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Postsynthetic Trimethylation of Histone H4 at Lysine 20 in Mammalian Tissues Is Associated with Aging*

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Methylation of the N-terminal region of histones was first described more than 35 years ago, but its biological significance has remained unclear. Proposed functions range from transcriptional regulation to the higher order packing of chromatin in progress of mitotic condensation. Primarily because of the recent discovery of the SET domain-depending H3-specific histone methyltransferases SUV39H1 and Suv39h1, which selectively methylate lysine 9 of the H3 N terminus, this posttranslational modification has regained scientific interest. In the past, investigations concerning the biological significance of histone methylation were largely limited because of a lack of simple and sensitive analytical procedures for detecting this modification. The present work investigated the methylation pattern of histone H4 both in different mammalian organs of various ages and in cell lines by applying mass spectrometric analysis and a newly developed hydrophilic-interaction liquid chromatographic method enabling the simultaneous separation of methylated and acetylated forms, which obviates the need to work with radioactive materials. In rat kidney and liver the dimethylated lysine 20 was found to be the main methylation product, whereas the monomethyl derivative was present in much smaller amounts. In addition, for the first time a trimethylated form of lysine 20 of H4 was found in mammalian tissue. A significant increase in this trimethylated histone H4 was detected in organs of animals older than 30 days, whereas the amounts of mono- and dimethylated forms did not essentially change in organs from young (10 days old) or old animals (30 and 450 days old). Trimethylated H4 was also detected in transformed cells; although it was present in only trace amounts in logarithmically growing cells, we found an increase in trimethylated lysine 20 in cells in the stationary phase.

In vivo methylation of the side chains of specific lysines, histidines, and arginines in proteins is a very common phenomenon in nature involving numerous classes of proteins in both prokaryotic and eukaryotic cells (1, 2). During the last several years, studies on the methylation of proteins have yielded many important observations. While these studies were under way, it was generally realized that protein methylation is far more complex and has more ramifications than originally assumed.

Methylation is also a well known posttranslational modification reaction of histone proteins on lysine and/or arginine residues with a site selectivity for lysine methylation at specific positions in the N termini of histones H3 and H4. In combination with other posttranslational modifications, *i.e.* acetylation and phosphorylation, methylation seems to play a significant role in regulating nuclear functions. Thus, it has been suggested that distinct combinations of covalent histone modifications, also referred to as "histone code," provide a specific mark on the hydrophilic histone tails, which, when read by other proteins, cause specific downstream events finally inducing transitions in chromatin structure (3, 4). These chromatin changes are essential prerequisites for important cellular processes such as transcription, replication, recombination, etc. In contrast to acetylation and phosphorylation, which represent short term signals of the histone code, histone methylation is regarded as a more long term epigenetic mark with a relatively low turnover of the methyl group (5).

Histone H3 is typically methylated *in vivo* at lysines 4, 9, and 27 (6) and most probably also at arginine 17 (7). Concerning the biological significance of H3 methylation, recent papers have shown that methylation of lysine 4 and arginine 17 is correlated with active gene expression, whereas lysine 9 methylation is linked to gene silencing (7–11).

Each of the lysine residues can accept up to three methyl groups forming mono-, di-, and trimethylated derivatives, thus adding a further potential complexity to the posttranslational status of histone H3 (12, 13). The detailed biological role of mono-, di-, and trimethylation, however, is completely unknown to date.

Histone H4, also a major acceptor of methyl groups, was found to be methylated at positions 3 (arginine) (14) and 20 (lysine) (2, 15). In contrast to histone H3 lysine methylation, H4 lysine 20 is described as being maximally dimethylated in mammals (16). To date, very little is known about the biological outcome of methylated H4. An increase in H4 methylation has been linked to the termination of liver growth in rats, and when methylated H4 was found to be present in low amounts in active chromatin it was proposed that this covalent modification may not be associated with transcription. A recent paper (17) reported arginine 3 of H4 as the target for methyltransferase PRMT1 *in vivo* and *in vitro* and assigned this specific site an important function in transcriptional regulation.

To elucidate the biological importance of histone H4 methylation, the methylation pattern of this core histone was investigated in cells of various origins. Unlike histone modification by acetylation or phosphorylation, histone methylation does not greatly influence the charge of individual amino acids, thus making the electrophoretic separation of distinct methylated proteins from each other and from the unmethylated parent

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proteins a problematic part in histone analysis. Until now, methylated amino acids have often been determined in protein and tissue hydrolysates using amino acid analyzers and through cells radiolabeled with [*methyl*-^{3}H]methionine. To avoid these labor-intensive and time-consuming methods as well as the use of radioactivity, a high resolving chromatographic method was developed for separation and precise quantification of mono-, di-, and trimethylated H4 histones, including their distinct acetylated forms. Applying this procedure, we found for the first time *in vivo* evidence that lysine 20 of histone H4 is not only mono- and dimethylated but also trimethylated. A significant increase in this trimethylated form was observed in rat kidney and liver during aging. Trimethylated H4 was also detected in small amounts in logarithmically growing human tumor cells, *i.e.* Raji and K562. The proportion of trimethylated histone H4 increased when the cells were accumulated in the stationary phase. The possible biological significance of age-related accumulation of trimethylated forms of histone H4 is discussed in the light of our results.

