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# Different Types of Maize Histone Deacetylases Are Distinguished by a Highly Complex Substrate and Site Specificity<sup>†</sup>

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**ABSTRACT:** Enzymes involved in histone acetylation have been identified as important transcriptional regulators. Maize embryos contain three histone deacetylase families: *RPD3*-type deacetylases (HD1-B), nucleolar phosphoproteins of the HD2 family, and a third form unrelated to *RPD3* and HD2 (HD1-A). Here we first report on the specificity of deacetylases for core histones, acetylated histone H4 subspecies, and acetylated H4-lysine residues. HD1-A, HD1-B, and HD2 deacetylate all four core histones, although with different specificity. However, experiments with histones from different sources (hyperacetylated MELC and chicken histones) using antibodies specific for individually acetylated H4-lysine sites indicate that the enzymes recognize highly distinct acetylation patterns. Only *RPD3*-type deacetylase HD1-B is able to deacetylate the specific H4 di-acetylation pattern (position 12 and 5) introduced by the purified cytoplasmic histone acetyltransferase B after incubation with pure nonacetylated H4 subspecies. HD1-A and HD2 exist as phosphorylated forms. Dephosphorylation has dramatic, but opposite effects; whereas HD2 loses enzymatic activity upon dephosphorylation, HD1-A is activated with a change of specificity against acetylated H4 subspecies. The data suggest that different types of deacetylases interact with different and highly specific acetylation patterns on nucleosomes.

ε-amino groups of specific lysine residues within the N-terminal extensions of core histones are posttranslationally modified by acetylation. The recent discovery that numerous transcriptional regulators exert histone acetyltransferase (HAT)<sup>1</sup> or histone deacetylase (HD) activity stimulated the attention on the role of chromatin structure in gene regulation (1). It has been demonstrated that HATs and HDs can interact with specific DNA-binding activator or repressor proteins, thereby modulating transcriptional activity of specific promoters by locally changing chromatin structure. It is still unclear whether distinct acetylation patterns directly alter nucleosomal structure: the recent 2.8 Å resolution structure of the nucleosome core (2) rather argues for a role of lysine acetylation in the destabilization of the chromatin higher-order structure than of the nucleosome itself. Alternatively, histone acetylation could act as a signal, much like protein phosphorylation, to trigger chromosomal events by changing protein-histone interactions; this view was conceptually

supported by the recent findings that nonhistone proteins, like p53 or general transcription factors are acetylated by histone acetyltransferases in vivo and in vitro (3, 4).

In vertebrate cells, so far only deacetylases related to the *RPD3*-type have been identified (5) which are recruited by DNA-binding transcriptional repressors (6, 7). Moreover, *RPD3*-type deacetylases may be potential targets for transcription therapy in human cancer (8, 9). In plants, there are three distinct, well-characterized HDs; in maize embryos, an *RPD3*-type deacetylase has recently been identified (10); this enzyme corresponds to the HD1-B enzyme activity (11, 12; Lechner et al., manuscript submitted). Besides, a novel nucleolar phosphoprotein (HD2) has been identified as a deacetylase (13), which shares sequence homology with a class of peptidyl-prolyl cis–trans isomerases (14, 15), and a third deacetylase, HD1-A (16), which is unrelated to *RPD3* and HD2 (Brosch, Pipal and Loidl, unpublished results). In contrast to different classes of HATs which were shown to be highly substrate and site specific (17, 18), no detailed information on specificity is available for deacetylases. It was recently reported that recombinant HDAC1/*RPD3* is able to deacetylate all four core histones in vitro (19). Here we report that the different classes of HDs present in maize embryos exhibit distinct and specific substrate and site selectivities.

## EXPERIMENTAL PROCEDURES

**Purification and Enzymatic Assay of Maize HDs.** HDs were purified from germinating maize embryos at 72 h after start of seed imbibition. Enzyme purification was performed

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<sup>1</sup> Abbreviations: AUT-PAGE, acetic acid-urea-Triton polyacrylamide gel electrophoresis; HAT, histone acetyltransferase; HD, histone deacetylase.

according to established protocols (12, 16, 20). After incubation of highly purified enzymes with 8  $\mu$ g of either hyperacetylated MELC or [ $^3$ H]-acetate prelabeled chicken reticulocyte histones (12), the mixture was subjected to AUT-PAGE with subsequent Coomassie-blue staining and fluorography.

