

Sequence Analysis of Acetylation and Methylation in Two Histone H3 Variants of Alfalfa*

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Analysis of acetylation in the two histone H3 variants of alfalfa by acid/urea/Triton-polyacrylamide gel electrophoresis has established that the minor variant H3.2 has a 2-fold higher level of acetylation than the major variant H3.1. Purification and sequence analysis of both variants showed sequence identity across the complete amino-terminal domain, which contains the 6 modified lysines 4, 8, 14, 18, 23, and 27. The two proteins have different distributions for acetylation: mono-, di-, and tri-methylation. The higher level of acetylation of H3.2 was confirmed in a wider pattern across all 6 lysines. Lysine modification levels varied for all sites in both proteins between 5 and 95%, with combinations of one to four types of modification co-existing at each residue. Additional sequence analysis of the H3.1 and H3.2 proteins and of tryptic core peptides established that the two histones differ only in residues 31, 41, 87, and 90. This indicates that major histone H3.1 is the product of the major alfalfa histone H3 gene and makes it likely that H3.2 is the product of the minor H3 gene, known from a partial cDNA clone. The variant-specific differences in lysine modifications in protein domains with identical primary structures suggest that the pattern and level of lysine modifications may be directed by the distinct chromatin environments of the two histone H3 variants.

sufficient requirement to allow RNA polymerases to further unfold the chromatin for gene transcription (for reviews, see Matthews and Waterborg, 1985; Gross and Garrard, 1987, 1988; Pederson and Simpson, 1988; Vidali *et al.*, 1988). This model assumes that differences in histone sequence, observed as histone variants, play no role. However, this model cannot explain observed preferential localization of some histone variants in active chromatin (Allis *et al.*, 1986; Donahue *et al.*, 1986; Jasinskiene *et al.*, 1985, 1988) nor their exclusion from it (Halleck and Gurley, 1982). Moreover, it predicts that all histone variants of a certain histone type will have, on average, identical levels of histone acetylation, whereas the levels of histone H3 acetylation in the two H3 variants of alfalfa (Waterborg *et al.*, 1987, 1989) and tobacco¹ are very different.

This study was initiated to evaluate whether the primary structure of the alfalfa histone H3 variants differ sufficiently in the domain containing the acetylation sites to explain the observed differences in acetylation. Sequence analysis of substantial parts of purified H3.1 and H3.2 proteins revealed variant-specific patterns and levels of lysine acetylation and methylation in a protein domain that does not contain the variant-specific differences in primary structure. Histone H3 variant modifications may be determined by the chromatin environment.

EXPERIMENTAL PROCEDURES

Preparation of Alfalfa Histones—Friable calli of alfalfa, cultivar strain R4 of *Medicago sativa*, were grown for 4 weeks at 25 °C, 85% relative humidity, and constant light. Aliquots of 200 g of fresh callus were dispersed and homogenized in a precooled 1-liter Waring Blender with a rotor-stator knife assembly 3 times for 1 min at high speed with 200 ml of buffer G40 (40% guanidine-HCl, 0.1 M potassium phosphate, pH 6.8) as described previously (Waterborg *et al.*, 1987), with fresh addition of 2-mercaptoethanol to 2 mM to prevent oxidation of histone H2B and H3 variant proteins.¹ The homogenate was centrifuged for 5 min at 2200 × *g*, filtered through 2 layers of Miracloth (Calbiochem), clarified by centrifugation for 15 min at 22,000 × *g*, and acidified by addition of concentrated HCl to 0.25 N. The solution was stirred on ice for 15 min, clarified by centrifugation for 30 min at 22,000 × *g*, diluted with 0.1 M potassium phosphate at pH 6.8 to the refractive index of buffer G5 (5% guanidine-HCl, 0.1 M potassium phosphate, pH 6.8), neutralized with KOH to pH 6.8, clarified by centrifugation for 20 min at 6,200 × *g*, and adjusted to 0.5 mM 2-mercaptoethanol. Bio-Rex-70 resin (Bio-Rad), equilibrated with buffer G5, was added to a ratio of 1 ml of resin for each 20 g of homogenized callus. The suspension was stirred at room temperature overnight. The resin was allowed to settle, washed with buffer B (0.1% trifluoroacetic acid in acetonitrile) containing 1.5 mM 2-mercaptoethanol, packed in a column, and washed with 8 column volumes of buffer G5 with 2-mercaptoethanol. The crude histone preparation was eluted by 10 column volumes of buffer G40 containing 1.5 mM 2-mercaptoethanol, dialyzed in Spectra/Por 3 membranes (3500-Da cut-off) against three changes of 100 volumes of 5% (v/v) acetic acid

