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Identification of Five Sites of Acetylation in Alfalfa Histone H4†

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ABSTRACT: Radioactive acetylation in vivo of plant histone H4 of alfalfa, *Arabidopsis*, tobacco, and carrot revealed five distinct forms of radioactive, acetylated histone. In histone H4 of eukaryotes ranging from fungi to man, acetylation is restricted to four lysines (residues 5, 8, 12, and 16) possibly caused by a quantitative methylation of lysine-20. Chemical and proteolytic fragmentation of the amino terminally blocked alfalfa H4 protein, dynamically acetylated by radioactive acetate in vivo, allowed protein sequencing and identification of selected peptides. Peptide identification was facilitated by analyzing fully characterized calf histone H4 in parallel. Acetylation in vivo of alfalfa histone H4 was restricted to the lysines in the amino-terminal domain of the protein, residues 1–23. Lysine-20 was shown to be free of methylation, as in pea histone H4. This apparently makes lysine-20 accessible as a novel target for histone acetylation. The in vivo pattern of lysine acetylation ($16 > 12 > 8 \geq 5 = 20$) revealed a preference for lysines-16 and -12 without an apparent strict sequential specificity of acetylation.

Core histone acetylation has been suggested to allow transient displacement of histones from DNA by polymerases during gene transcription and DNA duplication and permanent displacement by protamines during spermatogenesis [for recent reviews, see Van Holde (1989), Grunstein (1990), and Csordas (1990)]. Multiacetylated core histones H3 and H4 appear the functional forms for these processes (Van Holde, 1989; Delcuve & Davie, 1989; Boffa et al., 1990; Walker et al., 1990). In both histones, the amino acid sequence of the histone amino-terminal protein domain with the multiple lysine residues which can be acetylated in vivo is highly conserved (Matthews & Waterborg, 1985). Experiments in yeast (Megee et al., 1990; Grunstein, 1990; Durrin et al., 1991), *Physarum* (Pesis & Matthews, 1986), *Tetrahymena* (Chicoine et al., 1986), *Drosophila* (Munks et al., 1991), and animal cells (Couppez et al., 1987; Turner & Fellows, 1989; Thorne et al., 1990) have revealed functional differences between lysine residues, a nonrandom order of lysine acetylation, and a nonrandom distribution of steady-state acetylation patterns in histone H4. Nonrandom distributions of lysine acetylation and methylation have recently been described for two distinct histone H3 variants in alfalfa (Waterborg, 1990). This paper presents the pattern of acetylation of histone H4 in alfalfa.

The highest level of histone H4 acetylation observed in animals and lower eukaryotes is four, restricted to lysines-5, -8, -12, and -16 in the amino-terminal domain of histone H4 (Matthews & Waterborg, 1985; Van Holde, 1989; Thorne et al., 1990). Methylation of lysine-20 in lower eukaryotes like *Physarum* (Waterborg et al., 1983) and in animals ranging from sea urchins and fishes to birds and mammals (Van Holde, 1989; Duerre & Buttz, 1990) precludes modification by acetylation. In contrast, when alfalfa cells were labeled in vivo with acetate, five radioactive, charge-modified forms of histone H4 were observed when histone H4 was fractionated in acid/urea/Triton (AUT)[1] gels (Waterborg et al., 1989, 1990). In vivo treatment of alfalfa cells with [^{32}P]phosphate failed to label histone H4 (Waterborg et al., 1989), suggesting that phosphorylation (Ruiz-Carrillo et al., 1975) is not involved. This paper describes experiments that identify lysine-20 of

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[1] AUT, acid/urea/Triton; BAP, 6-benzylaminopurine; BSA, bovine serum albumin fraction V; PTH, phenylthiohydantoin; TEMED, *N,N,N',N'*-tetramethylethylenediamine; TFA, trifluoroacetic acid; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; 2,4D, 2,4-dichlorophenoxyacetic acid.

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alfalfa histone H4 as a novel site of histone acetylation.

Materials and Methods

Preparation of Plant Histones. Callus cultures of *Arabidopsis thaliana* Redei, alfalfa (*Medicago sativa*) cultivar strain R4, carrot (*Daucus carota*), soybean (*Glycine max*) cv Williams 82, and tobacco (*Nicotiana tabacum*) cv Wisconsin 38 were initiated and maintained as described before (Waterborg, 1992). Alfalfa (*Medicago varia*) cv Rambler suspension cultures named A2 were obtained from D. Dudits (Szeged, Hungary) and maintained on MS medium (Murashige & Skoog, 1962) at 1 mg/mL 2,4D and 0.2 mg/mL BAP.

Radioactive acetate labeling in vivo of callus cultures was performed under shaking for 60 min at 25 °C with 1 mCi of high specific activity [^{3}H]acetate (ICN or NEN) per 15–20 g of callus fragments suspended in 60 mL of callus culture growth medium. Cells from log-phase alfalfa A2 suspension cultures were allowed to settle and were resuspended to a density of 10 mL of cell pellet per 20-mL total volume of growth medium. Per culture (approximately 2×10^8 cells), 1 mCi of tritiated acetate was added, and cell culture was continued for 60 min at 25 °C on a rotary shaker.

Histone was extracted from up to 60 g of callus fresh weight, solubilized, and fractionated by reversed-phase hplc on a Zorbax Protein Plus (4.6 × 250 mm) reversed-phase hplc column as described before (Waterborg, 1990) using a gradient of 96 min between 39 and 55% acetonitrile in water at 0.1% TFA and 1 mL/min. Histone H4 protein was localized in the eluate by the absorbance at 214 nm and by AUT gel electrophoresis of aliquots of lyophilized eluate fractions. Quantitative densitometry of Coomassie-stained gels, fluorography, and quantitation of properly exposed fluorographs were performed as described before (Waterborg et al., 1989). The relative specific radioactivities of the plant histone H4's in Figure 1 most likely reflect differences in label accessibility caused by differences in callus morphology rather than plant species-specific differences.

Purification of Histone H4 of Alfalfa and Calf Thymus. Alfalfa A2 cells (3×10^9 cells) were labeled with 17.5 mCi of tritiated acetate as described above and collected by centrifugation for 4 min at 150*g*. The cell pellet was homogenized with 2 volumes of 40% (w/v) guanidine hydrochloride, 0.1 M potassium phosphate, pH 6.8, and 2 mM 2-mercaptoethanol, clarified, and batchwise-treated with 2.1 mL of BioRex-70 resin as described before (Waterborg, 1990). The total histone extract was dialyzed exhaustively against solution A (2.5% acetic acid with 1.5 mM 2-mercaptoethanol), lyophilized, and solubilized at 10 mg/mL in solution B (7.2 M urea, 50 mM dithiothreitol, and 1 M acetic acid), as described before (Waterborg, 1990). Histones were fractionated by reversed-phase hplc on a Vydac large-pore C4 column (10 × 250 mm) at 5 mL/min by a 96-min gradient of 35–51% acetonitrile in water at 0.1% TFA. Chromatography was monitored by the absorbance at 214 nm and by liquid scintillation counting of 20 μL per 5-mL fraction. Fractions containing histone H4 together with histone H2A and some other minor proteins were identified by AUT gel electrophoresis and radioactivity, pooled, and lyophilized.