**Preparation of Hyperacetylated Histones, Purification of Acetylated H4 Subspecies.** The procedure to obtain [ $^3$ H]-acetate prelabeled chicken reticulocyte histones is outlined in detail elsewhere (12). Phenylhydrazine was administered to male white leghorn chicken to enrich the reticulocyte population. Blood was diluted in SSC buffer and centrifuged at 500g. The pellet was repeatedly washed with SSC buffer and resuspended in 100 mL of minimum essential medium, Eagle, HEPES modification. For labeling, the cell pellet of one chicken was resuspended in MEM containing 10 mCi [ $^3$ H]-acetic acid (10 Ci/mmol; Amersham International plc) and 0.4  $\mu$ M Trichostatin A. After incubation for 45 min at 37 °C and centrifugation at 1000g, the pellet was resuspended in 130 mL of MEM, containing 0.4  $\mu$ M Trichostatin A, and incubated for 1 h at 37 °C. Lysis of cells was achieved by resuspension in lysis buffer (10 mM NaCl, 5 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.5% (v/v) Triton x-100, 10 mM Tris-HCl, pH 7.4, 0.4  $\mu$ M Trichostatin A), and incubation was continued for 30 min at 4 °C. The pellet was washed repeatedly and finally incubated on ice in 0.5 M HCl for 12 h. The suspension was centrifuged for 20 min at 8000g, and the supernatant was stored on ice. The pellet was reextracted in 0.5 M HCl. Combined extracts were filtered through Whatman-GF-C filter, and eight volumes of acetone were added to the filtrate for precipitation of histones overnight at -20 °C. The collected histones were dried under vacuum. Histones were dissolved in distilled water (1.5 mg/mL). An aliquot of 10  $\mu$ L yielded 120000 cpm.

**MELC Histones.** Murine erythroleukemia cells (Friend cells, line B8) were grown in Dulbecco's modified Eagles medium supplemented with 10% fetal calf serum in humidified CO<sub>2</sub> (5%) at 37 °C. Exponentially growing cells (5  $\times$  10<sup>5</sup> cells/mL) were incubated for 6 h with Trichostatin A (0.1  $\mu$ g/mL). After incubation, cells were collected by centrifugation (800g; 10 min) and histones were extracted as described (21).

Acetylated H4 subspecies were purified from total core histones by preparative acetic acid-urea-triton polyacrylamide gel electrophoresis (AUT-PAGE) and SP-Sephadex chromatography as described in detail elsewhere (12,18).

**Acetylation of Nonacetylated H4 with Maize HAT-B.** Highly purified HAT-B (HAT: histone acetyltransferase) of maize embryos (22) was incubated with pure nonacetylated H4 isoform for 30 min at 37 °C in the presence of [ $^{14}$ C]-acetylCoA (18). After incubation, the sample was heated to 60 °C for 10 min before aliquots of highly purified maize deacetylases HD1-A, HD1-B, or HD2 were added and incubation was continued at 30 °C for 30 min. Finally, samples were subjected to AUT-PAGE and exposed on phospho-storage image screens.

**Immunoblotting.** After incubation of MELC or chicken histones with purified HDs (HD: histone deacetylase), the reaction mixture was subjected to AUT-PAGE (18). Gels were blotted onto nitrocellulose membrane and membrane strips were incubated with antibodies against H4 N-terminal peptides acetylated on lysine-5, -8, or -12 (23). Antibodies

