Mammalian linker-histone subtypes differentially affect gene expression *in vivo*

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Posttranslational modifications and remodeling of nucleosomes are critical factors in the regulation of transcription. Higher-order folding of chromatin also is likely to contribute to the control of gene expression, but the absence of a detailed description of the structure of the chromatin fiber has impaired progress in this area. Mammalian somatic cells contain a set of H1 linker-histone subtypes, H1 (0) and H1a to H1e, that bind to nucleosome core particles and to the linker DNA between nucleosomes. To determine whether the H1 histone subtypes play differential roles in the regulation of gene expression, we combined mice lacking specific H1 histone subtypes with mice carrying transgenes subject to position effects. Because position effects result from the unique chromatin structure created by the juxtaposition of regulatory elements in the transgene and at the site of integration, transgenes can serve as exquisitely sensitive indicators of chromatin structure. We report that some, but not all, linker histones can attenuate or accentuate position effects. The results suggest that the linker-histone subtypes play differential roles in the control of gene expression and that the sequential arrangement of the linker histones on the chromatin fiber might regulate higher-order chromatin structure and fine-tune expression levels.

Gene expression is regulated at the transcriptional level by differential expression of DNA-binding transcription factors and at the chromatin level because access of the DNA-binding transcription factors to their cognate binding sites is controlled by chromatin structure (1, 2). The nucleosomes, which consist of ≈200 bp of DNA wrapped around an octamer of core histones (H2A, H2B, H3, and H4), are the fundamental units of chromatin. Posttranslational modifications of the core histones, such as acetylation and methylation, and remodeling of the nucleosomes have been shown to play critical roles in gene activation and repression (3). H1 histones, also termed linker histones, bind DNA between the nucleosomes and represent another potential level of regulation. An important characteristic of these proteins is their heterogeneity (4). Organisms as diverse as plants and mammals have been shown to express multiple linker-histone variants or subtypes. In mice there are at least eight H1 histones that are differentially regulated during development and differentiation. H1a, H1b, H1c, H1d, and H1e, the somatic linker histones, are ubiquitously expressed but at different levels in different tissues (5, 6). H1 (0), the replacement linker histone, tends to be highly expressed in fully differentiated cells (7), and H1t and H1oo are expressed specifically in developing spermatocytes and oocytes, respectively (8, 9). This heterogeneity in expression pattern is matched by a strong divergence among the subtypes at the structural level. Although they all share the same basic organization of metazoan H1s consisting of a globular core flanked by two "unorganized" tails (2), both the globular domain and the tails exhibit significant differences among the various mammalian linker histones, with H1 (0) being the most divergent (6). These differences are thought to be important because they have been conserved among mammals and for certain subtypes even between invertebrates and vertebrates (6).

Early studies with chromatin reconstituted *in vitro* suggested that linker histones participate in chromatin condensation and have a generally repressive role in transcription (reviewed in ref. 2). However, more recent work carried out in *Tetrahymena* shows that the linker histone in the transcriptionally active macronucleus has much more specific effects, both positive and negative, on gene expression *in vivo* (10). To investigate the roles of the individual linker-histone subtypes in mammals, we have systematically deleted linker-histone genes in mouse embryonic stem cells and generated mice null for H1 (0), H1a, H1c, H1d, H1e, or H1t, as well as several double mutants. Surprisingly, mice lacking any one of these subtypes develop normally (11–14). Studies of chromatin in specific tissues of H1-null animals suggest that the lack of a phenotype in these mice is due to compensation by the remaining subtypes. These results left unanswered the question of whether the heterogeneity of the linker-histone subtypes is functionally important. To further address this problem, we have bred mice deficient in the production of specific linker-histone subtypes with transgenic mice in which the transgenes are subject to position effects.

Position effects, which can be broadly defined as variations in gene expression associated with changes in chromosomal location caused either by a chromosomal rearrangement or random integration of a transgene, have been observed in yeast, *Drosophila*, plants, and mammals (15–18). Classically, stable position effects are defined by an abnormal level of expression (usually lower) of a transgene in all of the cells of a given tissue. Variegating position effects (and the related silencing position effects in cell culture) are defined as variations in the level of expression of a gene in different cells of the same tissue resulting in mosaic expression patterns. In mice, position effects affecting transgenes have been reported in more than 15 tissues. For a subset of promoters, including those of the α- and β-globin genes in erythroid cells (19, 20) and the L-FABP gene in intestinal epithelial cells (21), the position effects are age-dependent and expression is silenced gradually in an increasing proportion of the cells as the transgenic mice age. It has been reported that in *Drosophil*a (22) and mice (23) integration next to heterochromatin is associated with position effect variegation. Strong regulatory elements such as the human β-globin locus control region (24) and the chicken HS4 insulator (25) have been reported to prevent position effects. However, we and others have shown that very large transgenes such as bacterial and yeast artificial chromosomes (YACs) containing the entire β-globin locus with all its known regulatory elements are subject to position effects even when located far from heterochromatin (26–28). Therefore, the causes of position effects remain poorly understood but are likely to be complex, because levels of

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Abbreviations: YAC, yeast artificial chromosome; wt, wild type.