Study of the two histone H3 variants of alfalfa (*Medicago sativa*) has revealed that the major variant, histone H3.1, representing two-thirds of the histone H3 protein, has a steady-state acetylation that is only half of that seen in the minor variant, histone H3.2 (Waterborg *et al.*, 1987, 1989). The correlation between high levels of histone acetylation and transcriptional activation of chromatin (Matthews and Waterborg, 1985; Hebbes *et al.*, 1988; Pfeffer *et al.*, 1988, 1989; Turner and Fellows, 1989) suggests that histone H3.2 plays a specific role in active chromatin in a way similar to other histone variant proteins with a specific location in active chromatin (Allis *et al.*, 1986; Donahue *et al.*, 1986).

The current model for gene activation within chromatin envisions that specific gene regulatory proteins induce a change in the conformation of chromatin. This change increases the accessibility of the core histones for dynamic acetylation and results in higher levels of histone acetylation. The increased acetylation levels appear a necessary but not

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¹ J. H. Waterborg, unpublished results.

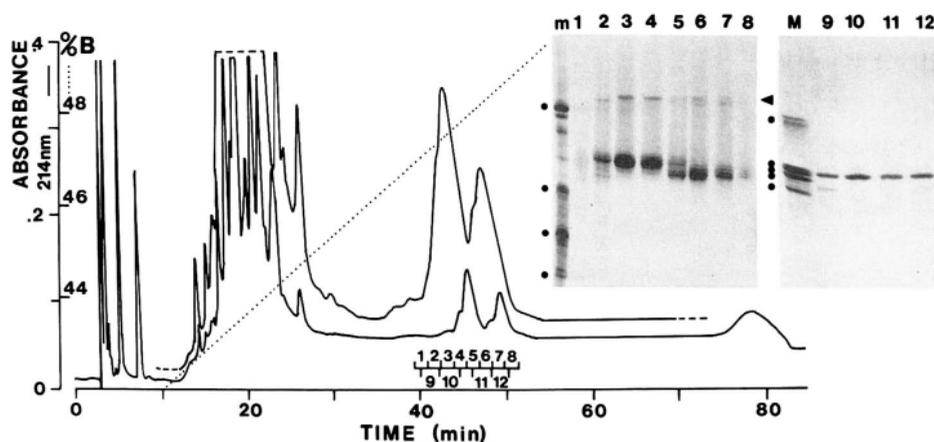


FIG. 1. Reversed-phase HPLC of alfalfa histone H3 variants. Histone H3 proteins, extracted from callus cultures of alfalfa, were prefractionated by Bio-Gel P60 chromatography, and extracts corresponding to 28 and 280 g of callus were chromatographed on Zorbax Protein Plus as described under "Experimental Procedures" and shown as the lower and upper registrations of the absorbance at 214 nm, respectively. Histone variants H3.1 and H3.2 eluted separately during a gradient elution between 42 and 52% buffer B (acetonitrile in water/0.1% trifluoroacetic acid, dotted line). Samples across the double elution peaks of the upper chromatographic registration were dried and analyzed by AUT (lanes 1–8) and SDS (lanes 9–12) gel electrophoreses, as indicated. All samples containing high concentrations of histone H3 show to a small and variable degree apparent oxidation to dimers (arrowhead). Calf thymus histones were used as markers in AUT (lane *m* with histones H2A, H1, H2B, and H4 indicated from top to bottom) and SDS gel electrophoreses (lane *M* with histones H1, H3, H2B, H2A, and H4 indicated from top to bottom). Samples corresponding to AUT, lanes 3 and 4, and 6 and 7, or SDS, lanes 10 and 11, were routinely used for sequence analysis. The basis for the peak heterogeneity observed for both histone H3 variant peaks has not yet been identified and is not detectable by AUT or SDS gel analyses.

