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Existence of two histone H3 variants in dicotyledonous plants and correlation between their acetylation and plant genome size

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Abstract

Histone H3 proteins were purified to near homogeneity from callus cultures of dicotyledonous plants alfalfa, soybean, *Arabidopsis*, carrot and tobacco to determine the number of histone H3 variants. In every species two histone H3 variants were identified by gradient gel electrophoresis and reversed-phase chromatography. They were named H3.1 and H3.2 in order of increasing mobility in acid-urea-Triton gels. Co-electrophoresis of histone H3.2 proteins of all species in this gel system and HPLC co-chromatography suggest that all histone H3.2 variants have a primary protein sequence identical to alfalfa H3.2. Two distinct H3.1 variant forms were identified, represented by alfalfa and *Arabidopsis* H3.1 proteins which differ only at residue 90. Soybean H3.1 resembles H3.1 of alfalfa. Carrot and tobacco H3.1 appear identical to the *Arabidopsis* H3.1 histone variant. All H3 proteins were acetylated to multiple levels and in each plant the histone H3.2 forms were more highly acetylated. An inverse relationship was observed between plant genome size and the relative abundance of histone variant H3.2 and also with the level of acetylation of both histone H3 variants. This correlation matches the general tendency that in plants with smaller genomes a larger fraction of the genome is transcriptionally active.

Introduction

Variants or isoproteins of core histones have been observed in many eukaryotic organisms but variant-specific functions have been identified in only a few cases [for reviews see 4, 34]. Histone H3 variants in mammals and birds appear to fall into two classes: H3.1 and H3.2 variants are cell cycle-regulated histones with replication-dependent synthesis; H.3 variant genes are constitutively expressed [12, 13, 18] and are presumed to function in the replacement of lost nucleosomes [4]. Our knowledge of histone H3 variants in lower eukaryotes is very fragmentary but sequence heterogeneities [6, 25, 26, 32] and gel analysis of histone proteins [1, 8, 23] generally indicate the existence of two or three H3 variant forms without providing information on differences in primary sequence, synthesis or function.

During a study of histone variants in the dicotyledonous plant alfalfa (*Medicago sativa*) two histone H3 variants have been identified [27], purified and sequenced [29, 30]. These two variants exhibited remarkable differences in histone

acetylation [27–30]. Genes for both histone H3 variants have been cloned [35]. The existence of H3 variants has been suggested for other dicot plants from protein gels of tobacco [11] and pea [11, 22], although protein heterogeneity was not detected in *Arabidopsis thaliana* [16]. In a recent study we have analyzed the histone H3 variant complexity in wheat, barley, rice and maize with results consistent with the observations reported here [31].

A. thaliana, soybean (*Glycine max*), carrot (*Daucus carota*) and tobacco (*Nicotiana tabacum*) were analyzed for the multiplicity of histone H3 variant proteins and in each dicot we detected two distinct histone H3 protein variants like in alfalfa [27, 29]. Clear differences in acetylation characterize the two types of H3 variants within each organism, and the absolute levels of modification appear to correlate inversely with the size of the plant genome.

Materials and methods

Alfalfa R4 callus and suspension cultures were maintained and used as described before [27–29]. Callus cultures of *Arabidopsis thaliana* Redei were initiated from hypocotyls of sterile seeds germinated on agar and maintained as described elsewhere [5]. Callus cultures of carrot (*Daucus carota*) and tobacco (*Nicotiana tabacum* cv. Wisconsin 38) were obtained from Carolina Biological Supply Company and cultured as instructed. Callus cultures of soybean (*Glycine max* cv. Williams 82) were cultured on Murashige and Skoog basal salt medium with 1 mg/l indoleacetic acid, 0.5 mg/l 2,4-dichlorophenoxyacetic acid and 0.3 mg/l 6-(γ, γ-dimethylallylamino)purine riboside. In all cultures rapidly growing, soft, friable calli were selected to facilitate the transfer into liquid medium for homogeneous radioactive labeling of histones. Amounts of 15–20 g callus cells in 60 ml medium were labeled *in vivo* with 1 mCi tritiated sodium acetate for 60 min, as described before for alfalfa cultures [29].