EXPERIMENTAL PROCEDURES

Materials—Sodium perchlorate, triethylamine (TEA),[1] acetonitrile (ACN), and trifluoroacetic acid were purchased from Fluka (Buchs, Switzerland). All other chemicals were purchased from Merck.

Animals and Tissues—Rat kidney and liver were obtained from several Sprague-Dawley rats aged 10, 30, 300, and 450 days.

Cell Cultures—Raji and K562 cells were cultured in RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum, penicillin (60 μg/ml), and streptomycin (100 μg/ml) in the presence of 5% CO_2. The cells were seeded at a density of 8×10^4 cells/ml and harvested after 3 days in the log phase or after 7 days to accumulate the cells in stationary phase.

Preparation of Core Histones—The core histones were extracted from organs and cells with sulfuric acid (0.2 M) according to the procedure of Lindner *et al.* (18) with slight modifications. The nuclear pellet was extracted in a first step with 5% $HClO_4$ (v/v) to remove the H1 histones. In a second step the pellet was extracted with 1 volume of 0.4 M H_2SO_4 and 4 volumes of 0.2 M H_2SO_4 for 1 h with occasional vortexing. H_2SO_4-insoluble material was removed by centrifugation at 10,000 rpm for 10 min, and soluble proteins were precipitated by adding trichloroacetic acid to a 20% (w/v) final concentration. The precipitated core histones were left on ice for 60 min and then centrifuged at 10,000 rpm for 10 min, washed with cold acidified acetone and with pure acetone, dissolved in 1 ml of water containing 0.1% 2-mercaptoethanol, lyophilized, and stored at −20 °C until used for HPLC.

High Performance Liquid Chromatography—The equipment used consisted of a 127 Solvent Module and a model 166 UV-visible region detector (Beckman Instruments, Palo Alto, CA). The effluent was monitored at 210 nm, and the peaks were recorded using Beckman System Gold software. The solvent compositions are expressed as v/v throughout this text.

Reversed-phase HPLC—The separation of core histones was performed on an Ultrapore RPSC C_3 column (250 × 10-mm inner diameter; 5-μm particle pore size; 30-nm pore size; end-capped; Beckman Instruments). The lyophilized proteins were dissolved in water containing 0.2 M 2-mercaptoethanol, and samples of ~600 μg were injected onto the column. The histone sample was chromatographed within 60 min at a constant flow of 1.5 ml/min with a two-step acetonitrile gradient starting at solvent A-solvent B (60:40) (solvent A: water containing 0.1% trifluoroacetic acid; solvent B: 70% acetonitrile and 0.1% trifluoroacetic acid). The concentration of solvent B was increased from 40 to 55% B during 40 min and from 55% to 100% B during 20 min. The histone H4 fraction was collected and, after adding 50 μl of 2-mercaptoethanol (0.2 M), lyophilized and stored at −20 °C.

The peptide samples obtained after digestion of H4 histones by endoproteinase Glu-C were separated using a Nucleosil 300–5 C_{18} column (150 × 2-mm inner diameter; 5-μm particle pore size; end-capped; Macherey-Nagel, Düren, Germany). Samples of ~100 μg were injected onto the column. Chromatography was performed within 65 min at a constant flow of 0.15 ml/min with an acetonitrile gradient starting at solvent A-solvent B (75:25) (solvent A: water containing 0.1% trifluoroacetic acid; solvent B: 85% acetonitrile and 0.093% trifluoroacetic acid). The concentration of solvent B was increased linearly from 25 to 50% during 65 min. The fractions obtained in this way were collected and, after adding 20 μl of 2-mercaptoethanol (0.2 M), lyophilized and stored at −20 °C.

Histone H4 peptide fractions obtained by endoproteinase Lys-C cleavage were injected onto a PepMap C_{18} column (150 × 1-mm inner diameter; 3-μm particle size; ICT, Vienna, Austria). Samples of ~3 μg were chromatographed within 55 min at a constant flow of 35 μl/min with a two-step acetonitrile gradient starting at solvent A-solvent B (90:10) (solvent A: water containing 0.1% trifluoroacetic acid; solvent B: 85% acetonitrile and 0.093% trifluoroacetic acid). The concentration of solvent B was increased linearly from 10 to 40% during 45 min and from 40 to 100% during 20 min. The fractions were collected and, after adding 10 μl of 2-mercaptoethanol (0.2 M), lyophilized and stored at −20 °C.

Hydrophilic Interaction Liquid Chromatography—The histone fraction H4 (150 μg) isolated by RP-HPLC was further separated on a SynChropak CM300 column (250 × 4.6-mm inner diameter; 6.5-μm particle size; 30 nm pore size; Agilent Technologies, Vienna, Austria) at 30 °C and at a constant flow of 1.0 ml/min using a multi-step gradient starting at solvent A-solvent B (100:0) (solvent A: 70% acetonitrile, 0.015 M TEA/H_3PO_4, pH 3.0; solvent B: 65% acetonitrile, 0.015 M TEA/H_3PO_4, pH 3.0 and 0.68 M $NaClO_4$). The concentration of solvent B was increased from 0 to 10% B during 2 min, from 10 to 40% during 30 min, and then maintained at 40% during 10 min. The isolated protein fractions were desalted using RP-HPLC. The histone fractions obtained in this way were collected and, after adding 20 μl of 2-mercaptoethanol (0.2 M), lyophilized and stored at −20 °C. The peptide fraction I (~120 μg) obtained by RP-HPLC of endoproteinase Glu-C- digested H4 histone was further separated by HILIC using the separation conditions described above.