Crude histone H4 (5×10^6 cpm) was solubilized overnight in 0.55 mL of solution C (8 M urea, 20 mM HCl, and 1% 2-mercaptoethanol) and fractionated in solution D (20 mM HCl, 50 mM NaCl, and 0.02% sodium azide) over BioGel P-60 (1.0 × 49 cm) at 0.25 mL/min, monitored by the absorbance at 230 nm (Von Holt & Brandt, 1977; Waterborg, 1990), liquid scintillation counting, and AUT gel electrophoresis. Fractions with pure histone H4 were pooled and dialyzed exhaustively against solution A. Approximately 1.1 mg of pure alfalfa histone H4 with 3.2×10^6 cpm was obtained, as estimated by Bio-Rad protein assay kit II, relative to BSA (bovine serum albumin, fraction V).

Calf thymus histones (200 mg of HLY, Worthington) were solubilized overnight at 50 mg/mL in solution C and fractionated in solution D over BioGel P-60 (2.5 × 100 cm) at 30 mL/h, monitored by the absorbance at 275 nm (Von Holt & Brandt, 1977; Waterborg, 1990). Aliquots of fractions were dialyzed into solution A, lyophilized, and analyzed by AUT gel electrophoresis. Fractions containing histone H4 were pooled, dialyzed, lyophilized, and solubilized at 50 mg/mL in solution B (Waterborg, 1990). Histone H4 (10 mg) was separated from low molecular weight protein material by reversed-phase hplc on a Vydac large-pore C4 column (10 × 250 mm) at 5 mL/min by a 30-min gradient of 38–43% acetonitrile in water at 0.1% TFA. Chromatography was monitored by the absorbance at 214 nm. Fractions containing pure histone H4 were identified by gel electrophoresis of lyophilized aliquots, pooled, lyophilized, and resolubilized in solution A. The concentration of essentially pure histone H4, as judged by AUT and SDS gel electrophoresis (Waterborg, 1990), was determined by Bio-Rad protein assay kit II, relative to BSA.

Modification of Histone H4. Histone H4 was exhaustively chemically acetylated with acetic anhydride. Histone H4 (1 mg) was solubilized in 1 mL of solution E (6 M guanidine hydrochloride and 0.2 M $NaHCO_3$, pH 8.7) in a polypropylene tube with a magnetic stirrer. Aliquots of 0.02 mL of a fresh solution of 0.5 M acetic anhydride in solution E were added with constant stirring, and measurement of pH until a final concentration of 50 mM acetic anhydride was reached. Subsequently, 0.002-mL aliquots of pure acetic anhydride were added to a final concentration of 100 mM. Multiple aliquots of 0.05 mL of 0.5 M Na_2CO_3 were added during these additions to maintain the pH between 8.5 and 8.8 during the total reaction time of approximately 30 min at room temperature. The reaction mixture was exhaustively dialyzed against solution A. All peptides derived from chemically acetylated histone H4 were named by the letter that identified the peptide followed by the ⟨ac⟩ suffix. This does not imply that every lysine residue in ⟨ac⟩ peptides is necessarily completely acetylated.

Acid Fragmentation of Histone H4. Aliquots of approximately 0.4 mg of purified histone H4, untreated or chemically acetylated with acetic anhydride, in solution A were placed into 1-mL Reactivials (Pierce), lyophilized, and resolubilized to a concentration of 0.5 mg of protein/mL into 0.25 N acetic acid which had been extensively purged with nitrogen. Vials were capped with a Mininert Teflon valve (Pierce), frozen, and evacuated, upon which time the valve was closed. Hydrolysis was for 40 h at 105 °C in a Reactitherm (Pierce) incubation block, covered with a polystyrene insulation cap. The extent of hydrolysis was determined by reversed-phase hplc and by peptide gel electrophoresis in a 15% polyacrylamide gel in 0.9 M acetic acid and 2.5 M urea and staining by Coomassie Brilliant Blue. The four main fragments of calf histone H4, which were identified in previous studies, were named by capital letters A, B, C, and D, in amino- to carboxy-terminal order. Equivalent fragments of alfalfa and pea histone H4 were named by the same capital letter nomenclature with the addition of an apostrophe sign. Fragmentation of histone H4 was essentially quantitative at H4 concentrations of 0.25–0.75 mg/mL when standardized on BSA in the Bio-Rad protein assay. Incomplete hydrolysis was observed at higher protein concentrations. Irreproducible results and generation of additional fragments were observed when oxygen

Table I: Quantitation of Radioactive Acetylation of Histone H4 from Several Plant Species[a]

	histone H4 AUT gel analysis				hplc H4 peptide A'
	Arabidopsis (0.2)	carrot (1.0)	alfalfa R4 (1.4)	tobacco (3.9)	alfalfa A2
(A) Coomassie (%)					(D) Coomassie (%)
Ac = 0	47.0	59.4	66.1	88.7	54
Ac = 1	33.0	27.6	21.1	8.7	34
Ac = 2	13.0	9.7	8.0	1.9	12
Ac = 3	4.3	2.6	3.6	0.5	-
Ac = 4	1.4	0.6	1.2	0.2	-
Ac = 5	1.3	-	-	-	-
Ac/H4	0.84	0.79	0.53	0.13	
(B) fluorography (%)					(E) cpm (%)
Ac = 0	-	-	-	-	4.5
Ac = 1	16.4	30.8	24.6	33.9	23.6
Ac = 2	32.9	35.4	29.1	28.9	28.0
Ac = 3	28.1	24.8	24.4	25.7	26.8
Ac = 4	16.3	6.8	14.5	8.9	11.7
Ac = 5	6.3	2.0	7.5	2.6	5.4
*Ac/H4	2.63	2.13	2.51	2.17	2.34
(C) standardized Ac label per acetylated lysine					
Ac = 1	1.0	1.0	1.0	1.0	
Ac = 2	2.6	1.6	1.6	2.0	
Ac = 3	4.4	2.9	2.0	4.5	
Ac = 4	5.9	2.6	2.5	3.2	
Ac = 5	1.9	-	-	-	

[a] Histone H4 was purified from acetate-labeled callus cultures of plants with known nuclear genome size, expressed as picograms of DNA (*C* value, in parentheses) (Waterborg, 1992), fractionated by reversed-phase hplc, electrophoresed in AUT gels, stained with Coomassie, fluorographed (Figure 1), and quantitated. (-) Not detectable. The relative distribution (percent) of stained protein (A) and radioactivity (B) and the specific radioactivity, calculated as the average per acetylated lysine residue and standardized on the monoacetylated form of histone H4 (C). The calculated Ac/H4 value is an averaged value for the number of acetylated lysine residues per histone H4 molecule. An equivalent calculation, expressed as *Ac/H4, was calculated from the fluorographic data. This value gives a relative, standardized value for radioactive acetate incorporation in histone H4 molecules and may allow assessment of the level of histone H4 modification at which dynamic acetylation occurs. The relative distribution (percent) of alfalfa peptide A' (D) could not be determined accurately from the hplc absorbance profile (Figure 3B) due to contaminating peptide fragments. It was determined at a reduced accuracy without quantitation of forms with more than two acetylated lysines by densitometry of the Coomassie-stained acid/urea peptide (results not shown). The radioactivity associated with the peptides (E) was determined directly by liquid scintillation counting (Figure 3C) of the hplc eluent.

was incompletely removed. H4 fragments were recovered by lyophilization.

