Plant Physiol. (1989) 90, 237-245
0032-0889/89/90/0237/09/$01.00/0

Received for publication October 20, 1988
and in revised form December 26, 1988

Differential Histone Acetylation in Alfalfa (*Medicago sativa*) Due to Growth in NaCl[1]

Responses in Salt Stressed and Salt Tolerant Callus Cultures

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ABSTRACT

The steady state distribution of histone variant proteins and their modifications by acetylation were characterized in wild type and salinity stress adapted alfalfa (*Medicago sativa*). Isotopic labeling detected dynamic acetylation at four sites in the histone H3 variants and five sites in histones H4 and H2B. Histone variant H3.2 was the most highly acetylated histone with 25% higher steady state acetylation and a two- to threefold higher acetylation labeling than histone H3.1. Histone phosphorylation was limited to histone variants H1.A, H1.B, and H1.C and to histone H2A.3, which was also acetylated. Histone variant composition was unaffected by cellular exposure to NaCl. Histone acetylation was qualitatively similar in salt-tolerant and salt-sensitive cells under normal growth conditions. However, short term salt stress in salt sensitive cells or continued growth at 1% NaCl in salt tolerant cells led to major increases in the multiacetylated forms of histone H4 and the two variants of histone H3. These changes were more pronounced in the diploid than in the tetraploid alfalfa strains. The increase in multiacetylation of core histones serves as an *in vivo* reporter suggesting an altered intranuclear ionic environment in the presence of salt. It may also represent an adaptive response in chromatin structure to permit chromatin function in a more saline intranuclear environment.

Understanding salt stress adaptation at the cellular and molecular level will require detailed knowledge of the intracellular responses to ionic environment and its effect on chromatin function. Especially important current questions in this regard center about the potential intranuclear environment in stressed and tolerant cells, and if so, how this might affect cellular processes. Although these questions are particularly relevant to plants in terms of future strategies for engineering salt tolerance, they are also fundamental questions in the molecular biology of gene regulation.

[1] Supported in part by funds made available through Component 2, Nevada EPSCoR program of the National Science Foundation to the University of Nevada (I. W., J. H. W.), by U.S. Public Health Service grant GM 33435 (R. E. H.), and by Hatch Projects 131 (R. E. H.) and 103 (I. W.) from the College of Agriculture, University of Nevada Reno.

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It is known from *in vitro* experiments that chromatin structure is exceedingly sensitive to changes in ionic environment (2, 22, 23 and references therein). Major salt-dependent conformational transitions have been observed in and just above physiological ionic strength that appear to be due to loosening or unfolding of the chromatin structure (23; MR Riehm, RE Harrington, unpublished data). Thus, changes in the intracellular concentration of salt and osmoprotectant species may also be expected to influence the magnitudes and balance of electrostatic and hydrophobic interactions within cellular chromatin. Altered chromatin function may be part of the reason that salt sensitive cells die when exposed to NaCl.

An important polyelectrolyte property of chromatin is the basicity of the N-terminal regions of histones. Acetylation of certain lysine residues in these regions reduces the basicity by charge neutralization. There is now abundant evidence that postsynthetic acetylation of histones is associated with chromatin activation for gene transcription in eukaryotes (1, 14, 17, 28). *In vitro* studies of chromatin in solution have shown that *in vivo* patterns of histone hyperacetylation affect both chromatin stability (6, 14, 22, 23; MR Riehm, RE Harrington, unpublished data) and the cooperative manner in which it unfolds (22). Thus, changes in histone acetylation induced by salt stress may signal a change in transcriptional activity at the chromatin level. In this manner, histone acetylation levels may also serve as unique 'reporters' on the intranuclear ionic environment under *in vivo* conditions. The appearance of histone variants that differ in hydrophobicity may also indicate that adaptive changes in chromatin function are required in salt-tolerant cells growing in salt. The function of most of the histone variants is unknown (for reviews, see refs. 10 and 32). However, it is possible that a multiplicity of histone variants may constitute a reservoir of histone forms which allow functional chromatin to be maintained in different nuclear environments. We therefore wished to investigate histone variant distributions and levels of acetylation as potential indicators of *in vivo* responses to the nuclear ionic environment and to assess their role in the salt tolerance adaptive process.

Exposure of glycophytic, salt-sensitive cells to increased concentrations of salts results in multiple biochemical changes, usually with lethal consequences. Many glycophytic plants, including alfalfa (*Medicago sativa*), do not exclude

NaCl from their cells (5, 27). Thus, wild-type alfalfa callus cultures die when exposed to 1% (0.17 M) NaCl in their growth medium. Isogenic salt tolerant cell lines have been developed in medium containing 1% NaCl (4, 18, 19). These cells exhibit a multiplicity of stable changes in nuclear and chloroplast transcription and translation patterns (31; I Winicov, JD Button, unpublished data) and do not exclude salt ions, as determined by radioactive ion flux measurements (27). A recent study on the subcellular localization of salt in tobacco cell lines similarly selected for salt tolerance suggests that the majority of the Na^+ is compartmentalized into cell vacuoles, but that it is balanced by a significant increase of as much as 50 to 100 mM in cytoplasmic Na^+, K^+, and Cl^- ions (5). In addition, concentrations of other osmotic solutes such as proline and glycinebetaine and nonionized sugars (24, 33) are known to increase in tolerance and salt stress, but it has been difficult to detect changes in subcellular ion concentrations in individual compartments such as the nucleus.