Endoproteinase Glu-C Digestion—Whole histone H4 (~100 μg) obtained by RP-HPLC fractionation was digested with *Staphylococcus aureus* V8 Protease (Roche Molecular Biochemicals; 1:20 w/w) in 50 μl of 25 mM NH_4HCO_3 buffer (pH 4.0) for 1 h at room temperature. The digest was subjected to RP-HPLC.

Endoproteinase Lys-C Digestion—N-terminal peptides obtained by Glu-C digestion were further cleaved with endoproteinase Lys-C (Roche Molecular Biochemicals; 1:5 w/w) in 15 μl of 25 mM Tris-HCl buffer (pH 8.7) for 2 h at 37 °C. The digest was subjected to RP-HPLC.

Amino Acid Sequence Analysis—Peptide sequencing was performed on an Applied Biosystems Inc. model 492 Procise protein sequenator.

Mass Spectrometric Analysis—Determination of the molecular masses of individual histone H4 peptides obtained after protease digest was carried out with an electrospray ion-mass spectrometry (ESI-MS) technique using a Finnigan MAT LCQ ion trap instrument (San Jose, CA). The samples (5–10 μg) were dissolved in 50% aqueous methanol containing 0.1% formic acid and injected into the ion source.

RESULTS

HILIC Separation of Acetylated and Methylated Forms of Histone H4—Sulfuric acid-extracted core histones were fractionated using RP-HPLC with a semi-preparative Ultrapore 300–5 C_3 column and a two-step water/acetonitrile gradient. An example, the separation of core histones from rat kidney, is given in Fig. 1*A*. The identity and purity of the fractions obtained were checked by means of SDS- and acid-urea-PAGE (data not shown). The histone H4 fraction eluted at about 41 min as a single peak despite the presence of a number of distinctly modified forms. The fact that various posttranslationally modified forms of one and the same protein coelute in RP-HPLC with its unmodified parent protein has previously been demonstrated for several histone proteins (18–20).

Excellent separations of such modified histone proteins, recently achieved in our laboratory (20–22) and by other groups (23) using hydrophilic interaction chromatography, prompted us to also evaluate the potential utility of this technique for the separation of methylated proteins. Therefore, the histone H4 fraction obtained in the RP-HPLC run (Fig. 1*A*) was subjected to HILIC. Under optimized conditions (pH 3.0 and 30 °C) using a SynChropak CM 300 column with a triethylammonium phos-

[1] The abbreviations used are: TEA, triethylamine; RP, reversed-phase; HPLC, high performance liquid chromatography; HILIC, hydrophilic interaction liquid chromatography; ESI-MS, electrospray ion mass spectrometry.