Proteolytic Fragmentation of Histone H4 Peptides. Histone H4 fragments, purified by reversed-phase hplc, were dissolved at a concentration of 1–2 mg/mL in solution F (0.1 M ammonium bicarbonate, pH 8.0). Digestion by TPCK-treated trypsin (Worthington) was for 16 h at 37 °C at an enzyme:substrate ratio of 1:100. Lower case single letters were used to identify the predicted tryptic peptides produced from fully chemically acetylated calf histone H4 fragments. The equivalent plant histone H4 peptides were again designated by the apostrophe addition.

Analysis of Peptides. Acid hydrolysates were lyophilized and solubilized in water with 0.1% TFA. Protein fragments were fractionated by reversed-phase hplc (4.6 × 250 mm column of C18 on small-pore silica, Vydac) during gradient elution of 70 min between 0 and 70% acetonitrile in water at 0.1% TFA and 0.5 mL/min, monitored by the absorbance at 214 nm. Aliquots of 20 μL were taken from 0.25-mL fractions for analysis by liquid scintillation counting.

Proteolytic digests were injected directly onto the Vydac C18 small-pore reversed-phase column. Digests of fragments A and A' from acetylated and nonacetylated calf and alfalfa histones H4 were developed at 0.5 mL/min by a 40-min gradient of 0–20% acetonitrile in water at 0.1% TFA with absorbance detection at 214 nm. Digests of all other fragments were developed at 0.5 mL/min by a 40-min gradient of 0–20% acetonitrile followed by a 50-min gradient from 20 to 70% acetonitrile in water at 0.1% TFA.

In vivo acetate-labeled (120000 cpm), chemically acetylated alfalfa peptide b'⟨ac⟩ was subjected to standard automated protein sequencing on an Applied Biosystems 477A/120 pulsed-liquid-phase sequencer, and radioactivity in the on-line hplc eluent was determined by liquid scintillation counting of 5-min fractions. Distribution of in vivo acetylation at lysine residues 5, 8, 12, and 16 were determined by the radioactivity specifically associated with PTH-acetyllysine (Waterborg, 1990), excluding low amounts of radioactivity eluting earlier in each hplc run caused by peptide washout from the Porton peptide support disk. Initial yields of glycine and acetylated lysine were approximately 3 nmol. Repetitive yields calculated from glycine and acetylated lysine were in excess of 97%. Recovery of radioactivity in acetylated lysine was 80000 cpm, without correction for material that was not injected onto the on-line hplc.

Selected peptides were sized with a VesTec 210 electrospray mass spectrometer at the Core Facility of the Medical Center of Louisiana State University in New Orleans.

Results

Plant Histone H4 Has Five Sites of Acetylation. When alfalfa callus cultures are incubated with tritiated acetate, radioactive label becomes rapidly incorporated in core histones, specifically in the forms which are postsynthetically modified by acetylation of lysines (Waterborg et al., 1990) and which are detected by their characteristic stepwise reduction in electrophoretic mobility in AUT gel electrophoresis as levels of acetylation increase (Figure 1). Only a fraction of histone H4 appears involved in this dynamic modification as high incorporation of label is observed in faint or undetectable bands of multiacetylated histone H4 (Table I). The number of labeled, charge-modified forms of histone H4 in alfalfa was previously reported as five (Waterborg et al., 1989, 1990), a value confirmed in the fluorograph overexposure of Figure 1G.

Acetate labeling of *Arabidopsis* (Figure 1C), carrot (Figure 1E), tobacco (Figure 1I), and soybean (results not shown)

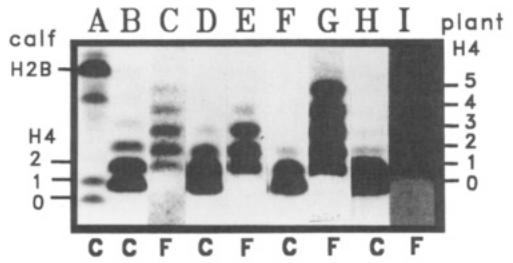

FIGURE 1: AUT gel analysis of acetylated histone H4 of plants. From left to right, Coomassie-stained (C) and fluorographed (F) AUT gel lanes of reversed-phase hplc-purified histone H4 of calf (A), *Arabidopsis* [B; 91-day fluorograph exposure (C)], carrot [D; 35-day exposure (E)], alfalfa R4 [F; 65-day (overexposed) fluorograph (G)], and tobacco [H; 32-day exposure (I)]. All plant histone H4 species had an AUT gel mobility like *Arabidopsis* H4 (B) relative to calf H4 (A). Numbers 0–5 indicate levels of histone acetylation.

demonstrated that histone H4, purified by reversed-phase hplc and fractionated into its distinct acetylated forms by AUT gel electrophoresis, appeared qualitatively similar with five apparent levels of acetylation.

Quantitative analysis showed that the steady-state level of histone H4 acetylation was highest in plant species with small genomes and lowest in species with large ones (Table IA). A similar correlation has been observed for the histone H3 variants of these species (Waterborg, 1992). In contrast, the distribution of acetate label over the various levels of acetylation is remarkably similar for all species (Table IB). The relative specific activity of the label per acetylated lysine residue, a measure of the dynamic character of the acetylation, increases with the level of acetylation (Table IC). These are characteristic observations for transcriptionally active chromatin in which especially multiacetylated forms of histone H4 are prominent (Matthews & Waterborg, 1985; Van Holde, 1989; Delcuve & Davie, 1989; Boffa et al., 1990; Walker et al., 1990).

Lysine-20 in Alfalfa H4 Is Acetylated. The absence of phosphate labeling of alfalfa H4 (Waterborg et al., 1989) suggested that all five levels of charge modification that were labeled with acetate are due to conversion of charged lysines residues into neutral acetylated lysines. The lysine residues that might be acetylated fall into two structural domains of histone H4. The amino-terminal domain of histone H4 appears unstructured when the protein is in solution (Van Holde, 1989) and contains residues 1–20. This includes five lysines (5, 8, 12, 16, and 20), the first four of which have been shown in animal cells to be acetylated in vivo and to be unmethylated (Van Holde, 1989). Lysine-20 typically appears quantitatively methylated (DeLange et al., 1969a; Waterborg et al., 1983), although methylation was not observed at this residue in pea seedling histone H4 (DeLange et al., 1969b; Duerre & Buttz, 1990).