Total crude histones were prepared as described for alfalfa [30] using 1 ml BioRex-70 resin per extract from every 20 g of callus cells. Histone H3 variant proteins were purified by Zorbax Protein Plus (DuPont) high-performance liquid chromatography (HPLC) [29, 30]. Total and purified histone preparations were analyzed by acetic acid-urea-polyacrylamide gel electrophoresis with a constant concentration of 9 mM or a transverse gradient of 0 to 10 mM Triton X-100, as described previously [28, 30]. Fluorography was performed as before [27, 28].

Results

To evaluate the complexity of histone H3 variant proteins in dicotyledonous plants, *Arabidopsis*, carrot, soybean and tobacco were selected to complement our published analyses of alfalfa [27–30]. Criteria for selection included representation of various plant families, access to established tissue culture procedures, use in molecular and genetic research, the size of the plant genome, and agricultural importance. The existence and relative abundance of histone H3 variants was initially established by two complementary methods applied to histone preparations made by extraction of whole tissues culture cells with guanidine.HCl followed by BioRex-70 chromatography as described elsewhere [30].

Electrophoresis of histones in an acid-urea-polyacrylamide gel with a transverse gradient of the non-ionic detergent Triton X-100 identifies the four groups of core histones by a characteristic affinity for the detergent [12, 27], despite the presence of other, non-core histone proteins. Histone H3 proteins bind Triton more weakly than H2A species but more strongly than histones H2B or H4. Histone of all dicots tested displayed two distinct sets of histone H3 bands, as shown for tobacco in Fig. 1A. Two characteristics were observed at this stage that were confirmed and quantified after purification (Fig. 1B). The relative amount of protein in the slower H3 bands, always named H3.1, and the faster H3 bands, named H3.2, was distinct for every species. And in every species, as in alfalfa [27, 28, 30], the acetylation level of histone H3.2 appeared higher than that of the H3.1 variant.

In alfalfa a majority of tritiated acetate incor-

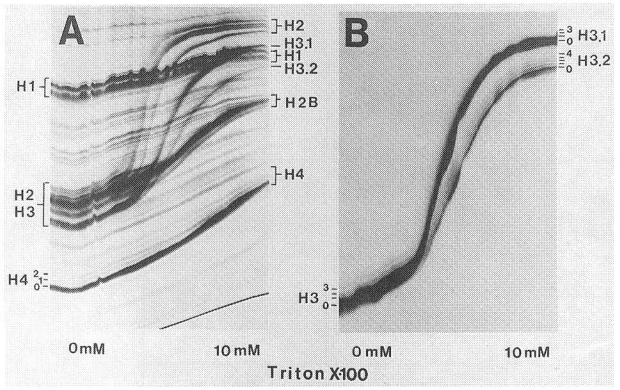

Fig. 1. Gradient gel analysis of tobacco histone H3. Electrophoresis (A) of total crude histones extracted from tobacco callus cells and (B) of histone H3 purified by reversed-phase HPLC on an acetic acid-ureagel with a transverse gradient of Triton X-100 between 0 and 10 mM. Histone species, identified by Triton affinity, are indicated with numerals denoting apparent levels of histone acetylation.

porated *in vivo* in histones is observed by fluorography of acid-urea-Triton (AUT) gels in the two histone H3 variants [28, 29]. The same was true for all other dicots and this has helped in the detection and identification of two acetylated histone H3 variants in each plant species (Fig. 2B) even in crude histone preparations (Fig. 2A). As