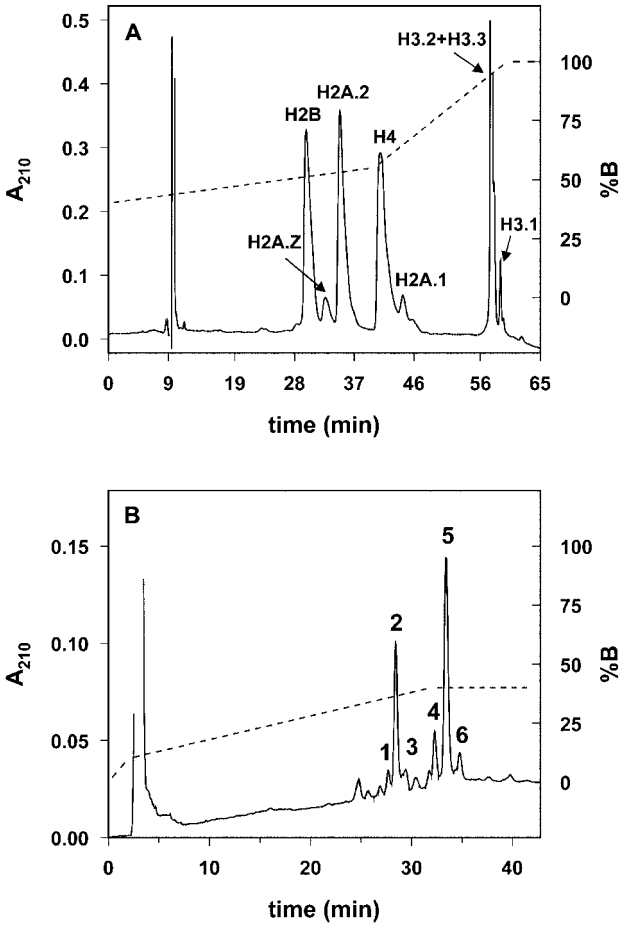


FIG. 1. **HILIC separation of rat kidney histone H4.** *A*, sulfuric acid-extracted core histones (~600 μg) from rat kidney were injected onto an Ultrapore RPSC C_3 column (250 × 10 mm). The histone sample was chromatographed using RP-HPLC and a two-step acetonitrile gradient starting at 60% A, 40% B (solvent A, water containing 0.1% trifluoroacetic acid; solvent B, 70% acetonitrile and 0.1% trifluoroacetic acid). The concentration of solvent B was increased from 40 to 55% B during 40 min and from 55 to 100% B during 20 min. Flow rate was 1.5 ml/min. The protein was monitored at 210 nm. The histone H4 fraction was collected and, after adding 50 μl of 2-mercaptoethanol (0.2 M), lyophilized and stored at −20 °C. *B*, the histone H4 fraction (150 μg) isolated with RP-HPLC (*A*) was analyzed on a SynChropak CM300 column (250 × 4.6 mm) at 30 °C at a constant flow of 1.0 ml/min using a two-step gradient starting at 100% A, 0% B (solvent A: 70% acetonitrile, 0.015 M TEA/H_3PO_4, pH 3.0; solvent B: 65% acetonitrile, 0.015 M TEA/H_3PO_4, pH 3.0, and 0.68 M $NaClO_4$). The concentration of solvent B was increased from 0 to 10% B during 2 min, from 10 to 40% during 30 min, and then maintained at 40% for 10 min. The isolated protein fractions (designated 1–6) were desalted using RP-HPLC. Histone fractions obtained in this way were collected and, after adding 20 μl of 2-mercaptoethanol (0.2 M), lyophilized and stored at −20 °C.

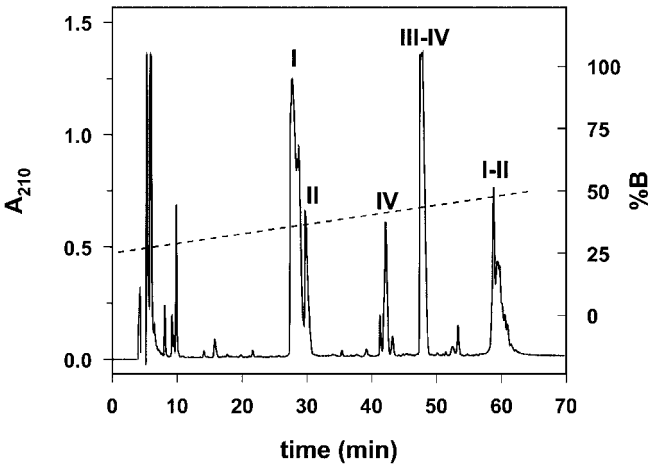
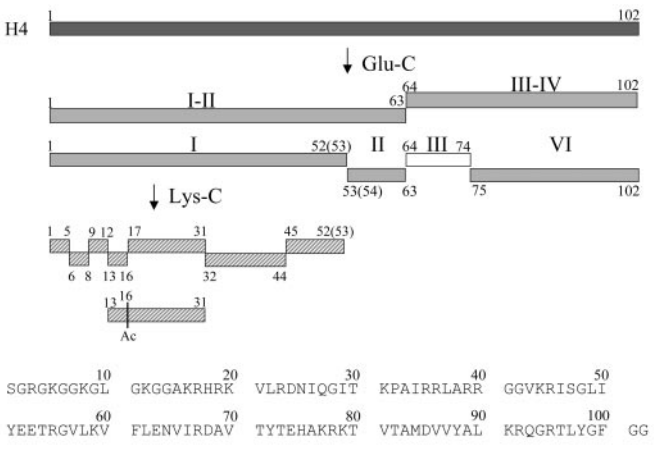


FIG. 2. **RP-HPLC of peptide fractions of endoproteinase Glu-C-digested rat kidney H4.** Whole histone H4 from rat kidney isolated with RP-HPLC (Fig. 1*A*) was digested with endoproteinase Glu-C as described under "Experimental Procedures." The digest (containing about 100 μg of peptides) was injected onto a Nucleosil 300–5 C_{18} column (150 × 2 mm). Analysis was performed at a constant flow of 0.15 ml/min using an acetonitrile gradient starting at 75% A, 25% B (solvent A: water containing 0.1% trifluoroacetic acid; solvent B: 85% acetonitrile and 0.093% trifluoroacetic acid). The concentration of solvent B was increased linearly from 25 to 50% during 65 min. The effluent was monitored at 210 nm. Peptide fractions I, II, I-II, III-IV, and IV were analyzed using high performance capillary electrophoresis, amino acid sequencing of the first three amino acids, and ESI-MS (data not shown). Fraction I was used for HILIC analysis (Fig. 3).

TABLE I
Peptide patterns obtained after endoproteinase Glu-C and Lys-C digestion of rat kidney histone H4

The peptide fractions of endoproteinase Glu-C-digested rat H4 were separated using RP-HPLC (Fig. 2) and analyzed with both amino acid sequencing of the first three amino acids and with ESI-MS. Peptide I, blocked N terminus (residues 1–52, 1–53); peptide II, *ETR* (53–63); peptide III-IV, *NVI* (64–102); peptide IV, *HAK* (75–102). Histone H4 sequence data were taken from rat H4 cDNA (43). Fraction I was further digested by Lys-C. The resulting peptides were identified using ESI-MS.



phate buffer system, a linear sodium perchlorate (0–0.68 M), and an inverse acetonitrile gradient (70–65%), the H4 fraction was separated into two major and some minor peaks (Fig. 1*B*).