It is not possible to determine the state of modification of these lysines by automated Edman degradation sequencing of the intact histone H4, as was possible for the two histone H3 variants of alfalfa (Waterborg, 1990). Calf histone H4 has been shown to contain an α-aminoacetylated serine (Van Holde, 1989). Histone H4 from alfalfa R4 was purified to homogeneity and subjected to automated sequencing, only to find that it too appeared to contain a blocked amino-terminal residue (results not shown). Acylamino acid releasing enzyme (Pierce) treatment of histone H4—and of the amino-terminal acid hydrolysis fragment of this histone—did not produce a polypeptide amenable to Edman degradation (results not shown). Fragmentation of histone H4 was required to determine the identity of the lysines that are acetylated in vivo.

A

fragment A

calf 1 a a a a m 24
aSGRGKGGKGLGKGGAKRHRKVLR-D-
pea a a a a +

fragment B

calf 25 + + +* 68
-NIQGITKPAIRRLARRGGVKRISGLIYEETRGVLKVFLENVIR-D-
pea + + +I

fragment C fragment D

calf 69 * + 85 + 102
-AVTYTEHAKRKTVTAM-D-VVYALKRQGRTLYGFGG
pea R + +

B

calf fragment A

1 a a a a m 24
aSGR-GKGGKGLGKGGAKR-HR-K=VLR-(D)
Peptides: a b c d

C

alfalfa fragment A'

1 ? ? ? ? ? 24
aSGR-GKGGKGLGKGGAKR-HR-KVLR-(D)
Peptides: a' b' c' d'

FIGURE 2: Sequences of calf and pea histone H4. (A) Histone H4 sequences. Calf histone H4 (top) (DeLange et al., 1969a; Von Holt et al., 1979) and pea (bottom) (DeLange et al., 1969b). a and m mark known in vivo sites of acetylation and methylation, respectively. Capitals A–D indicate the weak acid hydrolysis fragments produced by hydrolysis of peptide bonds involving aspartate (–). Asterisks mark sequence differences. Plus signs mark potential sites of chemical acetylation. Numbers indicate selected residues in the histone H4 sequence. (B) Acid hydrolysis fragment A of calf histone H4 with known modifications. Parentheses indicate fragment heterogeneity due to acid hydrolysis of the peptide bonds at the amino- or carboxy-terminal side of aspartate (Lewis et al., 1975). (–) mark cleavage sites for arginine-specific peptide bond cleavage. (=) marks a trypsin cleavage site which fails to become trypsin-resistant upon chemical acetylation. Lower case letters name tryptic peptide fragments. (C) Predicted acid hydrolysis fragment A' of alfalfa histone H4. Potential sites of lysine modification are indicated by (?). All other markings as in (B).

To aid in this fragment analysis, calf histone H4 was purified and analyzed in parallel to alfalfa histone H4. The sequence of calf histone H4, with known postsynthetic modifications, is shown in Figure 2. The primary sequence of alfalfa H4 has not yet been determined. To aid in design and analysis of the fragmentation of alfalfa H4, the sequence of pea histone H4 with known modifications (DeLange et al., 1969b; Van Holde, 1989) is also given in Figure 2. All plant histone H4 fragments and peptides were named after the known and predicted products from calf histone H4 but with the addition of an apostrophe. The results obtained throughout this study, including purification and identification of all tryptic peptides derived from alfalfa histone H4 by reversed-phase hplc fractionation and column retention and by amino acid analysis (results not shown), were consistent with the assumption that the primary protein sequences of pea and alfalfa histone H4 are identical. To date, no variation has been observed in plant histone H4 primary sequences, on the basis of protein (DeLange et al., 1969b) and gene sequence analysis (Wells & McBride, 1989; Wells & Brown, 1991) in pea, *Arabidopsis*, maize, and wheat.

In addition to the five amino-terminal lysines, the globular, hydrophobic domain of histone H4 (Van Holde, 1989) contains five or six additional lysines (Figure 2), some of which have been shown to be on the surface of the folded protein and are subject to postsynthetic modification (Waterborg et al., 1983).

To localize a fifth site of acetylation in either domain, alfalfa H4 was fragmented by weak acid hydrolysis of peptide bonds next to aspartate residues 24, 68, and 85 (Lewis et al., 1975).

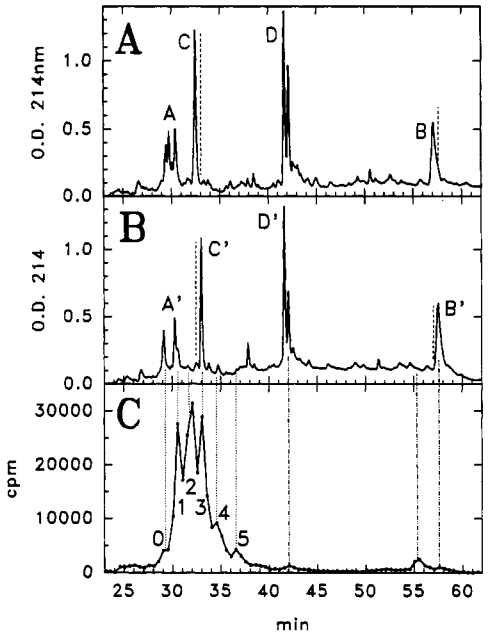

FIGURE 3: Hplc of protein fragments produced by acid hydrolysis of histone H4. Reversed-phase hplc fractionation between 6.5 and 45.5% acetonitrile of fragments produced by weak acid hydrolysis of 0.7 mg of calf histone H4 (A) and alfalfa histone H4 (B) with associated radioactivity from in vivo incorporated tritiated acetate (C). The presence of fragments A–D (calf) and A'–D' (alfalfa) in the indicated hplc peaks was determined by acid/urea peptide gel analysis, subsequent proteolysis, and peptide analysis. Differences in column retention between fragments C and C' and between B and B' are shown by dashed lines. The association of radioactivity (C) with optical density profiles, monitored at 214 nm (B), is indicated for the acetylated forms of the A' fragment by dotted lines and by numbers 0–5 that identify the level of acetylation, and for other fragments by dash–dot broken lines.

The resulting fragments were named by single capital letters (Figure 2) and were expected to be heterogeneous due to the cleavage of peptide bonds at the amino-terminal side and at the carboxy-terminal side of aspartate residues. They were fractionated by reversed-phase hplc (Figure 3B) and analyzed by acid/urea gel electrophoresis (results not shown). In order to identify protein fragments, calf thymus H4 was treated and analyzed in the same way by chromatography (Figure 3A) and electrophoresis. The identity and electrophoretic mobility of the major calf histone peptides (A–D) had been established before (Lewis et al., 1975; Waterborg and Matthews, unpublished results). The equivalent alfalfa peptides (A'–D') were tentatively identified by gel mobility and by reversed-phase retention times and elution profiles (Figure 3B).