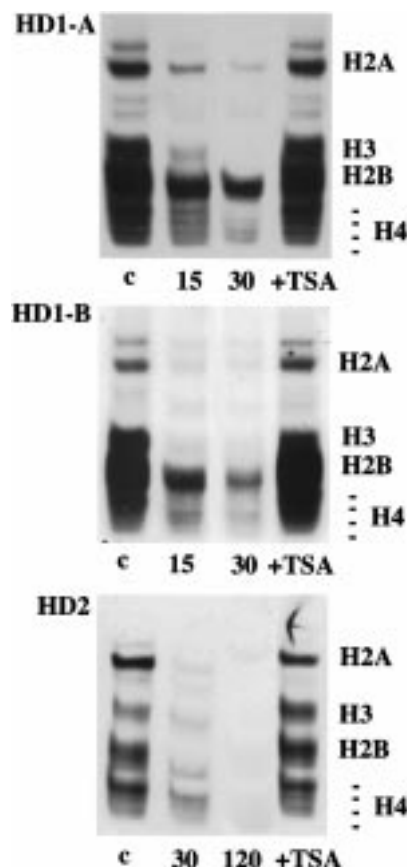


FIGURE 1: Substrate specificity of purified maize HDs. Aliquots of highly purified HD1-A, HD1-B, and HD2 were incubated with [ $^3$ H]-acetate prelabeled chicken reticulocyte histones (8  $\mu$ g). After incubation for the indicated time periods (15, 30, and 120 min) histones were subjected to AUT-PAGE with subsequent fluorography. Left lanes (c) show the histone control without enzymes, right lanes (+TSA) show incubations for 30 min (HD1-A, HD1-B) or 120 min (HD2) in the presence of the deacetylase inhibitor Trichostatin A (0.1  $\mu$ M). Positions of core histones and mono- up to tetraacetylated H4 subspecies are indicated.

were raised against the acetylated H4 N-terminal peptides: lysine-5, immunization with peptide SGRGacKGGKGL; lysine-8, with peptide SGRGKGGacKGLGK; lysine-12, with peptide GKGLGacKGGAK). Immunodetection was performed with the ECL detection system (Amersham International plc).

## RESULTS

We addressed the question of specificity of maize HDs by incubating highly purified enzymes (12, 16, 20) with purified [ $^3$ H]-acetate prelabeled hyperacetylated chicken reticulocyte core histones in vitro. HD1-A, HD1-B, and HD2 were able to deacetylate all core histones, yet with different selectivity (Figure 1). In all cases, H3 was the preferred substrate histone; HD1-A and HD1-B deacetylated H2A and H4 with almost equal specificity and were least active with H2B. Nucleolar deacetylase HD2 deacetylated H2A and H2B equally well, and was least active with H4. In all cases, addition of Trichostatin A completely inhibited the enzymatic reaction (Figure 1). When pure, individual histone species were used as substrate, the same specificity was observed (result not shown).

When we tested histones from different sources as substrate for maize HDs we recognized subtle differences