Characterization of the H4 Subfractions Obtained by HILIC—In a first attempt the H4 histone fraction obtained after the initial RP chromatography (Fig. 1*A*) was used to localize the domain responsible for the occurrence of multiple H4 forms in HILIC. For this purpose the H4 fraction was treated with endoproteinase Glu-C, an enzyme specifically cleaving proteins under appropriate conditions C-terminally of glutamic acid. As expected, the fragmentation yielded several peptides that were separated and isolated by RP-HPLC (Fig. 2). To identify these peptide fractions, amino acid sequencing and ESI-MS analyses were performed. As illustrated in Table I, fraction I-II (eluting at about 60 min) consisted of an H4 peptide ranging from residues 1 to 63. Fraction III-IV (eluting at 48 min) and fraction IV (42 min) consisted of C-terminal fragments (residues 64–102 and 75–102, respectively). Fraction II (30 min) was a pure fraction (residues 54–63), whereas fraction I consisted of two N-terminal fragments (residues 1–52 and 1–53, respectively). Although the peptide containing residues 64–74 (designated as peptide III in Table I) should also be present, it could not be detected. The subsequent HILIC analysis of the peptide fraction II and fractions III and IV revealed

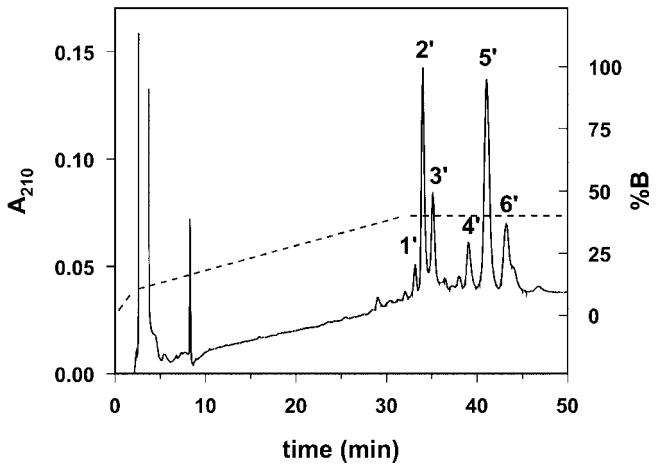


FIG. 3. **HILIC separation of peptide fraction I obtained with RP-HPLC of endoproteinase Glu-C-digested rat H4 histone.** The sample (~120 μg) was analyzed on a SynChropak CM300 column (250 × 4.6 mm) under the same conditions as described for Fig. 1*B*. The HILIC fractions (designated 1′–6′) were desalted using RP-HPLC. The peptide fractions obtained in this way were collected and applied on an electrospray mass-spectrometer (Table III).

that both fragments were homogeneous and, consequentially, excluded as possible sites for H4 modification (data not shown). Fraction I, however, analyzed by HILIC was heterogeneous and consisted of several subfractions (Fig. 3; designated 1′–6′); this chromatogram closely resembled that obtained by HILIC analysis of undigested H4 shown in Fig. 1*B*. To verify that fractions 1′–6′ in Fig. 3 indeed correspond to the same intact forms of the molecule, ESI-MS analyses of peaks 1–6 (Table II) and fractions 1′-6-′ (Table III) were performed. As can be seen from Tables II and III, similar mass differences were obtained. This result clearly indicated that under these chromatographic conditions the same modifications cause separation of multiple forms and that, furthermore, the structural alterations must take place in the N-terminal region of the H4 protein. Identification of peptide fractions 1′–6′ is shown in Table III. Whereas the mass of the blocked N-terminal peptide 1–52 of histone H4 was calculated to be 5636.7 Da, we found for fractions 6′, 5′, and 4′ significantly higher masses of roughly 14, 28, and 42 Da, respectively. Because a mass difference of 14 Da corresponds to a methyl group, we conclude that H4 peptide fragments 6′, 5′, and 4′ represent mono-, di-, and trimethylated forms, which were clearly separated under HILIC conditions. Fractions 3′, 2′, and 1′ differed from 6′, 5′, and 4′ by about 43 Da each, which very well matches with the mass of an acetyl group. Fractions 1′, 2′, and 3′, therefore, are the corresponding monoacetylated forms of fractions 4′, 5′, and 6′. Furthermore, it should be noted that ESI-MS analysis obviously revealed a contamination of fractions 3′ and 6′ with another peptide fragment (Table III). Whereas fraction 6′ was contaminated by the prolonged unacetylated but dimethylated N-terminal peptide 1–53, fraction 3′ was a mixture of the monoacetylated fragments 1–52 (monomethylated) and 1–53 (dimethylated). This finding also easily explains the slight differences in the relative amounts of HILIC fractions 1′–6′ (Fig. 3) as compared with that of the corresponding undigested H4 fractions (Fig. 1*B*).

Evidence for the Presence of Trimethylated Lysine 20 in Histone H4—To precisely determine the methylation sites in histone H4, the HILIC fractions (designated 4′–6′) shown in Fig. 3 were isolated and desalted using RP-HPLC. To localize the exact position of the methyl groups, the N-terminal peptides were further cleaved with endoproteinase Lys-C. The

TABLE II
ESI-MS data of peaks 1–6 (Fig. 1B)

The individual HILIC fractions 1–6 (Fig. 1B) were analyzed using electrospray ionization mass spectrometry. The mass for the unmethylated intact molecule was calculated to be 11278.2 Da. The mass differences found correlate with the molecular mass of an additional acetyl and three methyl (1), two methyl (2), and one methyl groups (3); the values found for fractions 4–6 correspond to the nonacetylated forms of fractions 1–3.