Analysis of the acetate label distribution in the hplc eluate by liquid scintillation counting (Figure 3C) clearly established that more than 90% of all label eluted in a multiple series of peaks. Acid/urea peptide gel analysis including fluorography was used to confirm that each labeled hplc peak contained peptide A', with stepwise incrementing levels of acetylation which caused the stepwise reductions in gel mobility similar to those shown in Figure 1. Increased reversed-phase column retention by increased acetylation has been reported before (Thorne et al., 1990). The nonradioactive form of peptide A'_0 (Figure 3B), the amino-terminal fragment of histone H4 in its unacetylated state, eluted prior to five radioactive peptide forms of A', A'_1 through A'_5 (Figure 3C). This result demonstrated that in peptide A' apparently all five lysines, including lysine-20 (Figure 2), can be acetylated in vivo. The quantitative distribution of acetylated peptide species (Table ID) and radioactivity (Table IE) during chromatography was very similar to that observed for Coomassie staining (Table IA) and radioactivity (Table IB) of intact alfalfa H4 in AUT gel electrophoresis.

Very small amounts of radioactivity eluted together with the major alfalfa H4 hydrolysis fragments B' (1.4 %), C' (undetectable), and D' (2.3 %) (Figure 3C). This demonstrated that acetylation of alfalfa histone H4 was completely restricted to the amino-terminal domain of this histone.

Purification of Amino-Terminal Fragment A' of Alfalfa Histone H4. The pattern of lysine acetylation in vivo and the presence of lysine methylation can be determined by automated protein sequencing (Waterborg, 1990). This requires the purification of amino terminally unblocked protein or peptide at least to near-homogeneity without selective loss of lower abundance, highly modified forms. Unacceptable losses were observed for multiacetylated A' fragments when large volumes of hplc eluent with all modified A' forms (Figure 3C) were pooled and concentrated. Separation of the amino-terminal fragment A' of alfalfa histone H4, acetate-labeled in vivo (Figure 3B,C), from fragment C' by ion-exchange hplc was attempted without success due to the heterogeneity of the acetylated A' peptides (results not shown).

Chemical acetylation of histone H4 offers the potential of converting the heterogeneity of the partially acetylated lysines in the amino-terminal protein domain into a homogeneous form. If histone H4 is acetylated in vivo with radioactive acetate prior to purification and chemical acetylation, analysis of the distribution of radioactivity within the homogeneously chemically acetylated A' fragment (named A'⟨ac⟩) allows determination of the pattern of in vivo acetylation. In this study, radioactive acetylation in vivo was performed for 1 h. It has previously been established that incubations for this length of time approach steady-state conditions of labeling (Waterborg et al., 1990) and thus provide information about the steady-state condition of acetylation of alfalfa histone H4.

Separation of weak acid hydrolysis protein fragments of in vivo radioactively acetylated, purified, and chemically exhaustively acetylated alfalfa histone H4 showed that the amino-terminal A'⟨ac⟩ fragment eluted primarily as a single sharp peak, separated from fragment C'⟨ac⟩ (Figure 4B), with a column retention identical to in vivo pentaacetylated A'_5 (Figure 3C). Acid/urea peptide gel analysis (results not shown) and tryptic peptide analysis (see below) of the minor, earlier eluting shoulder of radioactively acetylated fragment A'⟨ac⟩ (Figure 4C) with a column retention very similar to tetraacetylated fragment A'_4 showed that this form was not due to incomplete chemical acetylation or to lysine methylation. It contains a pentaacetylated form of fragment A'⟨ac⟩ that includes the carboxy-terminal aspartate residue (residues 1–24, Figure 2). The major A'⟨ac⟩ fragment of weak acid hydrolyzed histone H4 lacks aspartate-24. The major A⟨ac⟩ fragment of hydrolyzed calf histone H4, despite the lack of aspartate-24, demonstrated a column retention like a tetraacetylated product (Figure 4A), consistent with the quantitative methylation of lysine-20 in calf H4, which precludes chemical acetylation (Figure 2).

The difference observed in column retention between calf fragment C⟨ac⟩ and alfalfa fragment C'⟨ac⟩ is consistent with the presence of one more lysine residue, presumably lysine-77 (Wells & McBride, 1989; Wells & Brown, 1991), in calf histone H4 which by chemical acetylation increases the column retention relative to the alfalfa fragment (Figure 4A,B). The small heterogeneity in the carboxy-terminal fragments D and D' (Figure 3A,B), which has been demonstrated to be caused by the presence or absence of the terminal aspartate residues

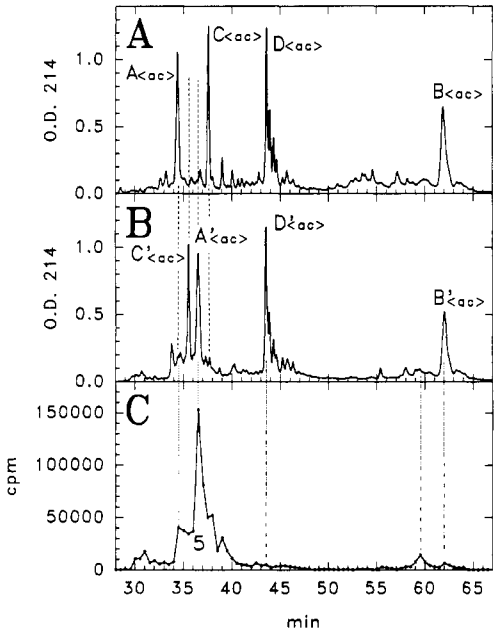

FIGURE 4: Hplc of protein fragments produced by acid hydrolysis of chemically acetylated histone H4. Reversed-phase hplc fractionation between 11.5 and 50.5% acetonitrile of fragments produced by weak acid hydrolysis of 0.8 mg of chemically acetylated calf histone H4 (A) and of approximately 1 mg of chemically acetylated alfalfa histone H4 (B) with associated radioactivity from in vivo incorporated tritiated acetate (C). The presence of fragments A⟨ac⟩ to D⟨ac⟩ (calf) and A'⟨ac⟩ to D'⟨ac⟩ (alfalfa) in the indicated hplc peaks was confirmed by acid/urea peptide gel analysis and by hplc analysis of tryptic peptides. Differences in column retention between fragments A⟨ac⟩ and A'⟨ac⟩ and between C⟨ac⟩ and C'⟨ac⟩ are shown by dashed lines. The association of radioactivity (C) with optical density profiles, monitored at 214 nm (B), is indicated for the main A'⟨ac⟩ fragment (1–23) with apparently 5 acetylated lysines and for the minor, earlier eluting shoulder (fragment 1–24) by dotted lines, and for other fragments by dash–dot broken lines.