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transgene expression are determined in part by the unique chromatin structure created by the juxtaposition of regulatory elements in the transgene and at each site of integration (16, 29). Despite this lack of mechanistic understanding, transgenes subject to position effect are exquisite indicators of chromatin structure and have been widely used to study epigenetic control mechanisms and to identify important chromatin proteins that can affect gene expression, first in *Drosophila* (30) and yeast and more recently in mice (31, 32).

In this study, we have used mice lacking specific H1 subtypes to investigate the role of linker histones in position effects on gene expression. We report that some but not all linker-histone subtypes can attenuate or accentuate position effects. The results suggest that the linker-histone subtypes play differential roles in the control of gene expression, and that the sequential arrangement of the linker histones on the chromatin fiber might regulate higher-order chromatin structure and fine-tune expression levels.

Materials and Methods

Breeding Strategy. Mice with the 4.4-kb β-globin transgene were C57/Bl6 syngenics (>8th generation backcross) and were a generous gift of Frank D. Costantini (Columbia University, New York). Mice with the 159-kb YAC were a generous gift of J. D. Engel (University of Michigan, Ann Arbor) and are in a mixed genetic background (FVB, C3H, 129/sv) but had been backcrossed between two and four times in the C57/Bl6 background. Histone knock-out mice were in a mixed (129/sv)/(C57/Bl6) background and had been backcrossed two to five times on average with C57/Bl6 mice at the time of the experiments. Mice of the appropriate genotypes were generated by crosses between mice heterozygous for a transgene and heterozygous for a linker-histone gene deletion with mice either heterozygous or homozygous for that linker-histone deletion. All comparisons were between littermates or between successive generations sharing at least one parent. Genotypes were determined by PCR as described (13, 33).

Isolation of Erythroid Cells. Hemolytic anemia was induced by phenylhydrazine injection as described (34). The spleens were harvested 5 days after the first injection. Spleens were disaggregated by squashing them between two microscope slides. Erythroid cells were further purified by using Ter-119 antibodies attached to magnetic beads by using the autoMACS system, as described by the manufacturer (Miltenyi Biotec, Auburn, CA).

Assays for β-Globin Expression. Staining of RBC with anti-human β-globin antibodies and reverse-phase HPLC to measure human β-globin levels were performed as described (26).

Histone Analysis. Purification of mouse bone marrow erythroid cells was performed on an autoMACS apparatus by using Ter119 microbeads (Miltenyi Biotec). HPLC to measure linker-histone content was performed as described (6, 13).

Fluorescence *in Situ* Hybridization Analysis. Mouse splenocyte culture, major satellite probe generation, and fluorescence *in situ* hybridization were performed as described (26). A bacterial artificial chromosome (kindly provided by T. J. Ley, Washington University School of Medicine, St. Louis) spanning the human β-globin locus was labeled with digoxigenin and detected with FITC, the mouse major satellite repeat with CY3. The cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Digital grayscale images for each fluorochrome were collected and merged to generate a final pseudocolored image.

Statistical Analysis. Statistical significance of the difference between the means level of expression of control and deletion mice was assessed by using Student's *t* test and one-way ANOVA.

Results

The first test transgene that we used is a 4.4-kb human β-globin gene that contains the entire coding sequence of the β-globin gene plus 2.1 kb of upstream flanking sequences and 0.8 kb of downstream coding sequences. Southern blot analysis revealed that ≈10 copies of the transgene are integrated in a head to tail array (data not shown). HPLC analysis on hemolysates showed that expression of the transgene was ≈14% that of the endogenous β-globin genes in young mice but that expression was gradually silenced reaching <4% in 6- to 7-month-old animals (Fig. 1*a*) and nearly undetectable levels in 2-year-old animals. To determine whether this silencing was due to a gradual decrease of expression of the transgene in every RBC or to a gradual decrease in the proportion of expressing RBCs, we characterized expression at the single-cell level by flow cytometry using a FITC-labeled monoclonal anti-human β-globin antibody (Fig. 1*b*). The results demonstrated that the silencing is due to a gradual decrease in the proportion of expressing cells. This transgene is therefore subject to an age-dependent variegating position effect. Similar position effects have been observed on transgenes composed of globin regulatory elements driving the LacZ reporter (20) but never on normal globin genes. To determine whether this silencing is associated with integration next to pericentromeric heterochromatin, we performed multicolor fluorescence *in situ* hybridization with probes specific for the human β-globin gene and for the mouse major satellite repetitive sequence that comprises pericentromeric heterochromatin. This study revealed that the transgene was inserted in the center of a large acrocentric chromosome far from the centromere or telomere (Fig. 1*c*). We conclude that silencing of this transgene is not associated with integration near pericentromeric heterochromatin.