Peak	ESI-MS	Difference from calculated mass of 11278.2 Da
	Da	
1	11365.3	87.1
2	11349.0	70.8
3	11335.3	57.1
4	11324.5	46.3
5	11307.5	29.3
6	11292.7	14.5

TABLE III
ESI-MS data of peaks 1′–6′ (Fig. 3)

The peptide fraction I of endoproteinase Glu-C-digested rat H4 was separated using HILIC (Fig. 3) and analyzed by means of electrospray ionization mass spectrometry. The mass for the unmethylated blocked N-terminal peptide 1–52 of rat histone H4 was calculated to be 5636.7 Da. Mass differences found correlate with the molecular mass of an additional acetyl and three methyl (1′), two methyl (2′), and one methyl groups (3′); the values found for fractions 4′–6′ correspond to the nonacetylated forms of fractions 1′–3′.

Peak	ESI-MS	Difference to calculated mass of 5636.7 Da	Identification
	Da		
1′	5721.5	84.8	1–52, ac1, me3
2′	5707.5	70.8	1–52, ac1, me2
3′	5693.3[a]	56.6	1–52, ac1, me1
	5836.8	200.1	1–53, ac1, me2
4′	5679.5	42.8	1–52, ac0, me3
5′	5665.3	28.6	1–52, ac0, me2
6′	5650.3[b]	13.6	1–52, ac0, me1
	5794.1	157.4	1–53, ac0, me2

[a] 25% of the total amount of peak 3′.
[b] 25% of the total amount of peak 6′.

digests were analyzed by RP-HPLC-ESI-MS using a 150 × 1.0-mm inner diameter microbore column. In each case, seven peptide fragments were detected (Table I). However, only the three fragments 17–31 revealed the mass differences expected. The peptide derived from fraction 4′ exhibited a molecular mass of 1876.0 Da, that from fraction 5′ exhibited a molecular mass of 1861.7 Da, and that from fraction 6′ exhibited a molecular mass of 1847.9 Da, indicating that the analyzed fragments were tri-, di-, and monomethylated. From these data, therefore, a methylation of arginine 3, which is known from the literature (14, 17), can be excluded. To prove our assumption that lysine 20 is trimethylated and also to eliminate other arginine and lysine residues present at positions 17, 19, 23, and 31 as possible candidates for methylation, Edman degradation of the three peptides was carried out (data not shown). Sequence analysis confirmed that HILIC fraction 6′ contained monomethyllysine, fraction 5′ contained dimethyllysine, and fraction 4′ contained trimethyllysine at position 20 of histone H4. Peptide fragments 3′, 2′, and 1′ are the analogously methylated peptides, however, bearing an acetyl group. It must be mentioned that in the case of digestion of the monoacetylated fragments 1–52 (53) the acetyl group bound to lysine 16 inhibits its cleavage at that position. The cleavage occurs N-terminally of lysine 12, yielding peptides 13–31 having an acetyl group at lysine 16. It should be noted that the appearance of a trimethylated lysine at position 20 was unexpected, because in contrast to histone H3, no evidence for a trimethylation of mammalian H4 has been found to date (24–26).