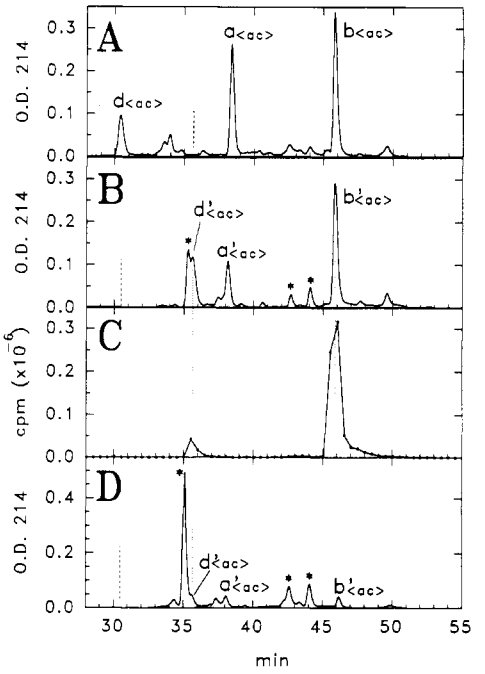

FIGURE 5: Tryptic digestion of chemically acetylated histone H4 A and C fragments. Reversed-phase hplc fractionation during a gradient from 8 to 20% acetonitrile of digests of (A) pooled chemically acetylated calf fragment A⟨ac⟩ (Figure 4A), (B) alfalfa fragment A'⟨ac⟩ (Figure 4B), and (D) alfalfa fragment C'⟨ac⟩ (Figure 4B) with minor fragment A'⟨ac⟩ (1–24) (Figure 4C) by trypsin at an enzyme:substrate ratio of 1:100 is shown by absorbance profiles. The profile of radioactivity associated with the digest of panel B is shown in (C) with dotted lines to indicate radioactivity associated with the major tryptic peptides. Differences in column retention between peptides d⟨ac⟩ and d'⟨ac⟩ in panels A and B are shown by dashed lines. The time of elution for a d⟨ac⟩ peptide (VLR) in panel D is indicated by a dashed line. Tryptic peptides derived from fragment C'⟨ac⟩ in panels B and D are marked by asterisks.

(Thorne et al., 1990), increased upon chemical acetylation (Figure 4A,B). This indicates that the denaturing conditions used for chemical acetylation failed to quantitatively acetylate each lysine residue to completion. A similar increase in heterogeneity was observed for fragment B. The difference in column retention for the calf B (Figure 3A) and alfalfa B' (Figure 3B) fragments, consistent with the predicted primary sequence differences of calf and alfalfa H4 (Figure 2), disappeared upon chemical acetylation (Figure 4A,B). However, chemical acetylation of the amino-terminal fragment appeared to be complete at each lysine residue, as suggested by hplc chromatography (Figure 4B) and as confirmed for tryptic peptide 4–17 (b'⟨ac⟩ in Figure 2) during automated protein sequence analysis (see below).

Preparation of Peptides from Histone H4, Amenable to Protein Sequence Analysis. Endoproteinase Arg-C (Boehringer) was evaluated as a method to produce stable limit peptides from the amino-terminal fragments A'⟨ac⟩ and A⟨ac⟩ of alfalfa and calf histone H4. As predicted (Figure 2), only the carboxy-terminal peptide was distinct. The calf peptide K_{me}VLR eluted before the alfalfa peptide K_{Ac}VLR during reversed-phase hplc (results not shown). Hplc analysis further showed that even before digestion was complete, nonspecific degradation and loss of peptide products were already observed. This failure of endoproteinase Arg-C to produce a stable limit digest, also noted by others (Pesis & Matthews, 1986), made it unacceptable to produce small peptides from the larger amino-terminal histone fragments for a quantitative analysis of lysine modification.

Tryptic digestion of alfalfa A'⟨ac⟩ and calf A⟨ac⟩ at enzyme:substrate ratios of 1:10 to 1:1000 gave identical stable products, as determined by peptide fractionation by reversed-phase hplc for all major histone H4 fragments. Figure 5 shows the peptide patterns obtained after preparative digestion (ratio 1:100) of calf A⟨ac⟩ (Figure 5A), alfalfa A'⟨ac⟩ (Figure 5B), and alfalfa C'⟨ac⟩ (Figure 5D).

As observed after endoproteinase Arg-C digestion, only the carboxy-terminal peptide d⟨ac⟩ of calf A⟨ac⟩ was distinct from the d'⟨ac⟩ peptide generated from alfalfa A'⟨ac⟩. The identity of peptide d'⟨ac⟩ was determined by mass spectroscopy (flight mass 556) as K_{Ac}VLR. The equivalent calf peptide is d⟨ac⟩ (VLR), generated by tryptic cleavage of the peptide bond to the carboxy side of the trypsin-sensitive methylated lysine residue. The absence of any VLR peptide upon tryptic digestion of alfalfa A'⟨ac⟩ (Figure 5B) demonstrated that lysine-20 in the main, highly labeled A'⟨ac⟩ fragment (Figure 4C) is completely unmethylated.

Tryptic digestion and hplc analysis of the minor, early eluting A'⟨ac⟩ fragment 1–24 with aspartate-24 (Figure 4C) showed the absence of any VLR peptide product (Figure 5D), confirming the complete absence of methylation of lysine-20 in alfalfa histone H4.

The major (1–23) and minor (1–24) forms of the A'⟨ac⟩ fragment (Figure 4C) were collected in such a way as to avoid potential selective loss of minor radioactively acetylated forms which might skew the quantitative analysis of lysine acetylation. As a result, fragment C'⟨ac⟩ was present in small amounts in the 1–23 A'⟨ac⟩ preparation (Figure 5B) and in large amounts in the 1–24 A'⟨ac⟩ preparation (Figure 5D). The tryptic peptides derived from C'⟨ac⟩, marked by asterisks,

Table II: Quantitative Distribution of Acetylation in Alfalfa Histone H4[a]

residue in histone H4	A (%)	B (%)	C (%)
1	0.5		
5	91.4	9.9	9.0
8		10.9	9.9
12		22.3	20.2
16		56.9	51.7
20	9.1		9.1

[a] Quantitative distribution of radioactivity (*A*) between hydrolysis fragments of in vivo labeled histone H4 (Figure 3C) and chemically acetylated histone H4 (Figure 4C) and (*B*) at residues 2, 5, 9, and 13 in fragment b'⟨ac⟩, equivalent to residues 5, 8, 12, and 16 in histone H4 (Figure 2), determined by automated protein sequencing (Figure 6). A composite distribution of in vivo acetylation of lysines is given in *C*.

are readily visible in both patterns.

Almost 10% of the acetate label, incorporated in vivo, was localized in chemically acetylated peptide d'⟨ac⟩ ($K_{Ac}VLR$) (Figure 5C). Less than 1% of the label was recovered in the amino-terminal peptide a'⟨ac⟩ which was identified as ^{Ac}SGR by mass spectroscopy and cochromatography with calf peptide a⟨ac⟩. Label incorporation into the a'⟨ac⟩ peptide most likely occurred cotranslationally (Ruiz-Carillo et al., 1975) during the 60-min in vivo treatment with tritiated acetate. The predicted peptides c⟨ac⟩ and c'⟨ac⟩ (Figure 2) were never recovered preparatively, most likely because the high hydrophilicity of a His-Arg dipeptide would prevent column retention during reversed-phase chromatography.