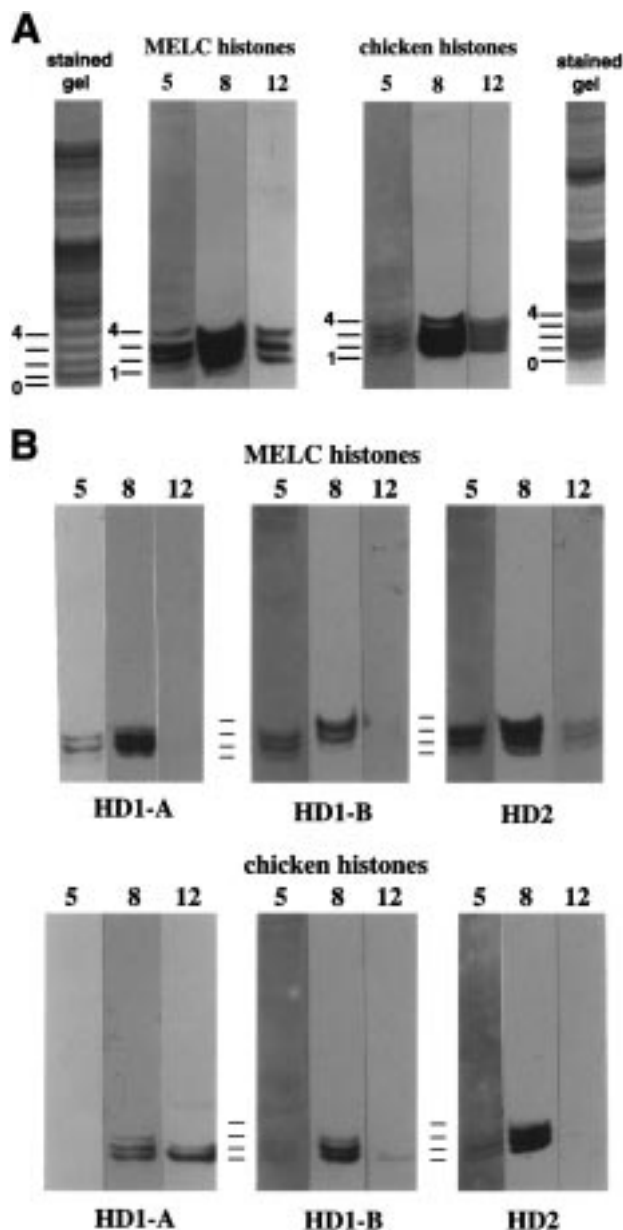


FIGURE 2: Specificity of purified maize HDs for acetylated lysine residues 5, 8, and 12 in acetylated H4 subspecies. (A) hyperacetylated histones from MELC (left part) and chicken reticulocytes (right part) were subjected to AUT-PAGE with subsequent blotting onto nitrocellulose membranes. Membrane strips were incubated with antibodies against H4 N-terminal peptides acetylated on either lysine-5, -8, or -12. Immunodetection was performed with the ECL detection system (Amersham). (B) MELC- or chicken reticulocyte histones were incubated with purified maize HD1-A, HD1-B, or HD2 for 1 h (HD1-A, HD1-B) or 2 h (HD2) at 30 °C. After incubation samples were processed as described for A. Positions of mono- up to tetraacetylated H4 subspecies are indicated.

in the specificities of the three enzymes (result not shown). This indicated that a different extent of acetylation and/or acetylation of different lysine sites may influence enzyme specificity. To further address this question we used antibodies against H4-N-terminal peptides differing in the acetylated lysine position and compared core histones from murine erythroleukemia cells (MELC histones) and chicken reticulocytes that had been hyperacetylated *in vivo* by Trichostatin A treatment (Figure 2A). The Coomassie-blue stained gel lanes demonstrate that equal amounts of input histones were used from both species; it is evident that the percentage of

acetylated H4 subspecies is different in MELC and chicken reticulocytes (Figure 2A). Antibodies directed against acetylated lysine-5, -8, or -12 were highly specific for acetylated H4 subspecies and did not crossreact with other core histones, as shown on immunoblots after AUT-PAGE of total core histones (Figure 2A). Antibodies against acetylated lysine-5 reacted predominantly with di- and triacetylated H4 (only weak label on mono- and tetraacetylated H4 subspecies) of MELC, whereas lysine-5 was equally acetylated in mono- to tetraacetylated H4 isoforms of chicken reticulocytes. Lysine-8 was acetylated in all four subspecies in both histone types, although this site was acetylated to a greater extent in highly acetylated H4 subspecies of MELC, whereas it was preferably modified in moderately acetylated subspecies of chicken. Lysine-12 was acetylated in di-, tri-, and tetraacetylated H4 of MELC and in mono- to tetraacetylated H4 of chicken reticulocytes (Figure 2A). The data demonstrate that MELC and chicken reticulocyte histone H4 populations, hyperacetylated by Trichostatin treatment, differ in their overall acetylation pattern.

Highly purified maize HD1-A, HD1-B, and HD2 were incubated with these hyperacetylated core histones; after incubation, AUT-PAGE was performed with subsequent immunoblotting using site-specific anti-acetyl-lysine antibodies (Figure 2B). HD1-A completely deacetylated lysine-12 of MELC H4 but only deacetylated lysine-12 of chicken reticulocyte H4 in tri- and tetraacetylated subspecies; it completely deacetylated lysine-5 of chicken H4 but only partially deacetylated this residue in MELC histones; HD1-A was equally active with lysine-8 in all acetylated chicken H4 subspecies, but it was predominantly active with the tetraacetylated form of MELC.

