THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 268, No. 7, Issue of March 5, pp. 4918–4921, 1993
Printed in U.S.A.

Dynamic Methylation of Alfalfa Histone H3*

(Received for publication, September 4, 1992)

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Dynamic lysine methylation of histone H3 in alfalfa tissue culture cells was studied by labeling with tritiated methionine, purification of variants H3.1 and H3.2 by reversed-phase high pressure liquid chromatography and amino acid analysis. Mono- and dimethyl-L-lysine were the major labeled amino acids. Within 100 h of continued growth conversion from $N\epsilon$-monomethyl-L-lysine (MML) to $N\epsilon$-dimethyl-L-lysine (DML) and $N\epsilon$-trimethyl-L-lysine (TML) was observed, consistent with steady-state histone methylation. During the same time 20% of the methylation label was lost from major variant H3.1 protein and more than 50% from the more highly labeled minor variant H3.2. A similar pattern of label incorporation and loss was observed during a study of histone synthesis and turnover. This conforms with the general observation in animal cells that lysine methylation is limited to newly synthesized histone. Increased methylation of the more highly acetylated forms of histone H3 protein indicates limited accessibility of chromatin for histone methylation. After loss of the labile fraction of newly synthesized H3 variants, stably methylated proteins with 30% of the label in MML, 40% in DML, and 25% in TML remain. Turnover of methyl modification groups independent of histone turnover was not detected.

Methylation of lysine residues is a common modification of histones H3 and H4 for which no clear function has been established (for review, see Ref. 1). A recent analysis of two histone H3 variants of alfalfa revealed very high levels of mono-, di- and trimethylation at specific lysine residues within the amino-terminal domain of both proteins in a variant-specific pattern (2). Widely different levels of methylation were observed at distinct lysine residues that also appeared to be targets for dynamic and reversible lysine acetylation. Earlier studies in animal cells have suggested that lysine methylation is an irreversible modification of histones that occurs in chromatin within hours after histone synthesis and deposition (1). Thus lysine methylation might provide a way to modulate the potential for histone acetylation, an apparent requirement for transcription of chromatin (for reviews, see Refs. 3–5).

Association of active histone methylation with histone acetylation has recently been described, suggesting that lysine methylation may be functionally important for gene transcription (6–9). Possibly, lysine methylation like acetylation might be a reversible modification of histones (1, 10). Here we present a first analysis of the dynamics of histone methylation in a plant species. Methylation of the two histone H3 variants in alfalfa that differ quantitatively in histone acetylation (2) appears limited to newly synthesized histones with an irreversible progression from mono- to trimethylation of lysines.

MATERIALS AND METHODS

Alfalfa (*Medicago varia*) cv. Rambler suspension cultures named A2 (11) were labeled with 0.75 mCi of L-[*methyl*-^{3}H]methionine (80 Ci/mmol, Du Pont-New England Nuclear)/100 ml culture at a density of 30% for 2 h during log phase growth at 25 °C. Cells were collected by centrifugation for 4 min at 800 × *g*, washed once with fresh medium, and diluted to a density of 10% in MS medium. One aliquot of 75 ml was taken immediately, and cells (2.75 ml) were collected by centrifugation at 800 × *g* and stored at −20 °C. Subsequent aliquots were taken after 17.5 h (4.0 ml of cells), 64 h (7.5 ml of cells), and 184 h (34 ml of cells). To maintain near logarithmic growth conditions for the last sample, 125 ml of fresh medium was added at 64 h. Histones were extracted from whole cells, and histone H3 variants were purified and separated by reversed-phase HPLC[1] on a Zorbax Protein-Plus column developed at 1 ml/min with a gradient from 30 to 60% acetonitrile in water with 0.1% trifluoroacetic acid as described before (2, 11). Elution of histone variants H3.1 and H3.2 was monitored by absorbance at 214 nm and by liquid scintillation counting. Variants were pooled separately excluding fractions containing both proteins, adjusted to 2 mM 2-mercaptoethanol, and lyophilized until dry. Aliquots of histone H3 protein obtained from approximately 2 g of cells were hydrolyzed in 0.28 ml of boiling HCl:neat trifluoroacetic acid:phenol (60:30:1) for 16 h at 120 °C (12), dried, and analyzed on an Applied Biosystems 420A automated amino acid analyzer. The eluent of the on-line Applied Biosystems 130A HPLC was collected in 0.5-min fractions, and radioactivity was quantitated by liquid scintillation counting. HPLC retention times (rt) of phenylthiocarbamyl (PTC) derivatives of modified lysines alone and mixed with amino acid standards were determined relative to arginine (rt = 8.6 min), threonine (rt = 8.9 min), valine (rt = 13.3 min), methionine (rt = 13.6 min), and lysine (rt = 17.9 min). The rt of $N\epsilon$-monomethyl-L-lysine (Sigma) was 18.7 min and of $N\epsilon$-dimethyl-L-lysine (Bachem) was 8.8 min. $N\epsilon$-Trimethyl-L-lysine (a gift from N. L. Benoiton, University of Ottawa) which showed, in addition to some DML, the presence of two peaks with retention times of 11.4 and 13.3 min. The latter peak was identified as TML by its coelution with one of the *in vivo* methylated lysine residues (see Fig. 3). It eluted seconds after PTC-valine, close to PTC-methionine, precluding independent quantitation of radioactivity in TML and methionine residues. An unknown, radioactively labeled peak, presumably a methylation-modified form of lysine (named *X*), was observed to coelute with lysine (see Fig. 3).