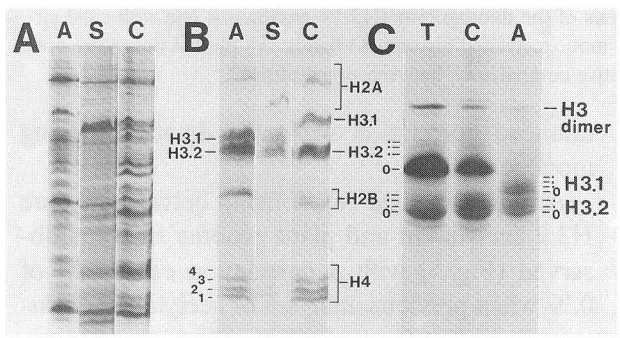

Fig. 2. Co-electrophoresis of histone H3 variants. Total histones of alfalfa (A), soybean (S) and carrot (C) callus cultures, labeled *in vivo* with radioactive acetate, were electrophoresed on AUT gels, stained for protein by Coomassie Brilliant Blue (Section A) and fluorographed (Section B). The names of all acetylated histone species are noted in section B, including mono- through tetra-acetylated histone H4. Section C shows the co-electrophoresis of HPLC purified histone H3 preparations of tobacco (T), carrot (C) and alfalfa (A) in AUT gels, stained by Coomassie. The detectable levels of histone H3 variant acetylation and a small amount of histone H3 dimer are indicated.

in alfalfa [29] more label was incorporated in H3.2 than in H3.1 bands.

The histone H3 variants of all species were purified to more than 90 percent homogeneity, as judged by AUT gel electrophoresis (Fig. 1B), by reversed-phase HPLC on a Zorbax Protein Plus column in water-acetonitrile with 0.1% trifluoracetic acid [29, 30]. The H3 elution profiles (Fig. 3, Row 1) for soybean (Fig. 3B) were very similar to alfalfa (Fig. 3A) [29, 30], and distinct from the pattern observed for histone H3 elution of *Arabidopsis* (Fig. 3C), carrot (Fig. 3D) and tobacco (Fig. 3E). In these latter species single, asymmetrical but incompletely overlapping elution profiles were obtained for absorbance and acetate label (Row 1). Determining the distribution of the two variant forms within these peaks required AUT gel electrophoresis (Row 2) and quantitation of the Coomassie staining of each variant (Row 3). Analysis of the same gels by fluorography (Row 4) and densitometry (Row 5) confirmed these variant distributions, supporting overall elution of incorporated acetate (Row 1) and showed that primarily mono- through tri-acetylated H3 variants were labeled (Row 4).

Comparison between the histone H3 variants of the various species revealed several qualitative and quantitative similarities and differences. Provided that analytical amounts of total histones were fractionated by reversed-phase HPLC, the retention of histone H3.2 from all species was identical. Histone H3.1 of *Arabidopsis*, carrot and tobacco eluted the same and failed to be separated from H3.2 variant protein during preparative chromotography (Fig. 3C–E). Histone H3.1 of alfalfa eluted as H3.1 of soybean, well resolved from H3.2 protein (Fig. 3A–B).

The same similarities could be observed upon AUT gel electrophoresis. Co-electrophoresis was observed between the H3.2 bands of all dicot species, shown for alfalfa, carrot and soybean in Fig. 2B and for tobacco, carrot and alfalfa in Fig. 2C. H3.1 of alfalfa and soybean displayed identical gel mobilities (Fig. 2B), distinct from the mobility of H3.1 prepared from carrot and tobacco (Fig. 2C) and *Arabidopsis* (results not shown).