To determine whether silencing is associated with age-dependent variation in methylation of the transgene in erythroid cells, we used methylation-sensitive restriction nucleases to determine the methylation status of two CpG dinucleotides that are present in the promoter of the β-globin gene at positions −266 and −406. A third CpG dinucleotide in the promoter of the β-globin gene cannot be tested by using this method. Splenic erythropoiesis in two 10-week-old and two 100-week-old mice was induced by phenylhydrazine injection, and spleens were isolated and mechanically disaggregated. Flow cytometry analysis with erythroid-specific Cy3-labeled Ter-119 antibodies revealed that ≈80% of the nucleated cells in the disaggregated spleens were from the erythroid lineage (Fig. 2*a*). Flow cytometry analysis with anti-human β-globin antibodies performed on RBCs obtained from the same animals just before they were killed revealed that the younger mice expressed the transgene in 70–80% of their RBCs, whereas transgene expression in the older animals occurred in <10% of the RBCs (Fig. 2*a*). Quantitative Southern blot analysis of genomic DNA from the disaggregated spleens after digestion with *Sna*BI and *Hga*I revealed that at these two sites the transgene is ≈70% methylated in both young and old animals (Fig. 2 *b* and *c*). Methylation analysis performed on DNA extracted from the brain of the same animals revealed that the transgene is 100% methylated in this nonerythroid tissue. To refine this analysis, we performed a similar analysis on highly purified erythroid cells. Splenic cells from phenylhydrazine-treated mice were obtained as before and the erythroid cells were separated from the lymphoid cells by immunomagnetic separation by using Ter-119 antibodies attached to magnetic beads. This procedure produced cell populations that were >90% erythroid (Fig. 2*a*). Southern blot analysis revealed that, as in whole spleens, the transgene is