More than 90% of the acetate label (Table IIA) was recovered in peptide b'⟨ac⟩. It showed cochromatography with calf peptide b⟨ac⟩ (Figure 5A,B), and the predicted primary protein sequence was confirmed by automated protein sequencing of several nanomoles of peptide b'⟨ac⟩ (Figure 6A–C). Lysine was quantitatively detected as the PTH derivative of acetylated lysine. At no time during the analysis were PTH derivatives observed of unmodified lysine or of mono-, di-, and trimethylated forms of lysine (Waterborg, 1990). This demonstrated that the chemical acetylation of this domain of histone H4 had been complete and confirmed the absence of methylation of any lysine.

The radioactivity specifically associated with the PTH-acetyllysine during on-line hplc analysis (Figure 5D), distinct from a small amount of radioactivity caused by peptide washout from the filter (Figure 5E), allowed a precise calculation of the distribution of in vivo acetylation of lysines-5, -8, -12, and -16 which was not compromised by repetitive sequencing losses since it could be calculated as cpm eluted per picomole of PTH-acetyllysine eluted (Table IIB). Combination of this distribution with the radioactivity distribution between the tryptic peptides of fragment A'⟨ac⟩ (Table IIA) gave the absolute distribution of in vivo acetylation of the lysines within the amino-terminal domain of alfalfa histone H4 (Table IIC).

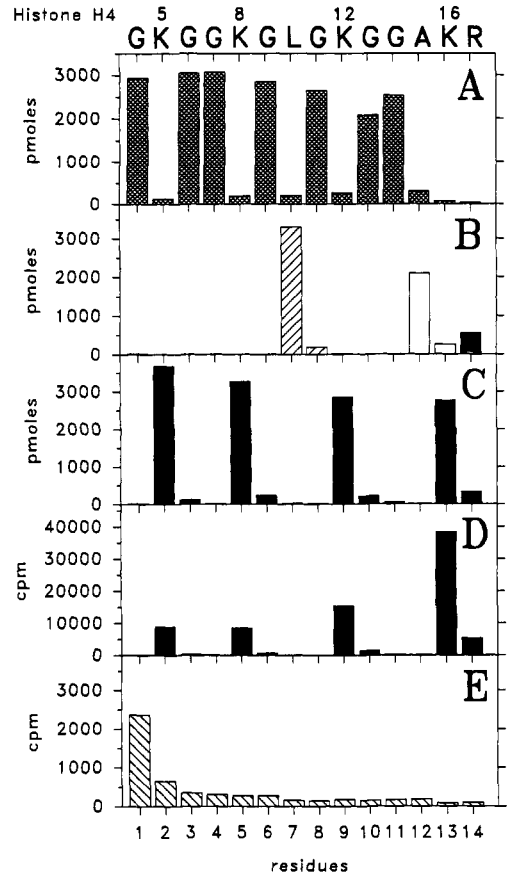

FIGURE 6: Automated sequence analysis of alfalfa tryptic peptide b'⟨ac⟩. Automated sequencing of alfalfa histone H4 peptide b'⟨ac⟩ (Figure 2), a tryptic peptide (Figure 5B,C) from the in vivo and chemically acetylated alfalfa histone H4 main fragment A'⟨ac⟩ (Figure 4B,C). The deduced sequence of peptide b'⟨ac⟩ with residue numbers for intact histone H4 is given at the top and the residue numbers in peptide b'⟨ac⟩ at the bottom. Sequencing yields (picomoles) in the on-line hplc analysis are given in panel A for PTH-glycine (cross-hatched), in panel B for PTH-leucine (hatched), PTH-alanine (white), and PTH-arginine (black), and in panel C for PTH-ε-acetyllysine (black) with a retention time slightly shorter than for PTH-alanine (Waterborg, 1990). Panel D shows the radioactivity (cpm) which coeluted with PTH-ε-acetyllysine during on-line hplc analysis of PTH-derivatized amino acids, and panel E presents washout of radioactive peptide observed as radioactivity associated with the void volume of the on-line hplc.

Discussion

Pattern of Acetylation in Plant Histone H4. Postsynthetic modification of histone H4 was studied by incorporation of radioactivity from tritiated acetate in vivo in a number of diverse plant dicot species. In every case, five radioactive forms of histone H4 were observed. Similar experiments in animals, from mammals to insects, and fungi have shown maximally four radioactive acetylated forms. The analysis of histone acetylation in alfalfa histone H4 has shown that all five lysines in the amino-terminal domain of this histone can be acetylated in vivo and that lysine-20 in alfalfa, as in pea (DeLange et al., 1969b), is devoid of the modification by methylation that is observed in other species.

Comparison of the average number of acetylated lysine residues per histone H4 protein molecule (Ac/H4) between various plant species (Table IA) revealed a sharp drop in the steady-state acetylation levels of histone H4 with increasing genome size. A similar correlation has been observed for the histone H3 variants of these species (Waterborg, 1992) and for a number of monocotylendonous plants (Waterborg, 1991). This may relate directly to the fraction of the genome present in transcriptionally competent and active, highly acetylated chromatin (Matthews & Waterborg, 1985; Van Holde, 1989).

The equivalent calculation of the relative level of radioactive acetylation in histone H4 (*Ac/H4) provides a standardized measure of the relative level of radioactively labeled histone H4 protein (Table IB). Across the species analyzed, this value was remarkably stable and appeared independent of the steady-state level of histone acetylation. It may provide a glimpse of the average pattern and level of acetylation of histone H4 in transcriptionally active and competent chromatin.

The next set of calculations (Table IC) provided an additional view on this chromatin. When the relative specific radioactivity of histone H4 was calculated for each level of acetylation and averaged for that level, a measure of the specific radioactivity of each level of histone H4 acetylation was obtained. The value increased in every case from the mono- to the tetraacetylated form. This is consistent with the hypothesis that especially multiacetylated histone H4 is localized in transcriptionally competent and active chromatin with high rates of turnover of the acetylation moiety (Matthews & Waterborg, 1985; Van Holde, 1989; Delcuve & Davie, 1989; Boffa et al., 1990; Walker et al., 1990).

Detectable levels of pentaacetylated H4 in *Arabidopsis* revealed that this trend does not probably extend beyond the tetraacetylated level (Table IC). This could mean that also in plants the tetraacetylated form of histone H4 is the highest modified functional species. Alternatively, the lower specific activity of pentaacetylated H4 may be the direct consequence of restricted accessibility of the acetylating enzyme to lysine-20, located at of near the hydrophobic, globular domain of histone H4.

Site Specificity of Acetylation in Alfalfa Histone H4. Extensive analyses of the order of lysine acetylation in histone H4 have been performed for several mammalian species (Couppez et al., 1987; Turner et al., 1989; Thorne et al., 1990). The typical transcription-correlated pattern of histone H4 acetylation in animals showed a first, highly site-specific acetylation of lysine-16, a second acetylation of lysine-12, with subsequent acetylation of lysines-8 and -5 with a reduced degree of site specificity. This could be represented by 16 > 12 > 8 > 5. Our analysis of the site occupancy of alfalfa histone H4 provides the first evidence that a similar pattern (16 > 12 > 8 > 5 = 20) may also exist in alfalfa (Table II) with the exception that lysine-20 is also a target for acetylation. Observation of a 9% radioactive acetylation of lysine-20 (Table II) when only 5% of the label is in pentaacetylated histone H4 (Table IE) is consistent with a reduced degree of site specificity when additional acetylation of acetylated histone H4 occurs.