In the present study, we report a systemic characterization of histone acetylation states and variants in both wild type and salinity stress adapted alfalfa cells. We have recently shown (30) that alfalfa cell cultures display remarkably high levels of histone acetylation and contain multiple histone variant species for histones H2A, H2B, and H3 that differ in hydrophobicity as judged by Triton affinity in acetic acid-urea gels. In the present work, we report that postsynthetic acetylation and relative composition of histone variants in both diploid and tetraploid strains of alfalfa which are sensitive and tolerant for growth at 1% NaCl. Increases in histone acetylation accompany culture of alfalfa callus at 1% NaCl during short-term salt stress and during continuous growth.

While the direct cause of this response remains unclear, the changes we observe suggest that the intranuclear environment may be altered when alfalfa cells are grown in the presence of salt. This is in agreement with other studies which suggest that salt is not excluded by tolerant cells (5). Since histone acetylation levels appear to respond sensitively to the presence of salt, our results suggest that the salt stressed and tolerant cells sense and respond to an ionic shift at the nuclear level. Thus, we believe that salt stressed alfalfa may be an excellent and in many respects a unique model system for further *in vivo* studies which utilize intranuclear reporters such as the histones to probe the role of chromatin structure and other molecular genetic factors in salt adaptation at the cellular level.

MATERIALS AND METHODS

Alfalfa Culture

Cultivar strains of *Medicago sativa* were grown as callus cultures as described (30) on nutrient agar containing 0 or 1% NaCl. HG2 is a diploid strain ($2n = 2x = 16$) derived from *M. sativa* (18) from which, by selection for continued growth on agar with 0.5 and 1% NaCl, the salt tolerant line HG2-N1 was derived (4). R4 is a natural autotetraploid line ($2n = 4x = 32$) of *M. sativa* from which, by the same process, the salt tolerant cell line R4-N1 was derived.

Suspension cultures in the same nutrient growth medium were initiated by dispersal of 12 to 18 g of callus (grown for 3 to 4 weeks to a size of approximately 2 cm) into 60 mL growth medium in a 125 mL Erlenmeyer flask. Growth was continued with gentle rotary shaking for a maximum of 4 d. Such cultures were labeled for acetylation by the addition of 0.5 mL growth medium with 1 mCi sodium [U-^{3}H]acetate (ICN Radiochemicals, 6 Ci/mmol). Labeling was terminated after 60 min by the start of the nuclear isolation procedure. Cultures, grown at a reduced phosphate concentration of 5 μM, were labeled from 2 to 36 h with 1 mCi of carrier free [^{32}P]orthophosphate (New England Nuclear).

Nuclear Isolation and Histone Preparation

Aliquots of fresh callus (12–20 g) or suspension cultures (60–90 mL) were homogenized in a precooled metal Waring blender (1 L cup with polytron knives) with 250 to 350 mL homogenization medium (0.25 M sucrose, 10 mM Tris, 5 mM $MgCl_2$, 50 mM NaCl, 12 mM $Na_2S_2O_5$, 50 mM butyric acid, 0.1% [w/v] Triton X-100, adjusted to a final pH of 7.2 with NaOH to neutralize the butyric acid). Just prior to use, 1/20 volume of 50 mM PMSF in isopropanol was added. Cells were homogenized five times for 1 min at the HIGH setting, with intermittent 1 min cooling in crushed ice. The homogenate was filtered through two layers of Miracloth (Calbiochem). The retentate was rehomogenized with 200 mL homogenization medium for 1 min and filtered. The combined filtrates were centrifuged in the cold for 20 min at 2000*g*. The pellet was washed three times in 25 to 50 mL homogenization medium and recentrifuged for 12 min at 1800*g*. The resulting pellet of nuclei and starch grains was used to prepare histones as described before (30), by addition of 1 mL of 40% guanidine-HCl in 0.1 M potassium phosphate buffer (pH 6.8) and 0.005 mL of settled BioRex-70 resin (Biorad) per gram of callus homogenized. 2-Mercaptoethanol was added to 1 mM in all guanidine HCl solutions and to 0.1 mM in all dialysis solutions to minimize oxidation of histone H2B methionine residues (15).

Histone Analysis

Histones were separated by electrophoresis in discontinuous SDS and AUT[3] polyacrylamide gels as described before (30). The protocol for the AUT gel with a transverse gradient of Triton at a constant concentration of 8 M urea was modified as follows. The linear gradient generated was from 10% glycerol and 0 mM Triton X-100 to 0% glycerol and 10 mM Triton X-100. The inclusion of the glycerol at the acid-urea side of the gel (at 0 mM Triton) was found to enhance the resolution of histone species, especially of histone H3. Gels were stained with Coomassie brilliant blue R250, scanned with an EC-910 densitometer, connected through a 12-bit, 16-channel Analog-Digital converter (Applied Engineering) to an Apple IIe computer, and data were quantitated by the Scan. Graph program (available on request from J. H. W.). Radioactively labeled histone species were quantitated in the same way from autoradiographic and fluorographic exposures of preflashed x-ray film, as described before (29). Calf thymus histones (Worthington) were used as reference in SDS and AUT gels.

[3] Abbreviation: AUT, acid urea Triton.

Histone Dephosphorylation

Histones were prepared from alfalfa suspension culture cells, labeled *in vivo* for 60 min with sodium [^{3}H]acetate as described above, and solubilized in water. Histones of 100 μg were dephosphorylated with 50 units of calf intestinal phosphatase (New England Nuclear) in 100 μL containing 50 mM Tris·HCl (pH 8.0), 1 mM $ZnCl_2$, and 1 mM $MgCl_2$ for 30 min at 37°C, frozen and lyophilized prior to AUT gel electrophoresis. Control samples were incubated without phosphatase or without histones in order to localize the phosphatase enzyme in AUT gels (Fig. 2).