with 1.5 mM 2-mercaptoethanol, and lyophilized.

Preparative amounts of histones from up to 1500 g of callus were fractionated on a 2.5×100 -cm column of Bio-Gel P60 (Bio-Rad) in 20 mM HCl, 50 mM NaCl, 0.02% sodium azide (Von Holt and Brandt, 1977) as described previously (Waterborg *et al.*, 1987). Fractions containing histone H3 were identified by acid/urea/Triton (AUT)²-gel electrophoresis, pooled, dialyzed into 2.5% acetic acid, 0.1 mM 2-mercaptoethanol, and lyophilized. Histone H3 variants labeled *in vivo* were prepared from 20 g of callus cells, dispersed in growth medium, and labeled for 60 min with 1 mCi [³H]acetate as described previously (Waterborg *et al.*, 1989), by the procedure described above, with the omission of Bio-Gel P60 chromatography.

Separation of Histone H3 Variants—Histones were solubilized in 7.2 M freshly deionized urea, 50 mM dithiothreitol, 0.75 M ammonium hydroxide, and 0.05% phenolphthalein for 5 min at room temperature, acidified by the addition of 1/20 volume of glacial acetic acid, and clarified by centrifugation for 5 min at $13,000 \times g$. Crude histones prepared from up to 20 g of callus or histone H3-enriched Bio-Gel preparations from up to 300 g of callus were injected on a 4.6-mm \times 25-cm Zorbax Protein Plus (Du Pont-New England Nuclear) reversed-phase column, equilibrated in buffer A (0.1% trifluoroacetic acid in water). The column was developed at 1 ml/min by 5 ml of buffer A, a gradient of 5 ml from 0 to 42% buffer B, a gradient of 60 ml from 42 to 52% buffer B, and a gradient of 5 ml from 52 to 70% buffer B followed by re-equilibration to buffer A. The two histone H3 variant proteins were localized by UV absorbance at 214 nm and by AUT gel analysis, and pooled separately, excluding the small overlap between the two peaks after preparative chromatography. Analytical retention times were 45 min for histone H3.1 and 49 min for H3.2 (Fig. 1). The pooled histones were judged to be homogeneous by SDS and AUT gel electrophoreses. A typical recovery of homogeneous H3 variants was 0.6 μ g of H3.1 and 0.3 μ g of H3.2 protein/g of callus. For the analysis on the total number of detectable acetylation sites by fluorography (Fig. 2), the small overlap between the two histone H3 variants was not discarded to avoid possible bias, but care was taken to avoid inclusion of H3.1 protein into the H3.2 fraction because this would have interfered with the detection of high levels of H3.2 acetylation. These considerations lead to a minor contamination of histone H3.2 into the H3.1 fraction (Fig. 2).

Isolation of the Tryptic Core of Histone H3—HPLC-separated histone H3 variants were dissolved in 50 mM NH_4HCO_3 , pH 8.2, to 1

mg/ml and digested with 1/40 weight of L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Worthington) twice for 2 h at 37 °C. The digest was lyophilized and fractionated on a 4.6-mm \times 25-cm Zorbax Protein Plus reversed-phase HPLC column in 0.1% trifluoroacetic acid in water which was developed at 0.5 ml/min with a 120-min gradient to 70% acetonitrile in water with 0.1% trifluoroacetic acid. The tryptic core peptide of both H3 variants (residues 84–115 (Brandt and Von Holt, 1982; Vanfleteren *et al.*, 1987)) eluted at 93 min and was identified by UV absorbance of tyrosine at 275 nm and by amino acid analysis.