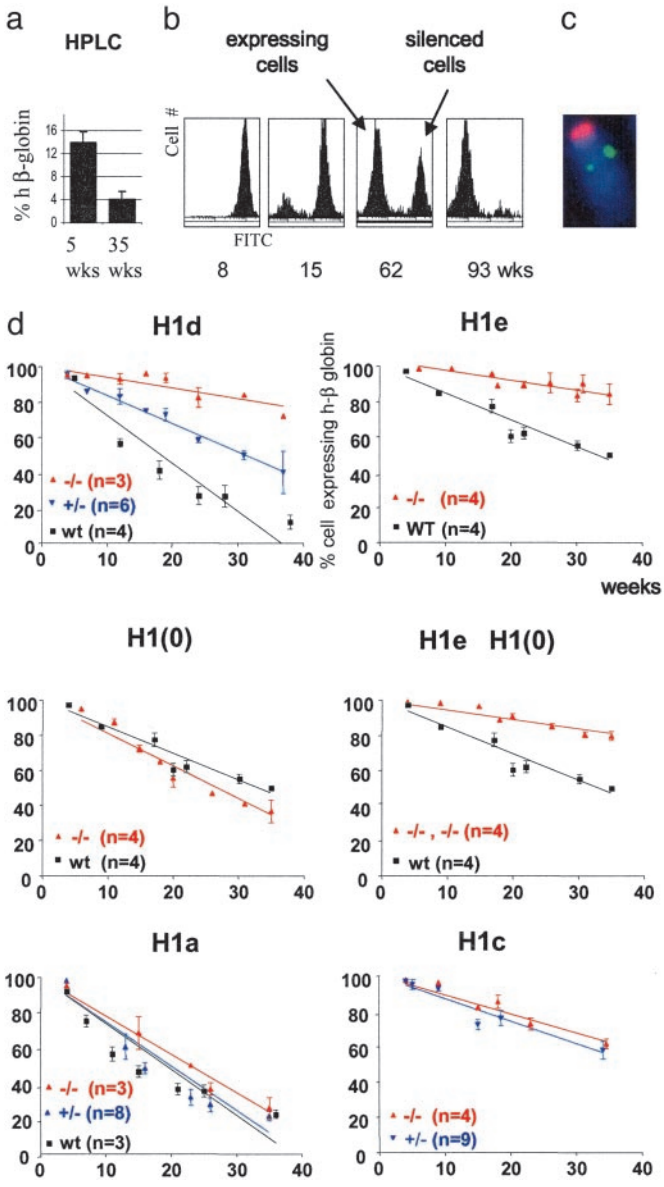

Fig. 1. Age-dependent silencing is modulated by specific linker-histone deletions. (*a*) A 4.4-kb human β-globin gene *Pst*I fragment (position 59859–64301 of GenBank HUMHBB) is subject to an age-dependent variegating position effect. Expression of the human β-globin gene was quantified by HPLC on RBC hemolysates and was found to decrease with time. % h β-globin = 100 × human β-globin/(mouse β-like chains + human β-globin). (*b*) Permeabilized RBCs were stained with FITC-labeled anti-human β-globin antibodies and periodically analyzed by flow cytometry. The proportion of RBCs expressing the human transgene decreases with age. (*c*) Metaphases from splenocytes were hybridized with a FITC-labeled human β-globin probe and a CY-3 labeled major satellite probe. In this mouse line, the human β-globin transgene is located far from pericentromeric heterochromatin. (*d*) Linker-histone subtypes differentially affect transgene expression. Silencing of the h-β-globin transgene in mice homozygous or heterozygous for deletions of linker histones was monitored over time by flow cytometry and compared with controls. Deletion of H1d, H1e, or H1e and H1 (0) dramatically attenuates the rate of silencing. Deletion of H1a, H1c, or H1 (0) has no effect on the rate of silencing. *n* = number of mice analyzed.

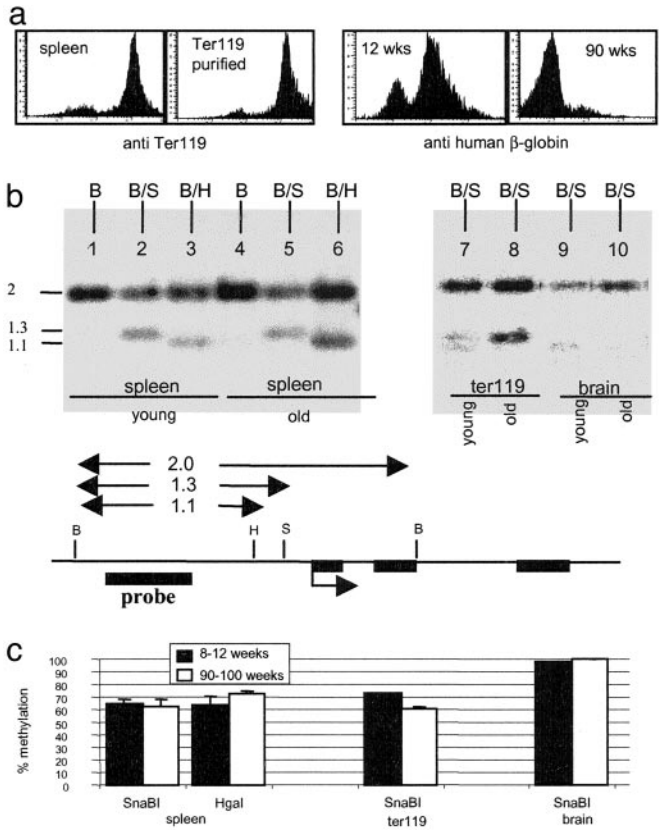

Fig. 2. Analysis of DNA methylation of the transgene during silencing. (*a Left*) Splenic cells from phenylhydrazine-treated mice were analyzed by flow cytometry with a Ter-119 antibody. About 80% of the nucleated cells are erythroid [nonnucleated RBCs were gated out by using the forward scatter (FSC) and side scatter (SSC) parameters]. A cell population >90% erythroid was obtained by further purifying the spleen cells on a magnetic column by using Ter-119 antibodies attached to magnetic beads. (*Right*) Permeabilized RBCs from 12- and 90-week-old animals were labeled with anti-human β-globin antibodies. About 75% of the younger animals and <10% of the older animals were expressing the transgene in their RBCs. (*b*) Southern blot analysis with methylation-sensitive restriction nucleases *Hga*I (H) and *Sna*BI (S). Lanes 1–6: Genomic DNA from spleen cells was digested with *Bam*H1 (B) *Bam*HI/*Sna*BI or *Bam*HI/*Hga*I and hybridized with a probe hybridizing in the 5′ flanking region of the β-globin gene (at position −1356 to −817 from the cap site of the β-globin gene). Lanes 1, 2, 3, 7, and 9: 12-week-old animals; lanes 4, 5, 6, 8, 10: 90-week-old animals. The human β-globin promoter in both the young and old animals is ≈70% methylated. Lanes 7 and 8: DNA from 90% pure erythroid cell population was digested with *Bam*HI/*Sna*BI and hybridized as above. Again ≈70% of the transgene DNA is methylated regardless of age. Lanes 9 and 10: Same as lane 7 and 8 but using brain DNA. In this tissue, the transgene is almost completely methylated. (*c*) Southern blots were quantified by phosphor-imaging, and the mean of the results from two or three independent determinations performed on at least two animals was plotted.