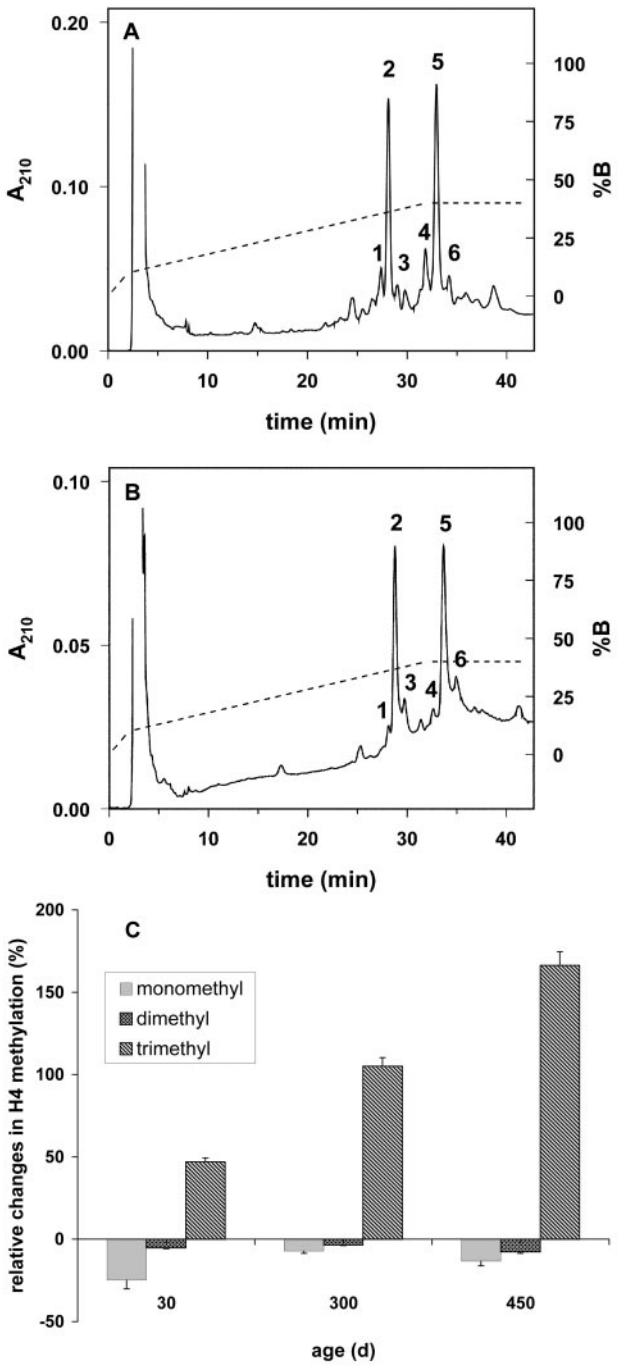


FIG. 4. **HILIC separation of rat liver histone H4 of young (10 days old) and old (450 days old) animals and age dependence of the different methylated histone H4 forms.** *A* and *B*, histone H4 fractions (~150 μg) obtained from liver of rats aged 450 days (*A*) and from liver of rats aged 10 days (*B*) were isolated using RP-HPLC and analyzed under the same HILIC conditions used in Fig. 1B. *C*, age dependence of the different methylated histone H4 forms ($H4ac_0$ + $H4ac_1$) from liver of rats aged 10, 30, 300, and 450 days was determined. The amount of each H4 modification was quantified using Beckman System Gold Software. The relative increase in H4 trimethylation of 30-, 300-, and 450-day-old rats was compared with the 10-day values (0%). The results represent the means ± S.D. for three to five independent experiments.

H4 Methylation Status in Liver and Kidney from Rats of Various Ages—To verify that trimethylated histone H4 is not only present in kidney, H4 was also prepared from rat liver and subjected to HILIC. The resulting chromatogram (Fig. 4*A*) closely resembled that obtained by HILIC analysis of histone

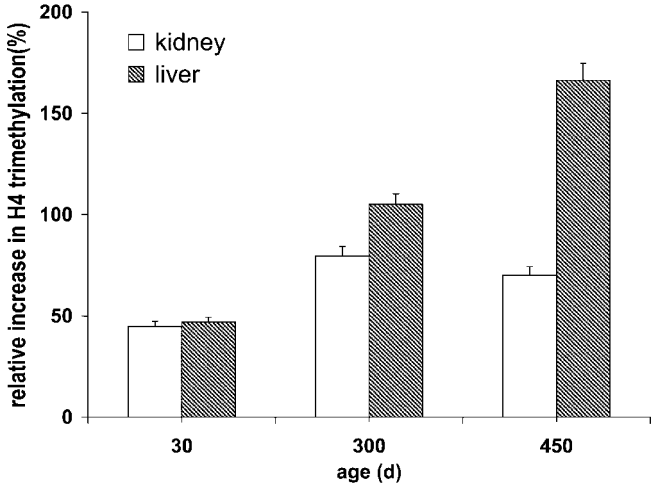


FIG. 5. **Age dependence of the trimethylated histone H4 forms in rat liver and kidney.** H4 was obtained from liver and kidney of rats aged 10, 30, 300, and 450 days. The H4 fractions (~150 μg) isolated with RP-HPLC were analyzed under the same HILIC conditions used in Fig. 1*B*. The amount of trimethylated H4 was quantified using Beckman System Gold Software. The relative increase in H4 trimethylation of 30-, 300-, and 450-day-old rats was compared with the 10-day values (0%). The results represent the means ± S.D. for three to five independent experiments.

H4 from kidney (Fig. 1*B*), clearly indicating the presence of trimethylated H4 in this tissue also. Preliminary data of H4 from very young rats, however, indicated a separation pattern (Fig. 4*B*) different from the one shown in Fig. 1*B*. These results prompted us to examine possible alterations of the H4 methylation status in the course of aging. For this purpose, liver H4 histones from rats aged 10 days, 30 days, 12 months, and 15 months were isolated and separated by HILIC. The result clearly showed that trimethylated histone H4 was present in all samples. Furthermore, no significant change in the relative amount of dimethylated H4 and only a slight increase in monomethylated H4 was found in the course of aging. However, a substantial increase in trimethylated H4 was observed in old rats as compared with young rats. As can be seen from Fig. 4*C*, in senescent (450 days old) rat livers, trimethylation is about 150% higher than in young ones (10 days old). It should be noted, however, that an increase of about 50% was already observed in the liver of 30-day-old animals. Similar results were obtained with rat kidney of various ages, although the age-dependent increase in trimethylation was less prominent as compared with that in rat liver (Fig. 5). An increase of about 70–80% in rat kidney *versus* 150% for liver was observed in older animals.

H4 Methylation in Human Cell Lines—Because previous papers did not report the presence of trimethylated lysine 20 in histone H4 of, for example, HeLa cells (16, 27) or human leukemic cells (28), we also investigated the extent of H4 methylation in several human cell lines (Raji, K562) using the HILIC technique. In fact, we found trimethylated histone H4 in both cell lines. The amount, however, was very small, especially in logarithmically growing cells, but increased clearly in nongrowing cells (data not shown).

DISCUSSION

It has been suggested that distinct combinations of covalent histone modifications (acetylation, phosphorylation, and methylation), also referred to as the histone code, generate unique surfaces for the binding of proteins that conduct further chromatin-related processes responsible for silencing and activating of genes.