Analysis of Histone H3 Variants—SDS and AUT gel electrophoreses, fluorography, and gel quantitation were performed as described previously (Waterborg *et al.*, 1987, 1989). Histone H3 proteins and peptides were hydrolyzed for 24 h at 110 °C in 6 N constant-boiling HCl with 0.1% phenol, and the amino acid composition was determined by HPLC (Bio-Rad) and postcolumn *o*-phthalaldehyde derivatization, which precludes detection of proline. Prehydrolysis derivatization of cysteine to a stable derivative was not performed.

Automated Sequence Analysis—The standard protein sequencing protocol for Edman degradation and phenylthiohydantoin-derivative identification from an Applied Biosystems 477A/120 Pulsed-liquid Phase Sequencer was used with repetitive yields of 92% or higher. Extended proline digestion cycles were used at positions 16, 30, 38, and 43 during the sequencing of intact histone H3 proteins. Automated cysteine modification by 4-vinylpyridine was applied to the tryptic peptide core of histone H3.1 prior to sequencing. Histone H3.1 (300 pmol) was sequenced for 44 residues, histone H3.2 (1000 pmol) for 51 residues, the tryptic core of histone H3.1 (700 pmol) for 32 residues, and the tryptic core of histone H3.2 (500 pmol) for 11 residues. The NORMAL-1 HPLC gradient program was modified for the detection and quantitation of methylated lysines when 30 amino-terminal residues of H3.1 (2.5 nmol) and H3.2 (1.6 nmol) were analyzed for modification. Solvent B was kept at 90% for an additional 5 min to allow elution of mono- and trimethyllysine. Modified amino acids were identified as phenylthiohydantoin-derivatives by chromatography of known standards and quantitated relative to known amounts of lysine, using a relative color value (color) which is the ratio of the molar absorbance (by peak height) of modified lysine and unmodified lysine. Retention times (rt) and limits of detection and accuracy (limit) for the modified amino acids were established relative to alanine (rt = 13.2 min), arginine (rt = 19.4 min), tryptophan (rt = 24.5 min), and lysine (Sigma, rt = 26.8 min, color = 1.0, limit = 0.7 pmol); *N*-acetyllysine (Sigma, rt = 12.5 min, color = 0.545, limit = 1.3 pmol), *N*-monomethyllysine (Sigma, rt =

² The abbreviations used are: AUT, acid/urea/Triton X-100; SDS, sodium dodecyl sulfate; HPLC, high pressure liquid chromatography.