≈70% methylated in both young and old animals (Fig. 2 *b* and *c*). We conclude that the globin transgene is less methylated in erythroid cells than in nonerythroid cells but that the age-dependent silencing is not associated with a detectable increase in the methylation status of the β-globin promoter in erythroid cells of old mice.

To determine whether the silencing of the transgene is modulated by linker histones, we bred 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 RBCs expressing the transgene in circulating blood by flow cytometry after staining with FITC-labeled monoclonal anti-human β-globin antibodies (26). Three to four mice homozygous for each of the linker-histone gene deletions were compared with at least three control littermates. This analysis revealed that the absence of linker histones H1e or H1d strongly attenuated the silencing (Fig. 1*d*). At 35 weeks of

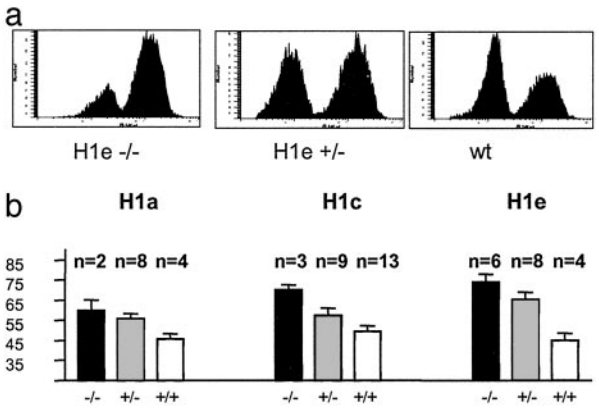

Fig. 3. Variegated expression of the human β-globin gene in a 150-kb YAC transgene is modulated by linker-histone deletions. (*a*) Representative FACS histograms obtained by analysis of permeabilized RBCs from littermates transgenic for YAC_{277w} and wt, and heterozygous or homozygous for deletion of the H1e linker histone were stained with a monoclonal FITC-labeled anti-human β-globin antibody. *x* axis, intensity of FITC labeling; *y* axis, cell numbers. The peak on the left represents nonexpressing cells; the peak on the right represents expressing cells. (*b*) Quantitation of the results for the H1a, H1c, and H1e series (see text). All three linker histones influence this position effect but H1e and has the strongest effect. $+/+$, $+/-$, and $-/-$ mice were derived from the same cross.

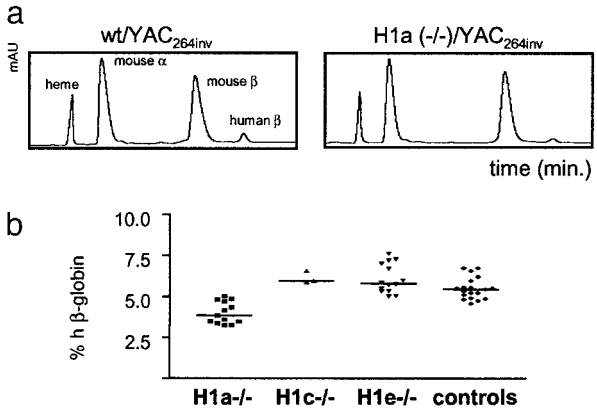

Fig. 4. Expression of the human β-globin gene in a 150-kb nonvariegating YAC transgene is modulated by H1a. (*a*) Typical reverse-phase HPLC chromatograms obtained by analysis of RBC hemolysates from mice transgenic for $YAC_{264\text{-inv}}$ and wt or null ($-/-$) at the H1a locus. (*b*) Quantitative analysis of the results: Percent human β-globin expression (% h β-globin) = $100 \times h\beta/(h\beta + m\beta)$ determined by HPLC in mice heterozygous for $YAC_{264\text{-inv}}$ and null for H1a, H1c, or H1e. Controls were littermates of the null mice that were either wt, H1a$+/-$, H1c$+/-$, or H1e$+/-$. Differences between H1a$-/-$ mice and all of the other types of mice are statistically significant (*t* test *P* value < 0.001 in all cases). Absence of H1a is therefore associated with a decrease in expression of the human β-globin gene in $YAC_{264\text{-inv}}$.