RESULTS

Histones of Alfalfa

A method for the preparation of nuclei from alfalfa callus and suspension cell cultures was developed which starts with the physical fragmentation of the cells. This method, rather than cell homogenization after enzymic protoplast formation, allowed effective termination of short-term radioactive labeling. Nuclear yields were estimated to be 60 to 80% of the amounts expected from DNA quantitation of callus cultures. Monovalent and divalent cations in concentrations effective to prevent chromatin solubilization were used to recover broken nuclei in the final nuclear preparation after differential centrifugation. Protease inhibitors PMSF and sodium bisulfite were used, as was the histone deacetylase inhibitor sodium butyrate at a concentration found to prevent histone acetylation turnover *in vivo* in alfalfa (JH Waterborg, unpublished data). Application of the BioRex-70 method for preparation of histones (30) to this nuclear preparation, which contained large amounts of starch grains, yielded a histone preparation which was judged to be essentially pure by acetic AUT gradient gel analysis (Fig. 1). The only major protein species that did not display an significant affinity for Triton binding, a characteristic of core histones, were the three proteins that have previously been identified as histone H1-like forms by solubility in perchloric acid and by reactivity with anti-H1 serum (30).

Identification of Posttranslational Modifications of Alfalfa Histones

Charge modifications were apparent on all major core histone species, especially on histones H3.2, H3.1, and H4. To identify histone acetylation, alfalfa suspension cultures were incubated with [^{3}H]acetate in medium without NaCl. The patterns of labeled histones in SDS and AUT gels were analyzed by fluorography. The majority of the label was incorporated in the two histone H3 variants with four apparent sites of modification each, and in histone H4 with five labeled species (Fig. 2). Label incorporation was not detectable in the least modified forms of histones H3.1 and H4, the bands with the highest AUT gel mobility within the charge modification pattern of each of the histones. This indicated that the labeling procedure employed was specific for posttranslational modifications (17, 25). Low levels of acetate incorporation were detected in AUT gels in all major and some minor H2B and H2A variant forms.

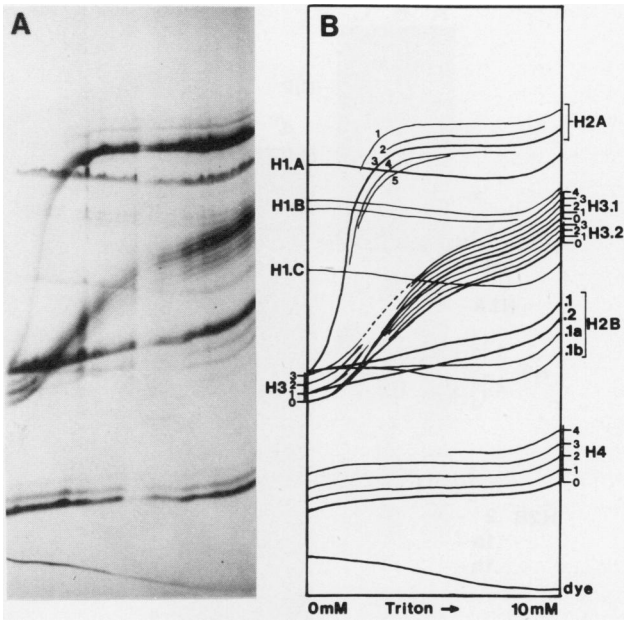

Figure 1. Triton gradient acid-urea gel analysis of alfalfa histones. Alfalfa histones were prepared from isolated nuclei of R4 calli and electrophoresed on an acetic acid gel with 8 M urea and a transverse gradient from 0 to 10 mM Triton X-100. Next to the Coomassie stained gel (A) a line drawing of detectable protein bands (B) is presented with identification numbering of histone variant forms. The pattern of all histone H3 variant protein bands could not be completely resolved between 0 and 10 mM Triton. The methylene blue marker dye position is indicated. Positive-charge modified forms of core histones H4 and of the H3 variants H3.1 and H3.2 were numbered relative to the unmodified forms (0–4). Mono- and di-oxidized forms of H2B.1 (H2B.1a and H2B.1b, respectively) were identified as described elsewhere (13).

To determine if the mobility shift in any of the acetylated histone species in AUT gels was due to additional phosphorylation events, we carried out separate analyses of [^{32}P] phosphate-labeled histone preparations. This was especially necessary for histone H4, which in all known species contains only four internal acetylated lysine residues, but is known to be modified by phosphorylation in animal cells (17, 25). SDS gel analysis of histones prepared from alfalfa suspension cultures labeled *in vivo* with [^{32}P]-phosphate showed phosphorylation of the three histone H1 species and of one or more proteins in the histone H2A-H2B band (Fig. 3A). AUT gel analysis confirmed phosphorylation of all histone H1-like species and identified H2A as modified by phosphorylation (Fig. 3B, lanes A′–C′). The phosphate labeling pattern of the histones was unchanged when labeling was extended from 10 to 24 h. Histone H2A appears to be charge modified by acetylation and by phosphorylation. No phosphate label incorporation was detected in histones H4, H2B, and H3, even in overexposed autoradiograms (Fig. 3B, lanes A″–C″). This observation suggested that all charge modifications observed in these histones were due to acetylation. This conclusion was supported by the observation that alkaline phosphatase treatment of [^{3}H]acetate-labeled histones failed to reduce the number of labeled bands of histones H4 and H2B (Fig. 2). Future analysis of the sites of acetylation in histones H4 and H2B