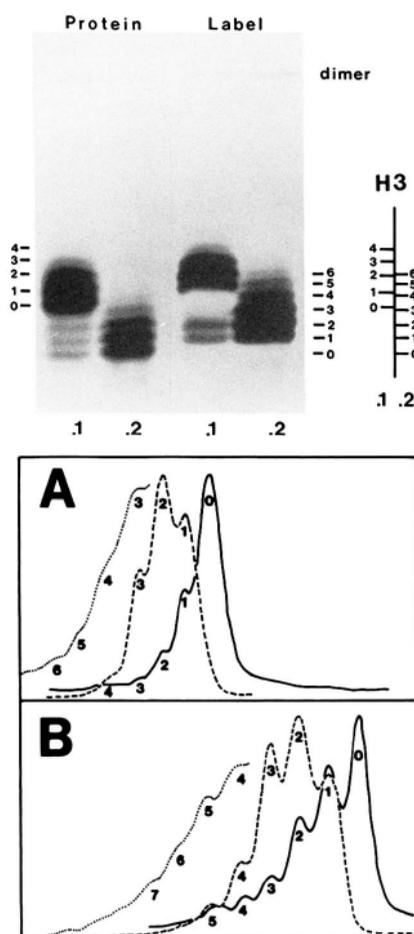


FIG. 2. Acetylation of histones H3.1 and H3.2 in AUT gels. Histone H3 variants were labeled *in vivo* with tritiated acetate and purified from a total histone preparation by reversed-phase HPLC. The H3.1 preparation (.1 and A) with a slight inclusion of histone H3.2 (see "Experimental Procedures") and the H3.2 preparation (.2 and B) were electrophoresed in parallel AUT gel lanes and stained for Protein by Coomassie and for radioactive Label by quantitative fluorography. The relative positions of non- through multiacetylated H3 bands are numbered separately and plotted to show their relative electrophoretic mobilities. A densitometric scanning analysis is shown after Coomassie protein staining (continuous line) and after fluorography for 4 days (broken line) and 67 days (dotted line). The overexposed portion of the 67-day exposure has been omitted. The scanning patterns of histone H3.1 (A) were aligned with the patterns of histone H3.2 (B) as observed in gel electrophoresis (from left to right).

28.8 min, color = 0.585, limit = 1.3 pmol), *N*'-dimethyllysine (Bachem, *rt* = 25.3 min, color = 0.158, limit = 14 pmol), and *N*'-trimethyllysine (gift from N. L. Benoiton, University of Ottawa, *rt* = 32.2 min, color = 0.074, limit = 30 pmol).

RESULTS

The two histone H3 variant proteins of alfalfa (H3.1 and H3.2) can be separated by water/acetone gradients with 0.1% trifluoroacetic acid ion pairing on a Zorbax Protein Plus reversed-phase HPLC column. The two histone H3 proteins elute later than any other histone and are separated from each other by approximately 0.7% acetonitrile. With analytical sample amounts, base-line separation is possible, and at the semipreparative sample amounts of crude histone H3 used in this study, routinely 75% of both variant proteins could be purified to homogeneity by excluding the small overlap between the peaks (Fig. 1).

As a source of histone H3, total cells were used (Waterborg

et al., 1987) rather than isolated nuclei (Waterborg *et al.*, 1989). Artificial heterogeneity arises during the preparation of alfalfa nuclei, probably due to some form of cysteine modification in the H3 proteins (Waterborg, 1990). The extraction of histones from total cells rather than from nuclei increased histone recoveries approximately 2-fold but decreased the histone content of the crude extract to below 50%. The maximal capacity of a Zorbax Protein Plus column (4.6 mm × 25 cm) for separation of the histone H3 variants was reached when 5 mg of total protein was applied. Fractionation of the total crude histone preparation by Bio-Gel P60 chromatography (Waterborg *et al.*, 1987) to obtain a histone H3-enriched preparation allowed separation and purification of 10-fold more histone H3 per reversed-phase HPLC run (see "Experimental Procedures"). Moreover, a small contamination of minor H2A variant protein and trace amounts of non-histone proteins were removed from the histone H3 preparations. A single band of identical molecular weight was visible for each preparation in SDS gels (lanes 10 and 11 in Fig. 1). The purity of each histone variant preparation was further proven by amino acid analysis (Table I) and by automated sequence analysis, which indicated a contamination level of less than 1% of non-histone H3 protein.

Analysis of the two purified histone variant proteins by AUT gel electrophoresis showed a series of multiple bands for each protein (lanes 3–4 and 6–7 in Fig. 1) in addition to a variable amount of presumably oxidized histone H3 dimer. To identify acetylation as the cause for this pattern of distinctly charged histone H3 protein forms and to establish the total number of detectable modification sites, alfalfa callus cultures were labeled *in vivo* with tritiated acetate, histone H3 variant proteins were fractionated by reversed-phase chromatography of the crude histone preparation, and the fractions were analyzed by AUT gel electrophoresis (Fig. 2). Histone H3.1 clearly showed three modified protein bands with increasing discrete reductions in gel mobility relative to the major protein form (Protein .1 in Fig. 2). These modified bands appear to be caused by acetylation, as judged by the acetate label incorporation pattern (Label .1 in Fig. 2), but extensive fluorographic analysis revealed the existence of six possible sites (Fig. 2A). Histone H3.2 had a 2-fold higher level