Acid:urea:Triton X-100 gel electrophoresis of purified histone H3 fractions, quantitative fluorography, and densitometry were performed as described (2).

* This work was supported by National Science Foundation Grant DCB-9118999. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: HPLC, high pressure liquid chromatography; rt, retention time(s); PTC, phenylthiocarbamyl; MML, $N\epsilon$-monomethyl-L-lysine; DML, $N\epsilon$-dimethyl-L-lysine; TML, $N\epsilon$-trimethyl-L-lysine.

RESULTS AND DISCUSSION

In a wide range of animal cells histone modification by methylation of lysines appears to occur at or shortly after histone synthesis (1). In a parallel study, histone synthesis in alfalfa was studied by radioactive labeling of A2 suspension cultures with tritiated lysine (13). In this plant the highly acetylated histone H3.2 protein variant appears to be synthesized continuously from constitutively expressed genes (14) at a rate much higher than that of the replication-dependent H3.1 variant. Remarkably, the most highly acetylated forms of the newly synthesized H3.2 protein appeared to be lost preferentially and at a high rate from chromatin (13).

In an attempt to analyze the dynamics of the high steady-state level of methylation of both histone H3 variants (2), alfalfa cells were labeled with tritiated methionine, and histone H3 variant proteins were prepared and separated by reversed-phase HPLC (Fig. 1*A*). The relative labeling of the histone H3 forms was very similar to that observed after lysine labeling for histone *de novo* synthesis. To determine the relative labeling of methionine in *de novo* synthesized histone protein *versus* label incorporation into methylated lysine derivatives in existing or newly synthesized histone protein, purified H3 variant proteins (Fig. 2*A*) were subjected to acid hydrolysis (to which methylated lysine derivatives are stable (15)) and amino acid analysis as PTC derivatives (Fig. 3). The elution behavior of $N\epsilon$-monomethyl-L-lysine (MML), $N\epsilon$-dimethyl-L-lysine (DML), and $N\epsilon$-trimethyl-L-lysine (TML) was established with standards. Significant amounts of MML were detected in histone H3.1 (Fig. 3*A*) and H3.2 (Fig. 3*F*). Coelution of PTC derivatives of DML with threonine and of TML with valine prevented direct quantitation of steady-state levels of the other methylated lysine forms.