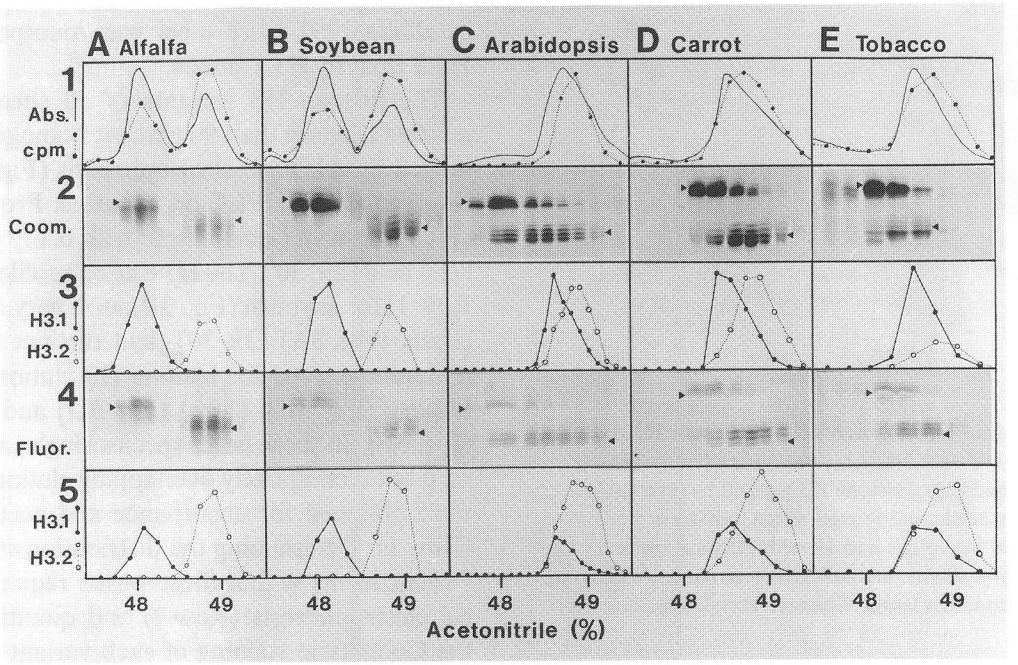

Fig. 3. Reversed-phase chromatography of histone H3 proteins. Zorbax Protein Plus reversed-phase chromatography of total crude histone preparations of (A) alfalfa, (B) soybean, (C) *Arabidopsis*, (D) carrot and (E) tobacco callus cultures, labeled *in vivo* with tritiated acetate. Displayed is a 12.5 min wide window during the chromatography at 1 ml/min and an increase in acetonitrile concentration of 10% per hour between 47.4 and 49.5% acetonitrile at 0.1% trifluoracetic acid in water. The concentrations of 48 and 49% acetonitrile in the fractions are indicated and correspond precisely to the data points in rows 1, 3 and 5. The photographs of rows 2 are aligned with row 4 but visual alignment between all five rows could not be achieved for columns C, D and E. Row 1: absorbance pattern at 214 nm (continuous line) and radioactivity per fraction, determined by liquid scintillation counting (dotted line). Row 2: Coomassie Brilliant Blue staining of collected fractions after AUT gel electrophoresis. Row 3: densitometric analysis of the stained gel lanes (H3.1 = continuous line with solid circles; H3.2 = dotted line with open circles). Row 4: fluorography of the AUT gel . Row 5: densitometric analysis of the fluorography (H3.1 = continuous line with solid circles; H3.2 = dotted line with open circles). Solid triangles indicate monoacetylated histone H3 bands. All plotted data were normalized to facilitate qualitative and quantitative comparisons between preparations.

These observations in the AUT gel system which resolves with high resolution even differences in single-charged residues or in residues that affect protein hydrophobicity and affinity for Triton X-100, suggest but do not prove (i) that the protein sequence of all histone H3.2 variants is identical to that determined for alfalfa H3.2 [30], (ii) that soybean H3.1 is identical to alfalfa H3.1 [30] and (iii) that the H3.1 variants of *Arabidopsis*, carrot and tobacco are identical but distinct from alfalfa H3.1.

Quantitative analysis showed that H3.2, the common H3 variant in all dicot species, is only a minor H3 variant in tobacco but it becomes a more prominent form in plants with smaller genomes and becomes the major variant in *Arabidopsis* (Fig. 4). A similar although less pronounced correlation was also detected among monocots [31].

An inverse linear relationship between histone H3.2 acetylation and plant genome size was observed (Fig. 5) with a correlation coefficient of 0.98 when previous analyses of acetylation of this conserved histone variant in monocots [31] are included. A similar linear correlation was observed for the other histone H3 variants which, in each plant species, contain on average 0.45 acetylated residues less per histone protein than the H3.2 variants (Fig. 5).