TABLE I
Amino acid analysis of histone H3.1 and H3.2

Amino acid	Experimental results		Predicted from gene sequences ^a			
	H3.1	H3.2	Major	Minor	Major	Minor
	mol%		amino acid no.		mol%	
Asx	4.4	4.5	5	5	3.7	3.7
Thr	7.3	8.0	10	11	7.4	8.1
Ser	4.4	3.4	6	4	4.4	3.0
Glx	12.4	12.4	15	15	11.1	11.1
Pro	ND ^b	ND	6	6	4.4	4.4
Gly	6.1	6.1	7	7	5.2	5.2
Ala	13.8	12.9	19	18	14.1	13.3
Val	4.9	5.0	6	6	4.4	4.4
Cys	ND	ND	1	1	0.7	0.7
Met	0.3	0.3	1	1	0.7	0.7
Ile	5.2	5.2	7	7	5.2	5.2
Leu	9.0	9.6	12	13	8.9	9.6
Tyr	1.3	2.0	2	3	1.5	2.2
Phe	3.4	3.0	5	4	3.7	3.0
Lys	10.4	10.3	14	14	10.4	10.4
His	2.0	2.2	2	3	1.5	2.2
Arg	15.1	14.8	17	17	12.6	12.6
Total	100.0	100.0	135	135	100.0	100.0

^a Prediction is based on Wu *et al.* (1989), assuming no sequence differences at residues 1–16.

^b ND, not determined.

of steady-state acetylation than did H3.1, as determined by quantitative Gaussian curve-fitting analysis of the Coomassie-stained pattern (Protein .2, and the continuous scan in Fig. 2B). The higher level of H3.2 acetylation was also supported by the larger number of detectable acetylation bands (Label .2 in Fig. 2) and their relatively higher abundance in the fluorography pattern with probably more than six sites of modification (Fig. 2B).

Automated sequence analysis of both histone H3 variant proteins for 30 residues revealed identical sequences (Fig. 3). At lysine residues 4, 9, 14, 18, 23, and 27, additional, unidentified phenylthiohydantoin-derivative peaks were observed at varying intensities. With the known postsynthetic modification of lysine by acetylation and methylation, the elution pattern of various modified forms of lysine was established using known standards. Extension of the NORMAL-1 HPLC gradient elution program enabled all previously unknown peaks to be identified as acetylated, mono-, di-, or trimethylated lysine. With quantitation of these modified residues relative to unmodified lysine, a complex pattern of acetylation and methylation was revealed with distinct quantitative and qualitative differences between histones H3.1 and H3.2 (Table II). It should be noted that the accuracy of quantitation of lysine, acetyllysine, and monomethyllysine is higher than that

of di- and trimethyllysine, which have lower apparent molar absorbance values (see "Experimental Procedures").

To establish the extent of primary structure differences for the two histone H3 proteins, additional sequence analyses were performed, guided by the observed differences in the amino acid analysis between the two variant proteins (Table I) and by the published cDNA sequence data for the major histone H3 gene of alfalfa (Wu *et al.*, 1989). This study identified a minor histone H3 gene from a partial cDNA clone (pH3c.11), predicting a histone H3 protein from amino acid residues 17–135 which would differ at residues 31, 41, 87, and 90 from the major gene (Fig. 3). Amino acid analysis of histone H3.1 and H3.2 showed differences only for threonine, serine, alanine, leucine, tyrosine, phenylalanine, and histidine (Table I), consistent with the major protein variant H3.1 as the product of the major histone H3 genes of alfalfa and the minor protein variant H3.2 as the product of the minor gene(s). This conclusion was supported by automated sequence analysis of histone H3.1 for 44 and of H3.2 for 51 residues, which showed the predicted sequences, including the differences at residues 31 and 41 (Fig. 3), and which supplied the amino-terminal sequence missing from the partial gene clone. Lysines 36 and 37 in both proteins appeared not to be modified by acetylation or methylation. Tryptic core peptides were prepared and sequenced to confirm the predicted sequence differences for residues 87 and 90 (Fig. 3).