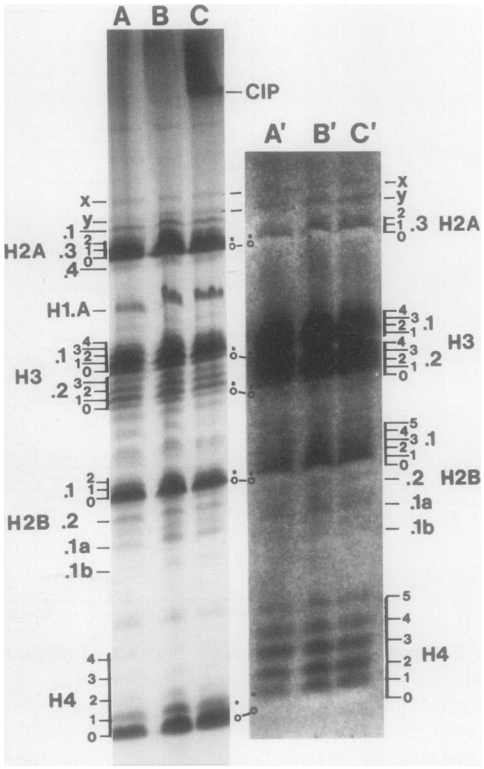

Figure 2. Dynamic acetylation of alfalfa histones. Histones were prepared from alfalfa R4 suspension cell cultures, labeled by [^{3}H] acetate in the presence (lane A) and absence (lane B) of 1% NaCl. Histones as in lane B were treated by CIP (calf intestinal phosphatase) (lane C) as described in "Materials and Methods." Lanes A to C show the Coomassie stained gel, and lanes A′–C′ the fluorograph. Individual acetylated forms could be identified as indicated for histones H3.1 (1–4), H3.2 (1–4), H2B.1 (1–5), and H4 (1–5). Resolution of H2A.2 and H2A.3 is not possible in single AUT gels. To facilitate comparison between stain (A–C) and label (A′–C′) patterns, the non- and mono-acetylated bands are indicated for histones H2A.3, H3.1, H3.2, H2B.1, and H4 by open circle and dot symbols, respectively. X and Y indicate unidentified acetylated protein species, although band Y could be identical with mono-acetylated H2A.1.

will have to show whether both histones contain five residues each which are acetylated *in vivo* and which, as in all known histones, reside in the unstructured amino terminal protein domain (17).

Histone Variants in Salt Sensitive and Salt Tolerant Lines

We wished to determine the composition of the histone variants of alfalfa in salt tolerant strains grown in the presence of salt ('Tolerant +'), and compare these results with those observed in the absence of salt in the salt sensitive strains from which the salt tolerant cell lines were derived ('Sensitive −'). The comparison of histone variant distribution (Table I) was necessary to assess the potential contribution to increased levels of histone acetylation by shifts in histone variants, especially since acetylation levels differed significantly among several variants, *e.g.* H3.1 *versus* H3.2 (30). Therefore, we analyzed the levels of histone variants in diploid HG2 and tetraploid R4 cell lines, salt adapted or salt-sensitive, in the presence or absence of salt.

To determine whether differences in histone composition associated with the tolerant phenotype required the continued presence of salt for expression, histone patterns were also analyzed from salt tolerant calli grown for 3 weeks on agar without added salt ('Tolerant −'). In addition, histones from salt sensitive strains were prepared from calli that had been exposed to 1% NaCl for 24 h to evaluate the effect of salt stress ('Sensitive +').

Quantitative histone analysis was performed in SDS gels (Fig. 4A) to measure the relative composition of the three histone H1 forms, which cannot be measured in AUT gels. The densitometric data are given in Table I. The data show a high degree of similarity in comparisons between individual preparations from independent parallel callus cultures. Quantitation of AUT gels in independent experiments was highly reproducible.

Histone variant distribution was not found to be affected by the presence of NaCl in the growth medium in diploid HG2 or in tetraploid R4 cells (Table I). The only differences observed were those between strains. Both R4 strains have a higher level of histone variants H1.C and H2A.4 and a lower level of variants H1.A and H2A.3 than any of the HG2 strains. Selection of the salt tolerant HG2-N1 strain was accompanied by increases in the level of H1.A expression from 47 to 55% and H3.2 expression from 33 to 38% at the expense of H1.C and H3.1 production, whose relative abundance decreased proportionally (Table I). Similar changes in histone variant expression were not observed when a salt tolerant strain was selected in the R4 background. However, the R4 tetraploid line showed a relatively higher proportion of histone H3.2 as compared to the diploid HG2 line.

Histone Acetylation in Response to NaCl

The steady state levels of histone acetylation in histone H4 and in the variants of histone H3 were quantitated from densitometric recordings of AUT gels (Fig. 4B). Quantitation of all acetylated species of the two histone H3 variants required resolution of the individual gaussian components in the overlapping pattern of the acetylated histone bands (16). The pattern of acetylation in histones H3 and H4 in callus cultures grown under the various conditions used for histone variant quantitation (see above) is shown in Figure 5. To facilitate comparison of the acetylation data among the independent histone preparations, we have numerically combined non- and mono-acetylated species and di- through tetra-acetylated species (Table II). The basis for this simplification is a functional one: multiacetylation of histones (Ac = 2–4) has been correlated with transcriptionally active chromatin (1, 3, 7, 14, 17, 28), while low levels of histone acetylation may have no direct effect on chromatin structure (7, 17). Insufficient gel resolution prevented quantitation of steady state levels of acetylation for histones H2A.3 and H2B.1.