age, >85% of RBCs in H1e-deficient mice expressed the transgene, whereas control littermates expressed the same transgene in less than half of their cells. At the same age, >70% of cells in H1d-deficient mice expressed the transgene, whereas <40% of the cells in the control littermates expressed it. Mice heterozygous for the H1d gene deletion display a rate of silencing intermediate between those of the wt and homozygous deletion mice, suggesting that even partial decrease in the amount of a linker histone can affect the rate of silencing. The differences between the H1e$-/-$, H1d$+/-$, and H1d$-/-$ mice and their control littermates were highly significant ($P < 0.001$). Absence of H1a, H1c, or H1 (0) had no effect on the rate of silencing (Fig. 1*d*). Mice lacking both H1e and H1 (0) silenced at the same rate as mice lacking only H1e ($P < 0.001$), confirming the results obtained with the H1e$-/-$ and H1 (0)$-/-$ mice. Comparisons were made between five H1e$-/-$ and five nonlittermates; age-matched controls (obtained from mice with similar genetic background) also demonstrated that absence of H1e is associated with a reduced rate of silencing (data not shown).

To extend these results, we used YAC_{277w}, a 150-kb-long transgene that encompasses the entire human β-globin gene cluster with all its known regulatory elements and that is inserted as a single copy in chromosome 3 far from the centromere or the telomere (26). YAC_{277w} was originally cloned by Gaensler *et al.* (35) and slightly modified by Tanimoto *et al.* (36). In wt mice, this transgene is silenced in ≈60% of the RBCs. Unlike the first transgene tested, the silencing of YAC_{277w} is not age-dependent. To determine whether linker histones H1a, H1c, and H1e play a role in the silencing of YAC_{277w}, mice hemizygous for YAC_{277w} and heterozygous for a linker-histone deletion were bred with mice heterozygous for the same linker-histone deletion. Littermates hemizygous for YAC_{277w} and wt, heterozygous, or homozygous for the deletion of histones H1a, H1c, or H1e deletion were identified by PCR, and the percentage of RBCs expressing the human β-globin transgene was determined as described above. The results clearly demonstrate that all three linker histones tested influence the silencing of YAC_{277w} (Fig. 3). Absence of H1e was associated with an almost 2-fold increase in the percentage of expressing cells (from ≈44.8 ± 12.1% to 74.6 ± 9.6%). Mice heterozygous for the H1e deletion had a phenotype intermediate between the wt and the H1e-null mice. Results with H1c- and H1a-null mice were similar to the H1e results, but the effect of the H1a deletion was smaller and did not reach statistical significance. The differences between the H1e$-/-$ and the H1c$-/-$ and their control wt littermates were highly significant ($P < 0.001$).

The first two test transgenes demonstrated that the linker histones have subtle but easily detectable effects on transgene silencing but did not address the question of whether the level of expression of the transgene in expressing cells is influenced by specific linker histones. To test this possibility, a third transgene, $YAC_{264\text{-inv}}$, which is inserted as a single copy in the middle of chromosome 2 (26), was tested against the panel of linker histone knock-out mice. $YAC_{264\text{-inv}}$ is identical to YAC_{277w}, except that it contains an inversion of the locus control region (26, 36), the major regulatory element of the β-globin cluster (37). As a result of the locus control region inversion, the human β-globin gene is expressed at only 40% of the level of $YAC_{264\text{-wt}}$, a control wt YAC inserted at the same site of integration (26). $YAC_{264\text{-inv}}$ was chosen for these studies, because expression of the human β-globin gene is relatively low but easily detectable and because its expression is not variegated and does not vary significantly with age. Use of YAC with a higher level of expression would have complicated the analysis, because high-level human globin expression in wt mice without a balancing α-globin gene can be toxic.

Mice heterozygous for $YAC_{264\text{-inv}}$ and homozygous for deletions of the genes for histones H1a, H1c, or H1e were generated by breeding. Expression of the human transgene was determined by HPLC on RBC lysates obtained from 6- to 8-week-old mice (38). This analysis revealed that absence of H1a is associated with a decrease in expression of the human β-globin gene of >50%, whereas absence of H1e or H1c has no effect or causes a slight increase in transgene expression (Fig. 4). These results confirm that specific linker-histone subtypes have differential effects on gene expression and demonstrate that this effect can be either positive or negative depending on the local chromatin structure.