DISCUSSION

This study identifies the three major sites of acetylation in alfalfa histone H3 as lysines 14, 18, and 23. Minor sites of acetylation were only quantitated in histone variant H3.2 as lysines 27 and 9, with a trace of modification detected at lysine 4 (Table II). This shows that the sensitivity for quantitation of acetylation by sequencing is similar to that by quantitative analysis of Coomassie-stained AUT gels (Fig. 2). Calculation from the sequence data of acetylated lysine residues per protein molecule for H3.1 and H3.2 yields values of 0.38 and 0.73 acetyllysine, respectively. These are similar to values determined from AUT gel analysis of 0.44–0.56 for H3.1 and 0.97–1.01 for H3.2. In both analyses, the relative level of acetylation is estimated to be 2-fold higher for histone variant H3.2 than for variant H3.1.

Fluorographic detection of the total number of acetylation sites of alfalfa histone H3 is more sensitive than either method, but it is limited to a qualitative assessment. At least seven sites of acetylation were detectable in histone H3.2 (Fig. 2), six of which are presumed to be the 6 lysines observed to be acetylated in histone H3.2 (Fig. 3). This result may indicate that, in trace amounts, lysines other than those in the amino-terminal region of histone H3 may become acetylated. An alternative explanation would be the generation of modified forms of histone H3 by modifications other than acetylation. The involvement of phosphorylation appears excluded by the failure to label histone H3 *in vivo* with radioactive inorganic phosphate (Waterborg *et al.*, 1989).

Histone H3 can be divided into two distinct structural domains. The amino-terminal domain of residues 1–27 contains all known lysine acetylation and methylation sites and appears highly accessible in chromatin as judged by its sensitivity towards proteolytic fragmentation, especially near residue 27 (Rill and Oosterhof, 1982; Marion *et al.*, 1983; Crane-Robinson and Boehm, 1985; Dimitrov *et al.*, 1986; Dumunis-Kervabon *et al.*, 1986; Encontre and Parello, 1988; Ausio *et al.*, 1989). The carboxyl-terminal part of histone H3 appears to be a structured, globular domain with major hydrophobic characteristics. The sequence differences between

H3.1	M	M	M	M	M	M	*		
ARTKQTARKS	TGGKAPRKQL	ATKAARKSAP	ATGGVKKPHR						
ALH3.1	artkqtarks	tggkaprkql	atkaarksap	atggvkkpkr					
	1			31					
	*							*	*
FRPG								FQSSAVS	
frpgtvalre	irkyqkstel	lirklpfqr	vrelaqdfkt	dlrfqssavs				87	90
	41								
	ALQEAEEAYL	VGLFEDTNLC	AIAHAK						
alqeaeeayl	vglfedtnlc	aihakrvtim	pkdiqlarri	rgera					135
H3.2	M	M	M	M	M	M	*		
ARTKQTARKS	TGGKAPRKQL	ATKAARKSAP	TGGVKKPHR						
pH3c.11	artkqtarks	rkql	atkaarksap	tggvkkpkr					
	1			31					
	*							*	*
YRPGTVALRE	I							FQSSAVL	
yrpgtvalre	irkyqkstel	lirklpfqr	vrelaqdfkt	dlrfqshavl				87	90
	41								
	ALQE								
alqeaeeayl	vglfedtnlc	aihakrvtim	pkdiqlarri	rgera					135

FIG. 3. Alignment of histone H3 protein sequences with predicted gene products. Top, protein H3.1 automated protein sequence results (upper case letters) were aligned with the protein sequence predicted for major alfalfa H3 gene ALH3.1 (lower case letters). Bottom, protein H3.2 sequence results (upper case letters) were aligned with the protein sequence predicted by cDNA clone pH3c.11 of the minor alfalfa H3 gene (lower case letters). The sequence numbering of the amino and carboxyl-terminal residues and of all residues that differ between the two histone H3 variant proteins (*) is indicated. M, modified lysine.