Recent studies in yeast by site-directed mutagenesis have suggested that acetylation of lysine residues 5, 8, 12, and 16 is an essential function as replacement by arginine or glutamine was detrimental or lethal (Megee et al., 1990). The importance of lysine-20 was not analyzed. Distinct phenotypes were observed when lysines at different sites were replaced, suggestive of a functional site specificity of histone acetylation. However, the importance of this specificity remains unclear. Different patterns of lysine acetylation, distinct from the mammalian pattern (16 > 12 > 8 > 5), have been observed in cuttle fish (12 > 5 > 16 > 8) (Couppez et al., 1987), *Drosophila* (5 = 8 = 12 > 16) (Munks et al., 1991), *Tetrahymena* (8 > 5 > 12 > 16) (Chicoine et al., 1986), and *Physarum* (8 > 12 > 5 > 16) (Pesis & Matthews, 1986).

As yet unidentified factors must play a role to confer the pattern of histone acetylation. The histone acetyltransferase may have some effect but appears not to be the major determinant. In alfalfa, the pattern of acetylation in histone H4 (16 > 12 > 8 > 5 = 20) (Table II) is quite distinct from that detected in histone H3 (14 > 18 > 23 > 27 = 9 > 4) (Waterborg, 1990). Less clear differences have been observed between mammalian H4 (16 > 12 > 8 > 5) and H3 (14 > 23 > 18 > 9 > 4) (Thorne et al., 1990; Marvin et al., 1990) and between *Tetrahymena* H4 (8 > 5 > 12 > 16) and H3 (9 = 14 > 18 > 23) (Chicoine et al., 1986).

Methylation of Histone H4. The failure to detect peptide VLR upon tryptic digestion of fragment A'⟨ac⟩ of alfalfa histone H4 (Figure 5) suggests the complete absence of methylation of lysine-20. This is remarkable as methylation of lysine-20 in H4 has been reported in the lower eukaryote *Physarum* (Waterborg et al., 1983), and in all animals ranging from sea urchins and fishes to birds and mammals (Van Holde, 1989; Duerre & Buttz, 1990). This study also did not reveal any indications that lysines-5, -8, -12, or -16 in alfalfa histone H4 might be targets for lysine methylation in vivo.

The absence of methylation of lysine-20 had been observed before in pea in the only plant histone H4 protein analyzed before (DeLange et al., 1969b), but the novel acetylation of lysine-20 reported here for alfalfa was not detected. The absence of methylation of lysine-20 in alfalfa is probably not caused by the absence of lysine methyltransferase enzyme activity because high levels of methylation of both histone H3 variant proteins have been observed at multiple amino-terminal lysine residues (Waterborg, 1990).

This methylation of histone H3 includes lysine-27. It has been speculated (Duerre & Buttz, 1990) that lysine-27 in histone H3 may be equivalent to lysine-20 in histone H4 and that both modified residues could provide a transition or anchor between the unstructured amino-terminal domain and the globular, hydrophobic domain of these proteins (Van Holde, 1989). However, this function has never been proven. The observation that lysine-20 in plant histone H4 is not methylated would suggest that this modification is not required for nucleosomal function, at least in plants.

Registry No. Lysine, 56-87-1.

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Isolation and Sequence of a cDNA Encoding Porcine Mitochondrial NADP-Specific Isocitrate Dehydrogenase[†,‡]

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ABSTRACT: The cDNA for porcine mitochondrial NADP-specific isocitrate dehydrogenase was isolated from a λgt11 library using polymerase chain reaction. Translation of the DNA sequence gave a 413-residue amino acid sequence and a calculated molecular weight of 46 600 for the mature polypeptide. Previously determined peptide sequences for the amino terminus and for internal tryptic peptides were all contained within the translated sequence. The porcine protein was found to share 63% residue identity with yeast mitochondrial NADP-specific isocitrate dehydrogenase and to be immunoreactive with an antiserum against the yeast protein. Highly conserved regions include residues which have been implicated in substrate and cofactor binding in previous studies of the porcine enzyme. The two eucaryotic enzymes exhibit only minimal homology with the NADP-dependent isocitrate dehydrogenase from *Escherichia coli*, with the exception of a striking conservation of residues implicated in formation of the metal–isocitrate site of the procaryotic enzyme.

Isocitrate dehydrogenase (IDH)[1] catalyzes the oxidative decarboxylation of isocitrate to form α-ketoglutarate. While a single NADP-specific form of this enzyme is responsible for this reaction in *Escherichia coli* (Plaut, 1963; Ragland et al., 1966), multiple isozymes of IDH that vary in subunit structure and cofactor specificity have been reported for both lower and higher eucaryotes (Colman, 1968; Plaut, 1970; Keys & McAlister-Henn, 1990; Haselbeck & McAlister-Henn, 1991).

In the yeast *Saccharomyces cerevisiae*, distinct NAD- and NADP-specific isozymes are both located in the mitochondrial matrix (Keys & McAlister-Henn, 1990; Haselbeck & McAlister-Henn, 1991). The NAD-specific IDH has recently been shown to be a significant contributor to tricarboxylic acid (TCA) cycle activity (Keys & McAlister-Henn, 1990; Cupp & McAlister-Henn, 1991) and is allosterically responsive to the adenylate energy charge as well as to key TCA cycle metabolites (Hathaway & Atkinson, 1963; Barnes et al., 1971). The yeast mitochondrial NADP-specific isozyme has been proposed to provide an alternative TCA cycle activity (Machado et al., 1975), although its actual relative contribution to TCA cycle function or to other cellular processes remains unclear.

Partial characterization of the mitochondrial NADP-IDH (IDP1) of *S. cerevisiae* and cloning and sequencing of the corresponding structural gene have recently been described (Haselbeck & McAlister-Henn, 1991). IDP1 is a dimer of identical subunits with a subunit molecular weight of approximately 46 000. Its activity has been determined to not be allosterically regulated (Kornberg & Pricer, 1951). In these respects, yeast IDP1 resembles the IDH of *E. coli* (Reeves et al., 1972; Burke et al., 1974) as well as the mitochondrial NADP-IDH isolated from porcine heart tissue (Colman, 1968). While the primary structures of the *E. coli* and yeast enzymes are not significantly homologous (Thorsness & Koshland, 1987; Haselbeck & McAlister-Henn, 1991), key residues of *E. coli* IDH identified in crystallographic studies to be involved in the binding of isocitrate may also be conserved in IDP1.

Comparison of amino acid sequences obtained from tryptic peptides of porcine mitochondrial NADP-IDH (Smyth & Colman, 1991) with the predicted amino acid sequence of

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[‡] The DNA sequence reported in this paper was submitted to GenBank under Accession Number M86719.

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[1] Abbreviations: IDH, isocitrate dehydrogenase; TCA, tricarboxylic acid cycle; PCR, polymerase chain reaction; NEM, *N*-ethylmaleimide.

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