol. Cell. Biol.*[19] with permission from the American Society of Biology.

[@] Data are from HPLC analyses of wild-type and H1 compound knockout mice. Values are means standard deviations of individual determinations made on each of the indicated genotypes.

[*] Determined by ratio of *A*214 of the indicated H1 peak to total *A*214 of all H1 peaks. The *A*214 values of the individual H1 peaks were adjusted to account for the differences in the number of peptide bonds in each H1 subtype.

[#] Determined by the ratio of the total *A*214 of all H1 peaks to half of the *A*214 of the H2b peak. The *A*214 values of the H1 and H2b peaks were adjusted to account for the differences in the number of peptide bonds in each H1 subtype and H2b.

The techniques of histone gene manipulation described in the earlier sections also open the way for the generation of other types of interesting mutant mice. For example, homologous recombination at histone loci can be used not only to delete the gene, but also to replace it with a mutant version of the same gene or different histone variant, for example, another H1 subtype. Providing that important control elements are not present in the replaced coding sequences, this should allow for normal regulation of the substituted histone protein. In this way, for example, it may be possible to study the consequences of expressing highly variant H1 subtypes, such as $H1^0$, H1t, and H1oo, at times or places during development when they are not ordinarily expressed.

As discussed briefly above, methods for creating defined chromosomal deletions are also now available and they could be used to eliminate all of the H1 histone genes on mouse chromosome 13. Based on our studies of the triple H1 knockout embryos, it is expected that homozygous deletion of all of the H1 genes on chromosome 13 will be lethal for the embryo. However, it is not known whether ES cells with such a deletion are viable. Even if they are not, it might be possible to rescue them with a conditional expressed H1 gene. This approach might allow for production of cells and possibly mice expressing only a single H1 subtype, and then mutants of that subtype. Alternative approaches, such as RNA interference, are much more limited when dealing with a multigene family with members that diverge in sequence.