TABLE II

Modification of histone H3 (residues 1–30)

Modified residues in histone H3, residues 1–30, as determined by automated protein sequencing. AcL, *N*-acetyllysine; MML, *N*-monomethyllysine; DML, *N*-dimethyllysine; TML, *N*-trimethyllysine. —, below the detection limits for AcL, MML, DML, or TML (see "Experimental Procedures"); tr, trace (less than 0.5%).

Residue	Histone H3.1					Histone H3.2				
	Lys	AcL	MML	DML	TML	Lys	AcL	MML	DML	TML
	%					%				
4	76.1	—	4.3	3.8	15.7	50.3	tr	24.4	6.4	18.9
9	11.2	—	73.5	15.2	—	31.5	1.6	43.4	8.7	14.8
14	66.2	20.2	—	—	13.6	45.3	37.0	—	—	17.6
18	71.2	12.8	—	—	15.9	59.6	24.7	—	—	15.7
23	95.1	4.9	—	—	—	57.9	9.0	—	—	33.1
27	5.2	—	31.0	21.5	42.3	36.2	1.6	24.1	—	38.1

histone H3.1 and H3.2 at residues 31, 41, 87, and 90 all reside in the globular domain. This makes it highly unlikely that these primary structure differences could be the determining factor in the distinct differences in lysine acetylation and methylation within the amino-terminal domain of the histone H3 variants (Table II).

The pattern of acetylation of histone H3.1 covers three contiguous lysines at positions 14, 18, and 23. Acetylation of histone H3.2 is similar, with higher levels of modification of these lysines and low levels in additional, contiguous lysines 27, 9, and 4 (Table II). This pattern suggests that the histone-acetylating enzymes lack a strict sequence specificity and that their action may be directed more by steric determinants. Lysines within the globular domain beyond residue 27, such as lysines 36 and 37, remain completely unmodified. The decrease in acetylation levels from lysine 14 to lysine 27 may be caused by increasing steric interference for acetylation by the neighboring globular histone domain. However, a severe restriction towards acetylation of lysines 9 and 4 is apparent. It is tempting to speculate that this area of histone H3 is closely associated with some chromatin structural element when histone H3 is part of chromatin. Highly methylated lysine 9 may be important for this interaction, because in histone H3.2 this residue is less methylated and, as predicted, acetylated to a higher extent than in histone H3.1 (Table II).

The higher levels of acetylation of histone H3.2 with an apparently wider pattern of accessibility are consistent with the concept that histone H3.2 with its higher acetylation levels is part of transcriptionally active chromatin. Such chromatin is expected to be partially unfolded with increased accessibility for proteins. This speculation suggests that the specific or preferential localization of histone H3.2 in activated chromatin causes its higher level of acetylation. It leaves open the question on the mechanism by which histone variants may become localized in different chromatin environments.

Histone methylation is generally considered to be an irreversible modification of lysines in soluble histones and in chromatin for which no function has been established (Thomas *et al.*, 1975; Honda *et al.*, 1975b; Duerre and Buttz, 1990). In histone H3, partial methylation and acetylation at the same lysines (residues 4 and 9) has been observed during sequencing of bovine and trout histone H3 (DeLange *et al.*, 1972; Honda *et al.*, 1975a). Some histone methylation has recently been shown to change in *Drosophila* when heat shock genes were induced or transcription was inhibited (Tanguay and Desrosiers, 1988; Desrosiers and Tanguay, 1989). Histone methylation may thus be important in the regulation of chromatin activity. However, it is unknown whether mono-, di- and trimethylation are functionally identical. The distinct differences observed in the patterns for all three forms of lysine methylation (Table II) might suggest functional differences, but not enough is known for fruitful speculation. For this paper, it appears most important to realize that prior methylation of a lysine, especially of lysines 9 and 27, will limit the possibility for histone acetylation.

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