Increased multiacetylation of histones H3 and H4 was observed in all cultures that were exposed to NaCl irrespective of whether this exposure was continuous or short-term. The effect was observed in salt tolerant as well as in salt sensitive strains (Table II). The magnitude of the increase in response to salt appeared higher in the diploid HG2 strains (+4–+10%) than in the tetraploid R4 strains (−1–+5%). We speculate that the magnitude of this increase may be influenced by the

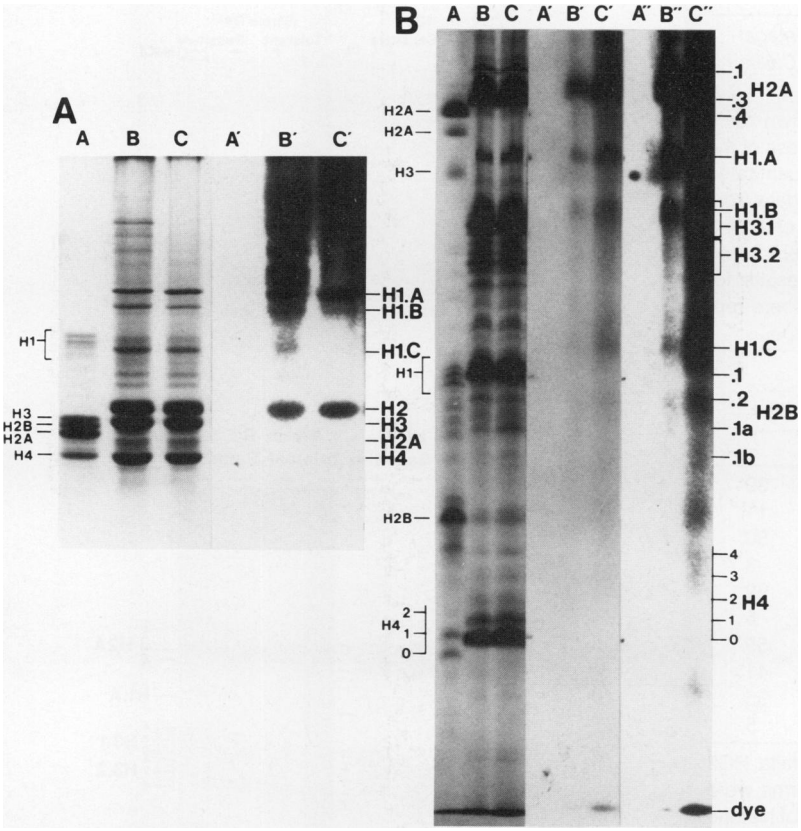

Figure 3. Phosphate labeling of alfalfa histones. Histones were prepared from alfalfa R4 suspension cell cultures, labeled for 2 h (B) and 10 h (C) with [^{32}P] phosphate, electrophoresed in SDS (panel A) and AUT gels at 8.5 mM Triton (panel B), stained by Coomassie (lanes A–C) and autoradiographed for 20 h (lanes A′–C′) or 50 h (lanes A″–C″). The AUT gel mobility of the three histone H1 forms was determined as described before (27). H1.B comigrated with tri-acetylated H3.1 in AUT gels. Calf thymus histones (lane A) were used as reference. Labeling of cultures for 36 h with [^{32}P]phosphate gave a somewhat elevated level of label incorporation relative to 10 h (C), without significant changes in the label pattern. SDS gel band H2 consists of histone H2A variant forms H2A.3 through H2A.5 and H2B.1. SDS gel band H2A contains histone H2A.1 and H2A.2 forms. Limits of resolution are as in Figure 2.

differences in genome size and the relative levels of each genome which is actually expressed.

To establish relative levels of acetylation as a function of NaCl in the growth medium, the average number of acetylated residues in both histone H3 variants and in total histone H3 and H4 was calculated (Table III) from the relative amount of histone variants (Table I) and their individual pattern of histone acetylation (Fig. 5). Salt induced a 4 to 12% increase in the average number of acetylations in the histone H3 variants in diploid HG2 strains and a 1 to 7% increase in tetraploid R4. The ratio of H3.2 to H3.1 acetylation appeared not to be affected by salt exposure in a recognizable pattern (Table III).

Salt exposure had a very marked effect on histone H4 acetylation in diploid lines. Histone H4 acetylation increased by 17 and 50% (Table III) and a major part of this increase was observed in the multiacetylated forms which rose from 10% to 14 and 16%, after exposure to salt, as shown in Table II. These increases may represent an absolute increase of chromatin with multiacetylated histone H4 molecules by 40 to 60%. This appears important since multiacetylated core histones appear to be preferentially located in transcriptionally active chromatin (7, 14, 17, 28).

DISCUSSION

Posttranslational Modifications of Alfalfa Histones

Alfalfa histone modifications have been characterized by separation and quantitation of acetate and phosphate labeled histones and their variants. Acetate labeling of alfalfa R4 histones detected 5 labeled, charge modified bands for histones H4 and H2B (Fig. 2). In all other known cases, this pattern signifies the presence of acetylation at four lysine residues in the amino terminal domain of histone H4 and H2B with an additional serine phosphorylation (11, 25). In contrast, we conclude that for alfalfa histones H4 and H2B all the charge modifications are due to acetylation. Phosphorylation of these histones was ruled out by phosphatase treatment (Fig. 2) and phosphate labeling experiments (Fig. 3). Purification of histone protein, sequence analysis, and identification of the sites of acetate labeling will be required to reveal which additional residue, presumably lysine, is modified by acetate in alfalfa histone H4 and H2B.