Site-specific methylation is catalyzed by conserved proteins known as the histone methyltransferases (24, 29–31). Histone lysine methylation has been shown to occur mainly in histones H3 (at lysines 4, 9, and 27) (6) and H4 (at lysine 20) (29). Whereas acetylation and phosphorylation on histone N termini represent short term signals of the histone code, histone methylation has been regarded as a long term epigenetic mark (5). At present it is unknown how the degree of methyl addition (mono-, di-, or trimethylation) is increased and whether one and the same histone methyltransferase is responsible for all of these methyl additions. The biological significance of the various methyllysine species also remains unclear. It is known, however, that methyl addition (mono-, di-, or trimethylation) increases the affinity between histone tails and anionic molecules (*i.e.* DNA) (32, 33), whereas acetylation and phosphorylation lead to a loosening of histone-DNA interactions. Interestingly, *in vitro* studies have shown that dimethylation of lysine 9 converts an unmodified H3 N-terminal peptide into a high affinity binding site for HP1 proteins (9, 10), whereas this high affinity is not significantly affected when dimethylated lysine 9 is replaced with mono- or trimethylated lysine (5).

Recent findings suggest that histone H3 methylation of lysine 9 plays a role in both transcriptional activation and silencing. In this context it was speculated that the differences in the methylated species of H3 lysine 9 (mono-, di-, or trimethylation) might explain these results, which at first glance seem to be contradictory (34). The latest findings by Jacobs *et al.* (35) and Bannister *et al.* (9) concerning methyl-Lys^9 H3 binding of HP1 (heterochromatin-associated protein 1), which is necessary for transcriptional repression, indicate that differences in the extent of methylation (di- or trimethylation) may result in differences in function. In detail, using an H3 peptide (residues 1–15) containing both dimethyl-Lys^4 and dimethyl-Lys^9 modifications, Jacobs *et al.* (35) found in their binding assay with the chromodomain of HP1 a K_D value of 268 μM. Using an H3 peptide (residues 1–16) containing both trimethyl-Lys^4 and trimethyl-Lys^9, however, Bannister *et al.* (9) reported a dissociation constant K_D of 70 nM, a value 1000-fold stronger than the K_D value reported by Jacobs *et al.* (35).

At present, little is known about histone H4 methylation. Several studies have described the occurrence of mono- and dimethyllysine at lysine 20 in H4 of different species and cell lines (15, 28, 36–38). Trimethyllysine, however, has been detected only in *Drosophila* (39), and not in mammals. Furthermore, many of the earlier observers did not distinguish the individual lysine derivatives or the lysine derivatives from arginine derivatives. This is due in part to the lack of convenient techniques for their resolution. In contrast to acetylation and phosphorylation, increasing methyl addition does not significantly alter the net positive charge of the histone molecule, thus limiting the applicability of the electrophoretic methods (acid-urea and acid-urea-Triton) commonly used for histone analysis. What does indeed change is the hydrophilicity, which decreases from mono-, di-, to trimethylation (31). This slightly altered hydrophilicity enables the HILIC system presented in this paper to discriminate between the individual modified proteins, because it separates solutes on the basis of differences in their hydrophilicity, *i.e.* the more hydrophilic the solute, the stronger the interaction with the hydrophilic column material. Therefore, trimethylated histone H4 is first eluted from the column followed by the di- and monomethylated forms. In addition to the separation of methylated H4, the distinctly acetylated derivatives are also clearly resolved by HILIC. As can be seen from Fig. 1*B*, lysine acetylation influences retention to a larger extent than does lysine methylation. It is possible, therefore, to assign a specific methylation pattern to each of the differently acetylated H4 histones, thus enabling the simultaneous analysis of acetylated and methylated H4. When applying this sensitive and high resolving HILIC method in combination with mass spectrometry, we found substantial amounts of trimethylated lysine 20 in H4 of rat liver and kidney, with traces also being detectable in H4 of human cell lines. Whether the increasing amounts of trimethylated H4 found in aged tissues ("normal cells") and in growth-inhibited cell lines ("transformed cells") are responsible for differences in transcriptional activity is presently unknown. However, the more pronounced increase in trimethylated H4 in liver as compared with kidney may be connected to the increased polyploidization that is characteristic for hepatocytes in aging liver. Moreover, there is emerging evidence that such a polyploidization leads to growth arrest, terminal differentiation, and tissue maturation (for review see Ref. 40). It has been reported that the decline of transcriptional activity is primarily due to changes in the chromatin structures. The methylation site at lysine 20 in H4 is positioned at the boundary between the very basic N-terminal tail and the more hydrophobic domain of the remainder of the molecule, which is folded within the nucleosome. Methylation at these sites may alter nucleosome and higher order chromatin structure (41). In any case it is likely that a histone code proposed for H3 also exists for histone H4 involving methylation (mono-, di-, or trimethylation) of lysine 20, phosphorylation of histidine 18, and acetylation of lysine 16 as well as methylation of arginine 3 and acetylation of lysine 5 (42).

In summary, our investigations provide the first evidence for *in vivo* alterations of a trimethylated lysine of mammalian histone H4 in aging organs. We favor the view that not only methylation in itself but also the degree of methylation (mono-, di-, or trimethylation) play a physiologically important role in remodeling chromatin and ultimately in regulating gene expression.

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