Discussion

This paper describes the use of reversed-phase HPCL and AUT gel electrophoresis to determine

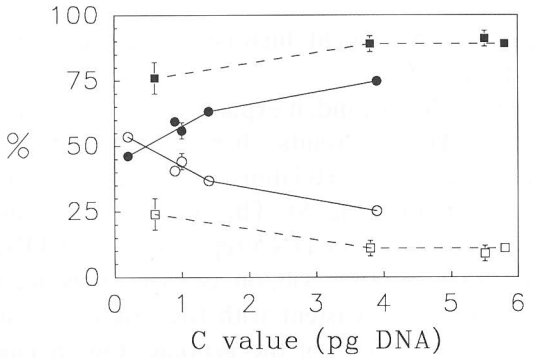

Fig. 4. Composition of histone H3 variants in plants. The relative histone H3 variant abundance (%) for dicot histone H3.2 (open circles), for the identical conserved histone variant form in monocots (open squares) [31], for dicot H3.1 (solid circles) and for the combined amount of non-conserved histone H3 forms in monocots (solid squares) [31] are plotted versus the plant genome size (C-value, expressed in pg DNA). The standard deviation (SD) is shown for species where 3 or more independent analyses were performed except for alfalfa, tobacco and wheat where the SD limits fit within the symbol used. Plant species, from left to right, are arranged in order of increasing genome size according to referenced sources: *Arabidopsis* [15], rice, soybean and carrot [2], alfalfa [2, 33], maize, tobacco, barley and wheat [2], with division of the C value for wheat to adjust for the fact that this hexaploid plant species is a co-existing mix of three distinct diploid genomes of approximately equal size [2].

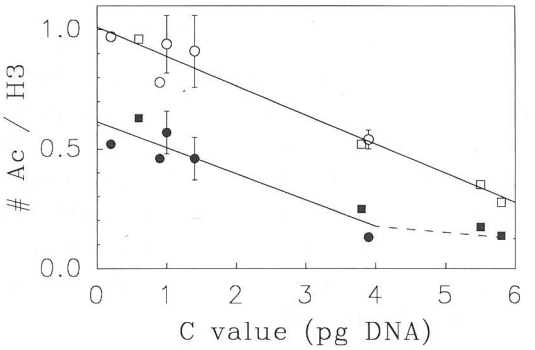

Fig. 5. Acetylation of histone H3 variants in plants. The number of acetylated residues per histone H3 molecule, as determined by AUT gel densitometry, for dicot histone H3.2 (open circles), for the identical conserved histone variant form in monocots (open squares) [31], for dicot H3.1 (solid circles) and for the combined amount of non-conserved histone H3 forms in monocots (solid squares) [31] are plotted versus the plant genome size (C value, expressed in pg DNA). The standard deviation (SD) is shown for species where 3 or more independent analyses were performed except for tobacco H3.1 where the SD limits fit within the symbol used. Last-squares linear regression calculations for all data plotted by open symbols (correlation coefficient 0.980) and for data plotted by solid symbols between 0 and 4 pg DNA C values (correlation coefficient 0.933) are shown as continuous lines. The solid symbol barley and wheat data are shown off this line although a correlation coefficient of 0.935 was obtained using all data. Plant species are arranged as described for Fig. 4.

the number of distinct histone H3 variants in several dicotyledonous plants. It predicts, although tentatively, the protein sequences of the observed plant histone H3 forms in soybean, *Arabidopsis*, carrot and tobacco on the basis of high-resolution co-electrophoresis and co-chromatography with plant histone H3 species of known sequence. The primary protein sequence of alfalfa histone variants H3.1 and H3.2 has been predicted from gene sequences [35] and has been determined directly by sequencing of the purified histone H3 variant proteins [30]. The sequence for one histone H3 protein of *Arabidopsis* has been predicted from cloned H3 gene sequences [3].