Acetate labeling of the variants of histone H3 confirmed the difference in modification between the two variant forms, which has been reported before (30). The relative level of steady state acetylation of H3.2 versus H3.1 ranged from 1.21 to 1.42 in the HG2 strains and from 1.23 to 1.27 in the R4 strains (Table III). Acetate labeling *in vivo* of R4 cells in which H3.2 represents 40% of the H3 protein (Table I), further demonstrated this difference. With 60% of the specific acetate label incorporation in H3.2, the relative steady state acetylation level was two to three times higher than that found in H3.1. Since the presence of dynamically multiacetylated histones have been shown to be a feature of chromatin transcriptional activation (7, 17, 30), the possibility that histone H3.2 is located in transcriptionally active alfalfa chromatin is intriguing. Further examination of this possibility is now in progress.

Phosphate labeling of alfalfa R4 suspension cultures re-

Table I. *Relative Composition of Histone Variants as Percentage Values of Individual Histone Classes in HG2 and R4 Alfalfa Callus Cultures*

All histone preparations were obtained from callus grown for 3 weeks with and without 1% NaCl, except in case of salt stress of the salt sensitive callus at 1% NaCl for 24 h. H1 data were quantitated from SDS gels, as shown in Figure 4A, and core histone data from AUT gels, as shown in Figure 4B. The relative amount of H2A.3 represents the combined amounts of the major variant H2A.3 and the unresolved minor variant H2A.2, and similarly the results for H2A.4 include those of unresolved variant H2A.5. All numbers represent the percent values of variants within each histone type.

Histone	Strain HG2				Strain R4			
	Tolerant		Sensitive		Tolerant		Sensitive	
	−[a]	+[b]	−	+	−	+	−	+
H1.A	55	54	47	47	40	35	35	39
H1.B	14	14	12	15	9	13	12	11
H1.C	31	32	41	38	51	52	53	50
H2A.1	3	4	3	4	4	4	4	3
.3	97	96	97	96	93	92	92	92
.4	[c]	[c]	[c]	[c]	4	4	4	5
H3.1	62	63	67	68	59	58	60	59
.2	38	37	33	32	41	42	40	41
H2B.1[d]	95	94	93	92	94	95	95	95
.2	5	6	7	8	6	5	5	5

[a] Without 1% NaCl. [b] With 1% NaCl. [c] In the alfalfa HG2 and HG2-N1 strains, histone H2A.4 and H2A.5 variant forms were present as traces totaling less than 1%. [d] The amounts of histone H2B.1 present in the oxidized forms H2B.1a and H2B.1b were combined with the values obtained from band H2B.1 (Fig. 4B).

vealed phosphorylation of histones H1 and H2A and of a large number of nonhistone proteins which are present in our histone preparation as low percentage contaminants (Fig. 3). High levels of nonhistone phosphorylation have been observed previously in other plant species (8). Histone phosphorylation was detected in the three protein bands that were previously tentatively identified as H1 by anti-H1° serum and by solubility in 5% perchloric acid (30). The phosphate labeling of these bands supports their previous assignment as histone H1 variants.

In the comparison of short-term acetate incorporation between histones H2A.3 and H2B.1, which showed similar levels of modified protein in Coomassie blue stained AUT gels, H2A.3 showed less labeling (Fig. 2). The constant ratio of labeling with time for these two histones (results not shown) suggests that the observed modification of histone H2A.3 was only partly due to acetylation. This idea was further supported by the observation that H2A.3 was the major histone phosphate acceptor (Fig. 3). However, little or no reduction in the charge-modification level of H2A.3 was observed after incubation with calf intestinal phosphatase as measured in Coomassie blue stained AUT gels (Fig. 2). Further investigations will be required to determine the relative contribution of acetylation and phosphorylation to the modifications of histone H2A.3.

Histone Variant Expression in Alfalfa Strains

The pattern of histone variant expression in callus cultures of tetraploid strains of *Medicago sativa* (R4) was qualitatively