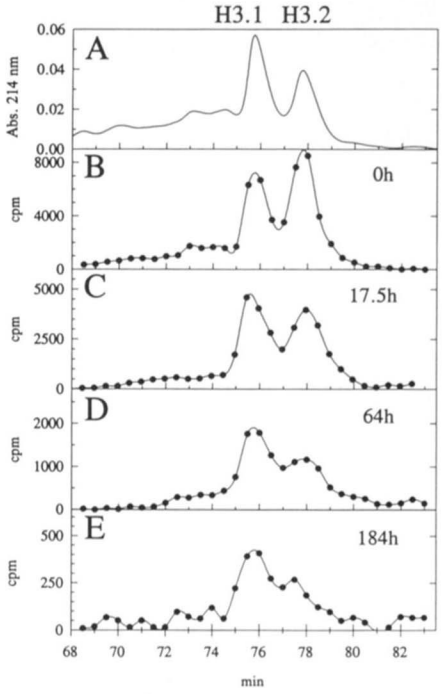

FIG. 1. **Fractionation of histone H3 variant proteins of alfalfa.** Absorbance elution profile during reversed-phase HPLC chromatography of histones extracted from a 1.4-ml cell pellet of A2 cells (*panel A*) and of similar amounts of histone labeled *in vivo* with tritiated methionine (see "Materials and Methods") after 0 (*panel B*), 17.5 (*panel C*), 64 (*panel D*), or 184 h (*panel E*) of continued growth. Histone variant H3.1 elutes prior to histone H3.2 at approximately 51–53% acetonitrile in the solvent. The radioactivity in cpm/0.5-ml fraction is given.

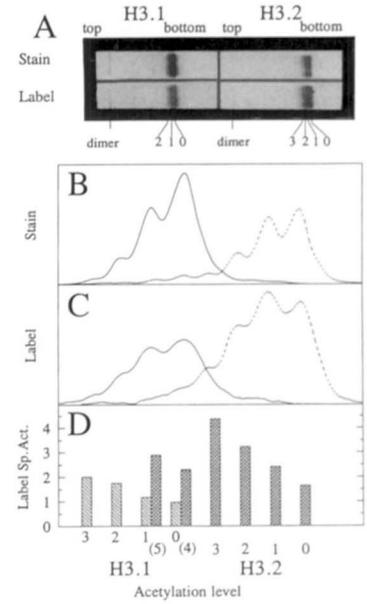

FIG. 2. **Acetylation of histone H3 variants.** Histone H3 variant proteins, pulse-labeled *in vivo*, were pooled separately from HPLC fractions (Fig. 1) and lyophilized. They were electrophoresed on acid:urea:Triton X-100 gels (*top* and *bottom* are indicated), Coomassie-stained (*panel A, top panels, Stain*) and fluorographed (*panel A, bottom panels, Label*). Histone acetylation levels are indicated. The patterns of Coomassie-stained protein bands (*Stain*; *panel B*) and radioactively labeled bands (*Label*; *panel C*) were determined by densitometry, and histone variant H3.1 is shown with *continuous lines* and H3.2 by *broken lines*. The specific radioactivity labeling (*Label Sp. Act.*) of each band was determined (in arbitrary units) and plotted for H3.1 (*diagonally marked bars*) and H3.2 (*cross-hatched bars*) in *panel D*. A small amount of histone H3.1 contaminated the histone H3.2 preparation in this experiment, detectable as elevated levels of bands that also contain tetra- and pentaacetylated H3.2 (indicated by the numbers *4* and *5* in *parentheses*). This contamination of H3.1 causes a reduction, relative to triacetylated H3.2, in the specific radioactivity for these bands in *panel D*.

Approximately 5% of the label eluted as methionine and/or TML and 95% as MML and DML. This established clearly that short incubation of alfalfa cells with tritiated methionine results almost exclusively in labeled methylated lysine residues. Thus the pattern of label incorporation into histone H3.1 and H3.2 protein (Fig. 1*B*) is not directly a result of different rates of histone synthesis (13). However, limitation of lysine methylation to newly synthesized histones, as observed in animal cells, could indirectly create a similar histone synthesis-like labeling pattern.