To control for the presence of genetic modifiers unlinked to the linker H1 locus that could complicate these studies because

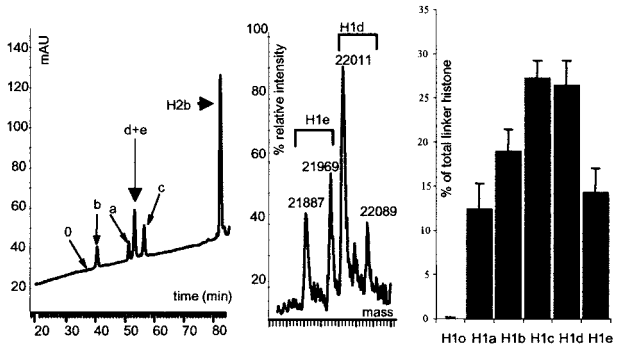

Fig. 5. Linker-histone composition in the murine erythroid lineage. (*Left*) Typical HPLC profile of total histone extract from bone marrow erythroid cells. The ratio of total H1 to core histones was determined by using the amount of H2b as a reference (13). (*Center*) Relative amounts of H1d and H1e (which cannot be resolved by HPLC) were determined by time-of-flight mass spectrometry on eluted HPLC peaks. Peaks of 21969 and 22089 are the phosphorylated forms of H1e and H1d, respectively. (*Right*) Summary of triplicate determinations of the distribution of the linker-histone subtypes in purified bone marrow erythroid cells.

the mice we used were not all 100% syngeneic with C57/Bl6 (see *Materials and Methods*), we generated and assayed multiple control littermates wt at the linker H1 locus. Small variations in the rate of silencing of the small globin transgenes (Fig. 1), the percentage of cells expressing YAC_{277w} (Fig. 3), or the level of expression of the human β-globin gene in $YAC_{264\text{-inv}}$ (Fig. 4) were observed between the various wt mice. The causes of these variations are probably a mixture of experimental errors, variations in environmental factors, and small differences in the genetic background of the mice. However, the fact that we observed an intermediate phenotype in the heterozygous littermates and the fact that the effects of H1e on age-dependent silencing and of H1a on expression of $YAC_{264\text{-inv}}$ could be seen when >10 mice were compared clearly demonstrate that the effects we have detected are due to the presence or absence of specific linker histones and not due to unknown unlinked modifiers.

We next determined the linker-histone subtype composition in the murine erythroid lineage as a function of age by isolating erythroid cells from bone marrow by using erythroid-specific Ter-119 antibodies attached to magnetic microbeads, and by performing HPLC and mass spectrometry on total histone nuclear extracts (6, 39). This analysis revealed that H1 (0) was virtually absent from the murine adult erythroid lineage and that levels of H1a, H1c, H1d, and H1e ranged from 12% to 27% (Fig. 5). No correlation was found between the expression levels of the two globin transgenes tested and the relative amounts of the linker H1 subtypes suggesting that the observed differential effects of the linker-histone deletions are not simply related to the abundance of each subtype in erythroid tissues. Interestingly, although no consistent age-dependent changes were observed in the relative amounts of the linker-histone subtypes (data not shown), the analysis reproducibly detected an age-related increase ($16.9 \pm 9.6\%$) in total linker-histone content relative to core histones in bone marrow erythroid progenitors of 90- to 100-week-old mice compared with 4- to 6-week-old mice. This observation suggests that the age-dependent transgene silencing might be caused in part by a progressive increase in the stoichiometry of linker histones and nucleosomes combined with a higher probability that specific linker-histone subtypes bind to the transgenes.

Discussion

Although each class of histones except H4 consists of primary sequence variants, the family of linker-histone proteins, consisting of at least eight members in the mouse, is by far the most diverse group. Previous studies of mouse mutants null for H1 (0), H1a, H1c, H1d, H1e, or H1t demonstrated that none of these linker-histone subtypes is essential for mouse development and that their absence did not result in a detectable phenotype (11, 13). In view of the diversity in sequence and regulation of the synthesis of these proteins, the absence of a phenotype in mice lacking H1 (0), H1a, H1c, H1d, H1e, or H1t is very surprising. One possible explanation for this apparent discrepancy is that the multiple H1 subtypes do not confer different structural properties on the chromatin fiber. Rather, the existence of multiple H1 genes may simply serve as a mechanism for differential regulation of H1 synthesis during development. An argument against this possibility is the high degree of amino acid sequence conservation of the mouse and human H1 subtypes. An alternative explanation might be that the subtypes are all able to fulfill the fundamental roles of linker histones in the structural properties of the chromatin fiber but that individual subtypes have additional nonredundant functions. This view is supported by the analysis of chromatin in the various strains of mice with deletion of an H1 gene, which showed that the H1-to-core histone ratio is unchanged in the null mice, indicating that the remaining subtypes can compensate for the missing H1 to maintain a normal H1-to-nucleosome stoichiometry. These results suggested that despite the very significant divergence in amino acid sequence of the subtypes, which can be as high as 62–64% in the case of H1 (0) vs. H1a through H1e, these subtypes can substitute for each other in the laboratory mouse. In this study, we tested the hypothesis that the different subtypes confer subtle differences in the properties of the chromatin fiber which allow for quantitative modulation of gene expression. Such subtle changes are important to detect because it is now well established that, for certain genes at least, even twofold differences in expression can have marked phenotypic consequences.