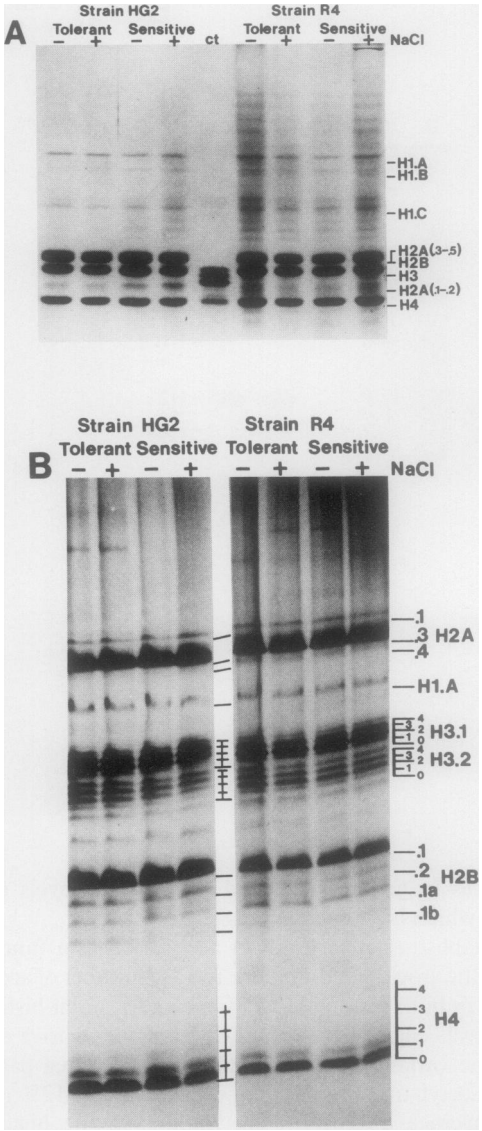

Figure 4. Comparison of histone modifications and variants. Histones were prepared as described in "Materials and Methods" from diploid HG2 and tetraploid R4 calli grown for 3 weeks on medium containing 1% (+) or 0% (−) NaCl, except that the salt sensitive strains indicated by (+) were grown without NaCl for 3 weeks and subsequently salt stressed for 24 h on medium containing 1% NaCl. Histones were electrophoresed in SDS gels with calf thymus histone markers (ct) (panel A) and in AUT gels (panel B), stained with Coomassie blue and quantitated by densitometry.

similar to that seen in the diploid *M. sativa* HG2 strains. The quantitative differences in histone variant composition between HG2 and R4 strains may have arisen during selection for salt tolerance by somaclonal variation. These differences are most likely not an essential part of development of salt tolerance in alfalfa because selection of the salt tolerant R4-N1 strain from R4 did not show any major changes in histone variant expression. However, the relative increase in histone H3.2 in the salt tolerant HG2-N1 cells leads to a constitutive increase in the level of H3 acetylation since this is the variant which is most highly acetylated. Thus, if increased levels of

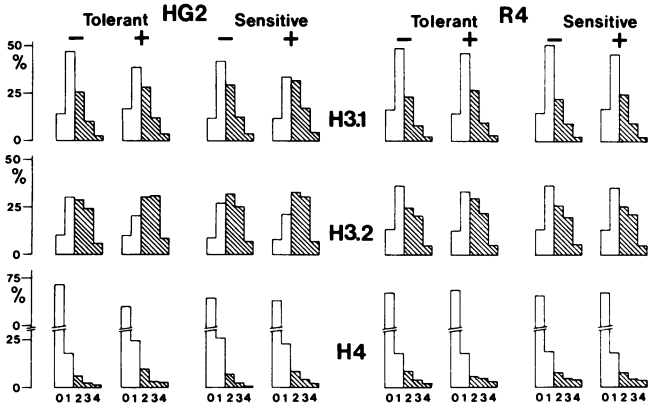

Figure 5. Identification of changes in steady state acetylation of histones H3.1, H3.2, and H4 by NaCl in the growth medium. Histogram representation of the relative percentage on individual acetylated species of histones H3.1, H3.2 and H4 obtained from densitometric registration of histone preparations electrophoresed on AUT gels as shown in Figure 4B. Numbers 0 through 4 indicate the level of histone modification. The multiacetylated forms are cross-hatched.

acetylation are important for the functional state of chromatin in salt exposed cells, the increase in the H3.2 variant in the salt tolerant HG2-N1 line will have a similar importance.

The expression pattern of histone variants appears unaffected by continuous or short-term exposure of callus cultures to salt. Comparison of the histone variant amounts observed in the absence and presence of salt (Table I) illustrates the accuracy of the histone variant determinations. The relative amounts of bands H1.A, H1.B, and H1.C were determined in several SDS gels for all strains and were found to be constant in each cell line, as shown in Table I. In a previous study we were unable to exclude the possibility that the smaller H1 bands, H1.B and H1.C, could have been derived proteolytically from the larger forms H1.A and H1.B (30). Thus, our observation that the ratio among the three bands was constant in each strain under different experimental conditions supports the idea that histone H1.A, H1.B, and H1.C variant proteins are indeed independent.

Salt Dependent Changes in Histone Acetylation in Alfalfa

Exposure of all alfalfa callus strains to 1% NaCl resulted in significant increases in acetylation levels for core histones H3 and H4 (Fig. 5; Tables II and III). This result was less marked in the tetraploid cell line. A contributing factor here may be the already high level of histone variant H3.2 in the tetraploid R4 strains (Table I). The H3.2 variant was acetylated to the highest extent of all alfalfa histones and was found to be increased during the selection of the tolerant diploid HG2-N1 line. Thus, similar salt induced increases in histone acetylation would have a larger effect in the HG2-N1 line. It is important to note that the response to salt was obtained both from salt sensitive and salt tolerant lines. Therefore, core histone acetylation levels can indeed serve as *in vivo* intranuclear 'reporters' for detection of environmental responses in chromatin, even after short-term exposure of alfalfa callus to salt. This is likely to be due to incomplete compartmentalization of salt ions in the vacuoles, even in the salt tolerant strains. Entry of salt ions into the cytoplasmic compartment of plant cells with partial accumulation into vacuoles has been shown previously (5), but the present results provide the first evidence for salinity effects in the nuclear environment.