To evaluate this possibility, a study was made of the loss of methylation label from the alfalfa H3 variant proteins during continued growth. It has been shown that a small fraction of new histone H3.1 and more than half of new histone H3.2, specifically that in a highly acetylated state, is rapidly lost from chromatin (13). Continued growth of methionine-labeled alfalfa cells yields histone H3 protein with continuously decreasing levels of specific radioactivity, especially of histone variant H3.2 (Fig. 1, *C–E*). When corrected for histone synthesis during continued growth, these values show a drastic decrease in label present in histone H3.2 (to 46.4 ± 6.2% (n = 3)) and a small but detectable decrease in histone H3.1 (to 78.3 ± 8.5% (n = 3)) (Fig. 4*A*). The time course and the extent of loss of label are identical to that observed for lysine (to 42.6 ± 3.8% (n = 5) for H3.2 and to 78.5 ± 3.8% (n = 5) for H3.1) (13). Quantitatively the initial methionine labeling of histone H3.2 was slightly higher than the lysine labeling when calculated relative to histone H3.2. Consequently, the

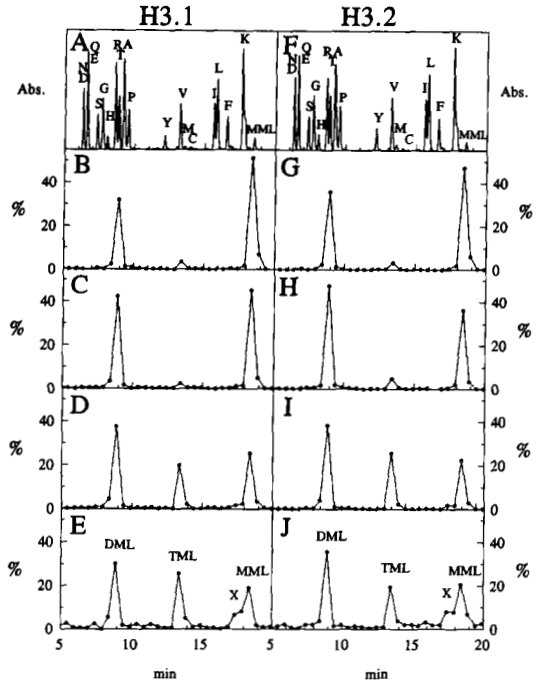

FIG. 3. **Quantitative analysis of histone H3 methylation labeling.** Purified H3 variant proteins (*panels A–E* = H3.1; *panels F–J* = H3.2) were hydrolyzed and analyzed as PTC derivatives by reversed-phase HPLC. The pattern of amino acid derivatives with single letter codes is shown for hydrolysates of 0.5 nmol of H3.1 (*panel A*) and H3.2 (*panel F*). The elution profile of methylation labeling, shown as the percentage of the radioactivity per fraction, is shown for purified histone H3 variant proteins chased *in vivo* for 0 (*panels B* and *G*), 17.5 (*panels C* and *H*), 64 (*panels D* and *I*), and 184 h (*panels E* and *J*). The radioactivity eluting at 13.5 min may consist of a mixture of the closely eluting tritiated methionine and tritiated TML amino acid derivatives. The identity of the labeled methylated lysine derivatives shown in *panels E* and *J* is based on coelution with known standards. The identity of the radioactive compound marked by *X* is unknown.

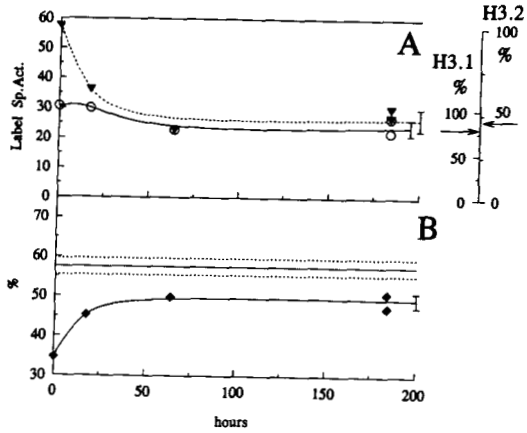

FIG. 4. **Loss of methylation label (*panel A*) and changes in relative methylation labeling (*panel B*) of alfalfa histone variants H3.1 and H3.2.** *Panel A*, the specific radioactivity (*Label Sp. Act.*) of histone variants H3.1 and H3.2 was determined from radioactivity (cpm) and amount of protein (214 nm absorbance) measured during reversed-phase chromatography (Fig. 1) and adjusted for the increase in cell mass caused by continued cell proliferation. Standard deviations of this calculation for histone H3.1 (*circles on continuous line*) and H3.2 (*triangles on broken line*) are shown. The relative loss of methylation label from each variant is shown on the *right*. *Panel B*, the relative distribution of label between H3.1 and H3.2 is presented as the percentage of label (*diamonds* with standard deviation error) present in histone H3.1. For comparison a *line* (with error limits as *broken lines*) is shown for the percentage of variant H3.1 that is part of total histone H3 protein.