Identical gel and chromatographic mobility of histone H3.2 species of all dicotyledonous plants analyzed suggests that all have a primary protein sequence identical to that of alfalfa H3.2 [30]. A recent analysis of several monocot plant species suggests that this form of histone H3 variant exists in all plants [31].

The histone H3.1 variants in dicotyledonous plants appear to exist in two distinct forms that differ only at residue 90. Alfalfa histone H3.1 has serine at position 90 in its primary sequence [30] at which position alanine is predicted for a histone H3 of *Arabidopsis* [3], which form, by elimination of H3.2, must represent histone variant H3.1. The change of ser-90 in alfalfa to ala-90 in *Arabidopsis* predicts an increase in protein hydrophobicity. This predicted change is detected as increased retention during reversed-phase chromatography (Fig. 3) and as increased affinity for Triton X-100 and thus decreased gel mobility during AUT gel electrophoresis (Fig. 2). Soybean H3.1 behaves in both systems like alfalfa H3.1 and thus should have the same primary protein sequence as alfalfa H3.1. Similarly, the sequences of carrot H3.1 and tobacco H3.1 should be identical to that of *Arabidopsis* H3.1.

Northern analysis of the mRNA levels for the

major and minor histone H3 variants in cell cycle-synchronized alfalfa suspension cultures (*Medicago varia* cv. A2) has revealed S-phase dependence for transcription of the major histone H3 genes coding for the H3.1 variant and a rather constant level of expression of the minor H3 genes that code for the conserved H3.2 histone form [10]. Extrapolation of this observation would suggest that the dicot H3.1 variant is a replicative histone H3 variant, similar to H3.1 and H3.2 in mammals and birds, and that dicot H3.2 is a replacement histone H3 variant, similar to H3.3 in mammals and birds [4]. In animals the ratio between the two types of histone H3 variants is clearly dependent on the replicative status of the cell: H3.3 is a minor variant in rapidly cycling cells but becomes the major H3 form in terminally differentiated tissues [4, 12].

In the realization that such dependence might also exist for higher plants, we chose to use in this study only callus cultures, grown in parallel under identical conditions with similar rates of callus growth. However, we have recently compared the ratio of H3 variants and their steady-state level of acetylation in embryogenic alfalfa RA3 cells in suspension culture, in roots and in stems and leaves and have obtained results indistinguishable from alfalfa R4 callus cultures or from each other (J.H. Waterborg and D. Dudits, unpublished results). Other experiments to test directly the identification of histone H3.2 in alfalfa as a replacement histone variant are in progress.

The identification of histone H3.1 as a replicative variant and of H3.2 as a replacement variant may provide the explanation for the observed correlation between the relative levels of these two H3 forms and the size of the plant genome (Fig. 4). The rate of nucleosome loss requiring replacement appears higher on transcriptionally active sequences with passage of RNA polymerases than for inactive chromatin [9, 19]. It has been observed that in plants the genome fraction in presumably inactive heterochromatin rapidly increases with the size of the genome [17]. Thus in plants with small genomes a larger fraction of the chromatin is expected to be transcriptionally active and this would require the higher level of replacement histones observed in this study (Fig. 4).

If this idea is valid, it explains why replacement histone H3.2 variants display consistently a higher level of acetylation than the replicative H3.1 variants (Fig. 5). The replicative variants are deposited during DNA replication on all DNA sequences and thus will, on average, show acetylation levels consistent with the extent of transcriptional activity of the genome: low in large genomes like tobacco and wheat and high in small genomes like *Arabidopsis*. Replacement variant deposition preferentially on transcriptionally active DNA sequences, which are thought to be characterized by histone multi-acetylation [7, 14, 20, 21, 24], will cause consistently a higher level of acetylation. This increase in the level of H3 acetylation by, on average, 0.45 more acetylated lysine residues per H3 protein molecule, is observed, superimposed on the correlation between genome size and overall histone H3 acetylation (Fig. 5).

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