It is not clear from our results whether increased histone acetylation represents an immediate, direct response of the cell to increased ion concentrations in the intranuclear environment or an indirect result of more complex changes induced by the salt. However, the observed increase in multiacetylated histone H4 by 40 to 60% (Table III) is likely to exert significant influence on chromatin regions which con-

Table II. *Effects of NaCl on the Relative Amounts (%) of Low and High Levels of Histone Acetylation*

The percent distribution of acetylated forms of histones H3.1, H3.2, and H4 was obtained from Coomassie stained AUT gel scans. The relative amounts of histones with 0–1 acetylated residues and of multiacetylated forms with 2–4 acetylated residues were calculated from data as shown in Figure 5. In most AUT gels the individual gaussian component of tetra-acetylated histone H3.2 could be resolved from the nonacetylated form of H3.1 and quantitated. The values in brackets represent the differences in the multiacetylated bands observed in 1% NaCl grown cultures relative to the amount measured in cultures grown in the absence of NaCl.

Histone	No. of Acetates	Strain HG2						Strain R4					
		Tolerant			Sensitive			Tolerant			Sensitive		
		–[a]	+[b]	Change by NaCl	–	+	Change by NaCl	–	+	Change by NaCl	–	+	Change by NaCl
				%			%			%			%
H3.1	0–1	61	56		54	46		66	61		66	63	
	2–4	39	44	(+5)	46	54	(+8)	34	39	(+5)	34	37	(+3)
H3.2	0–1	41	31		36	30		50	46		49	48	
	2–4	59	69	(+10)	64	70	(+6)	50	54	(+4)	51	52	(+1)
H4[c]	0–1	90	84		90	86		85	86		85	85	
	2–4	10	16	(+6)	10	14	(+4)	15	14	(−1)	15	15	(+0)

[a] Without 1% NaCl. [b] With 1% NaCl. [c] Penta-acetylated histone H4, which could be identified in acetate labeling patterns (Fig. 2), could not be measured reproducibly in Coomassie stained AUT gels.

Table III. *Effects of NaCl on the Average Number of Acetylated Residues per Histone Molecule*

The average number of acetylated residues in histone H3.1, H3.2, and H4 molecules was calculated from the steady state pattern of histone acetylation as shown in Figure 5. The percentage differences were calculated from the original peak areas in the densitometric registration of the pattern of the histones prepared from callus grown at 1% NaCl, relative to the values obtained at 0% NaCl. The ratio between the levels of acetylation of H3.2 and H3.1 is given in brackets. The average acetylation level for total histone H3 was calculated from the average number of acetylation sites of each of the histone H3 variant forms shown in this table and the relative amounts of the variant forms (Table I).

Histone	Strain HG2						Strain R4					
	Tolerant			Sensitive			Tolerant			Sensitive		
	−[a]	+[b]	Change by NaCl	−	+	Change by NaCl	−	+	Change by NaCl	−	+	Change by NaCl
			%			%			%			%
H3.1	1.40	1.46	+4	1.53	1.69	+10	1.31	1.40	+7	1.33	1.34	+1
H3.2	1.85	2.07	+12	1.95	2.05	+6	1.67	1.43	+3	1.69	1.71	+1
Ratio of $\frac{Ac(H3.2)}{Ac(H3.1)}$	[1.32	1.42]		[1.27	1.21]		[1.27	1.23]		[1.27	1.27]	
H3	1.57	1.68	+7	1.67	1.81	+8	1.46	1.54	+5	1.48	1.49	+1
H4	0.44	0.66	+50	0.51	0.59	+17	0.56	0.57	+3	0.62	0.59	−4

[a] Without 1% NaCl. [b] With 1% NaCl.

tain these histones. Increased histone hyperacetylation could result indirectly from changes in gene expression in response to the salt stimulus. Such changes in gene expression have been documented for many plants including alfalfa (12, 19, 26). An increase in chromatin activation might result in increased histone multiacetylation as one of the required elements in such a response toward increased levels of gene transcription (17). The question whether the changes in gene expression in alfalfa induced by salt are consistent with the significant increases that we have observed in histone multiacetylation, particularly in the diploid strains (Table II), cannot be answered at this time.

Changes in the ionic nuclear environment could also lead directly to changes in histone acetylation if such acetylation compensates for salt-induced alterations in chromatin structure or the ability of chromatin to unfold during transcription and replication. Multiacetylation reduces the level of positive histone charges and is known to correlate with actively transcribing chromatin (3, 6, 7, 9, 14, 17, 28). It has also been shown to relax interactions between histones and DNA in chromatin, leading to a more localized or less cooperative unfolding transition (22) but with no gross conformational changes (3, 13, 20, 21, 22 and references therein). On the other hand, recent *in vitro* studies of salt effects upon chromatin structure and structural stability in quasi-intracellular, polyacrylamide sol diffusion limiting matrices have evidenced a complex interplay of electrostatic and hydrophobic forces on chromatin structure under physiological and higher salt conditions (23; MR Riehm, RE Harrington, unpublished data). It is possible, therefore, that increased counterion screening of negative charges on the DNA phosphate groups under higher intranuclear salt conditions might be compensated by a reduction in positive charges on histones by increased acetylation. In this scenario, the nucleus can maintain localized electrostatic and hydrophobic balance by responding to increased intranuclear salt through reduction of histone basicity.

The concept of chromatin structure as a cellular component highly sensitive to ionic environment has been well documented by *in vitro* experimental data (2, 22, 23 and references therein). The experimental results reported here indicate for the first time that such sensitivity can be also detected *in vivo*. The hyperacetylation of alfalfa core histones which we have characterized in response to increased salt concentrations in the cellular environment may represent an intranuclear response by which chromatin structure constraints are overcome to enable continued function in an ionically altered cellular environment.

ACKNOWLEDGMENT

The authors gratefully acknowledge useful discussions with Dr. M. R. Riehm.

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