The findings reported here show that the H1 subtype composition of chromatin does indeed have important quantitative effects on gene expression. The effects were observed for three different transgenes integrated at three different sites in the mouse genome. Both positive and negative effects of specific H1 subtypes were observed. Absence of H1d or H1e, but not H1a, H1c, or H1 (0) was found to attenuate the rate of age-dependent silencing of a multicopy globin transgene. Examination of peripheral RBCs did not reveal any effects of the linker-histone deletions on expression of the endogenous globin genes (E.E.B., unpublished observations). However, detection of a small decrease in expression or of silencing of one allele in a fraction of the RBCs will require further studies.

Absence of H1a, H1c, or H1e was found to influence the proportion of cells expressing a variegating YAC transgene. H1a but not H1c or H1e was found to be required for maximal expression of a nonvariegating YAC transgene. In some cases, we were even able to detect an effect on gene expression in mice heterozygous for a linker-histone gene deletion. Together, these results strongly suggest that the other subtypes do not fully compensate for the loss of a specific subtype in the null mice. Studies in *Xenopus* have shown that a switch from the cleavage stage H1 subtype (B4) to adult H1s is important, both for repression of the oocyte-type 5S gene expression and for loss of mesodermal competence (40, 41). Overexpression of the mouse H1 (0) and H1c subtypes in cultured mammalian cells also showed that these two subtypes can have differential effects on gene expression and progression into the cell cycle from a quiescent state (42). Along with these earlier reports, our results support the view that the heterogeneity of linker histones seen in a wide array of organisms from plants to mammals indicates that the different subtypes play different roles in chromatin structure.

The question of whether the linker-histone effects that we have detected are direct or indirect is not answered by this study and will probably require the development of high affinity subtype-specific antibodies that can be used in chromatin immunoprecipitation studies. Nevertheless, the observation that linker-histone deletions modulated three different types of position effects likely to be caused by independent mechanisms suggests that the observed effects are not caused by an indirect effect of the histone on a single modifier gene. We therefore propose that the observed changes in the level of transgene expression in the linker-histone knock-out mice are caused by subtle changes in higher-order chromatin structure of the transgenes that are induced by replacement of the deleted histones by other subtypes. This effect of the linker-histone subtypes on expression could reflect the fact that each nucleosome preferentially contains a specific type of linker histone, raising the possibility that the subtypes are components of an elaborate "linker-histone code." Alternatively, linker-histone deposition could be regulated at a more regional level, with large chromatin domains preferentially associated with specific linker-histone subtypes. In both models, the heterogeneity in the H1 linker-histone family would serve to fine-tune the regulation of key genes during development or differentiation.

All three transgenes tested in this study are integrated far from pericentromeric heterochromatin. The position effects observed are therefore most likely linked to subtle variations in the rate of formation of facultative heterochromatin during erythroid differentiation or to differences between types of euchromatin. Regardless of the location of the transgenes, our results suggest that H1 subtypes can either increase or decrease gene activity. Based on our findings, we would predict that H1a is often associated with regions of active transcription and that H1d and H1e are associated with less active or inactive regions. This suggestion is consistent, at least for H1d and H1e, with studies in which the distribution of four human H1 subtypes on active and inactive DNA sequences in cultured human cells was studied by chromatin immunoprecipitation with subtype-specific antibodies (43, 44). H1d and H1e were found to be relatively depleted on actively transcribed genes, and H1d (and to a lesser extent H1e) was found to be relatively enriched on inactive genes. The H1a subtype was not studied. H1d also was observed to localize to the inactive Barr body in human cells (unpublished observations of Hong *et al.* cited in ref. 45).

Garrick *et al.* reported that variegated expression of a globin transgene correlates with chromatin accessibility but not methylation status (46). In accordance with this report, we did not find any evidence that position effect variegation of globin transgenes in mice correlates with DNA methylation. The specific linker-histone subtypes are therefore able to differentially influence gene expression by a mechanism other than that involving methylation of DNA. The properties of the mammalian H1 subtypes have been studied extensively, both *in vitro* and *in vivo* (reviewed in ref. 45). Nevertheless, a clear understanding of their functional differences awaits further studies. The systems described here should allow progress in understanding their roles in transcriptional regulation *in vivo*.

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