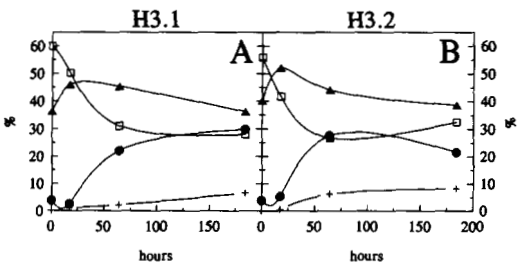

FIG. 5. **Composition of methylated lysines in histone H3 variants.** Quantitation in histone H3.1 (*panel A*) and H3.2 (*panel B*) of MML (*squares*), DML (*triangles*), TML (*circles*), and an unknown component *X* (*crosses*) by liquid scintillation counting of the HPLC eluent of the automated Applied Biosystems 420A amino acid analyzer.

amount of label that remains incorporated per stable H3.2 protein molecule is somewhat higher than for histone H3.1, resulting in a slight difference between the relative methionine labeling of stable H3 variant proteins (49.3 ± 1.8% (n = 3) as H3.1) and their mass distribution (57.7 ± 2.2% (n = 8) as H3.1) (Fig. 4*B*). Such a difference was not observed for lysine labeling (13).

To evaluate this difference the relative methylation labeling of each of the distinct acetylation states of both histone H3 variant proteins was determined by quantitative densitometry (Fig. 2, *B* and *C*). A correlation was observed between the level of histone acetylation and the extent of methylation labeling for both histone H3 variants, especially for histone H3.2 (Fig. 2*D*). Since highly acetylated histones are thought to be localized in transcriptionally active or competent and structurally less condensed chromatin (3–5), accessibility within the chromatin structure of the potential methylation sites for the methylating enzymes appears to be a major factor. Histone acetylation itself also appears to be determined to a large extent by accessibility (16). Histone acetylation with its site-specific pattern of lysine modification (2) may temporally precede and thus direct the extent and pattern of lysine methylation. These observations are fully consistent with the pattern and higher steady-state levels of lysine methylation observed in the more highly acetylated histone H3.2 variant (2).

Amino acid analysis of histone H3.1 (Fig. 3, *C–E*) and H3.2 (Fig. 3, *H–J*) protein samples obtained during continued growth revealed that the distribution between types of lysine methylation changed. Methionine labeling for 2 h incorporated 60% of the label in MML and 40% in DML. Subsequently some labeled MML appears to be converted to DML and ultimately into the increasing amounts of labeled TML (Fig. 5). This suggests a steady progression from lower to higher levels of methylation of modified lysines. A steady-state mixture of all three forms of methylated lysine was found previously in both histone H3 variant proteins (2). The notably high levels of TML in the more highly acetylated H3.2 variant are consistent with its continued greater accessibility to methylating enzymes. The time course of the continued methylation reaction appears similar for both histone H3 variants. The rate of conversion for H3.2 (Fig. 5*B*) appears somewhat higher than for H3.1 (Fig. 5*A*). The kinetics of lysine methylation are much slower than the apparent rates of histone synthesis (13) or dynamic acetylation (17).

The intermingled but mutually exclusive patterns of methylation and acetylation in the two histone H3 variants of

alfalfa suggest a functional link between these processes (2). Associations between histone acetylation and methylation have also been observed in mammalian, avian, and insect cells (6–9), but in all cases a direct link between histone methylation and gene transcription is absent (9, 18, 19).

Acknowledgment—I gratefully acknowledge W. Morgan for a critical reading of the manuscript and for frequent advice and discussions.

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