HD1-B almost completely deacetylated lysine-12 and 5 of MELC and chicken H4. HD1-B partially deacetylated lysine-8 of H4 from both sources, although a significant proportion of acetylated lysine 8 remained in the tri- and tetraacetylated subspecies of MELC. HD2 exhibited low activity for lysines-5 and -8 of MELC H4, but partially deacetylated these positions in chicken H4; lysine-12 was deacetylated in chicken mono- to tetraacetylated subspecies but only partially deacetylated in di-, tri-, and tetraacetylated H4 of MELC histones (Figure 2B). These results indicate that the maize HDs react with distinct H4 acetylation patterns since they differ with respect to specificity for the degree of H4 acetylation as well as for certain acetylated lysine positions. It should be mentioned that we also used an antibody raised against acetylated lysine-16; unfortunately, this antibody did not give us clear-cut results on acetylated H4 subspecies after AUT-PAGE (result not shown). It also has to be pointed out that the specificity of deacetylases as summarized in Figure 2 was identical when partially purified enzymes were compared with highly purified preparations.

It has previously been shown that the cytoplasmic histone acetyltransferase B is highly specific for nonacetylated H4, generating diacetylated subspecies by sequential modification of lysine 12 and 5 (18). Given this highly specific acetylation pattern, it was tempting to analyze whether one of the 3 enzymes was specific for deacetylation of this distinct acetylation type. After *in vitro* acetylation of highly pure nonacetylated H4 subspecies of chicken erythrocytes (12, 18) with purified maize HAT B, we incubated the diacetylated reaction product (modified in positions 5 and 12) with





FIGURE 3: Deacetylation of the specific diacetylation of H4 introduced by cytoplasmic HAT B. Highly purified maize HAT B was incubated with pure nonacetylated H4 subspecies of chicken erythrocytes for 30 min at 37 °C in the presence of [ $^{14}$ C]-acetylCoA. After incubation the sample was heated to 60 °C for 10 min before aliquots of highly purified maize deacetylases HD1-A, HD1-B, or HD2 were added and incubation was continued at 30 °C for 30 min. Finally, samples were subjected to AUT-PAGE and exposed on phospho-storage image screens. (c, control) Sample aliquot removed after incubation of nonacetylated H4 subspecies with HAT B. Positions of mono- and diacetylated H4 subspecies are indicated.

pure HD1-A, HD1-B, or HD2. Strikingly, only HD1-B was able to react with that specific acetylation, causing complete deacetylation (Figure 3).

It has been shown that two maize HDs (HD1-A, HD2) exist as phosphorylated enzyme forms (13, 20, 24). We could previously demonstrate that dephosphorylation of partially purified HD1 (mixture of phosphorylated HD1-A, dephosphorylated HD1-A, and HD1-B) resulted in a change of substrate specificity (24). In vitro dephosphorylation of HD2 by alkaline phosphatase completely abolished the enzymatic activity (result not shown); in contrast phosphatase treatment caused a considerable increase of enzymatic activity of highly purified HD1-A (Figure 4A). This effect was most pronounced for H2A and H4 which were completely deacetylated only after dephosphorylation of the enzyme. Native HD1-A, as purified from maize embryos, is a mixture of phosphorylated and nonphosphorylated enzyme forms (16, 24). To test whether dephosphorylation changes the specificity for acetylated H4 subspecies, we incubated purified native HD1-A or dephosphorylated HD1-A with hyperacetylated MELC core histones and analyzed the proportion of acetylated H4 subspecies after AUT-PAGE with subsequent protein staining. Hyperacetylated MELC H4 consisted of approximately 15% nonacetylated, 27% monoacetylated, 19% diacetylated, 20% triacetylated, and 19% tetraacetylated subspecies (Figure 4B). Incubation with native HD1-A caused partial deacetylation of tetra- and triacetylated subspecies with a corresponding increase of predominantly nonacetylated subspecies. Incubation with dephosphorylated HD1-A led to complete deacetylation of di-, tri-, and tetraacetylated subspecies and the accumulation of approximately 65% nonacetylated H4. Interestingly, the proportion of mono-acetylated subspecies practically remained constant, regardless of the enzyme incubation (Figure 4B).

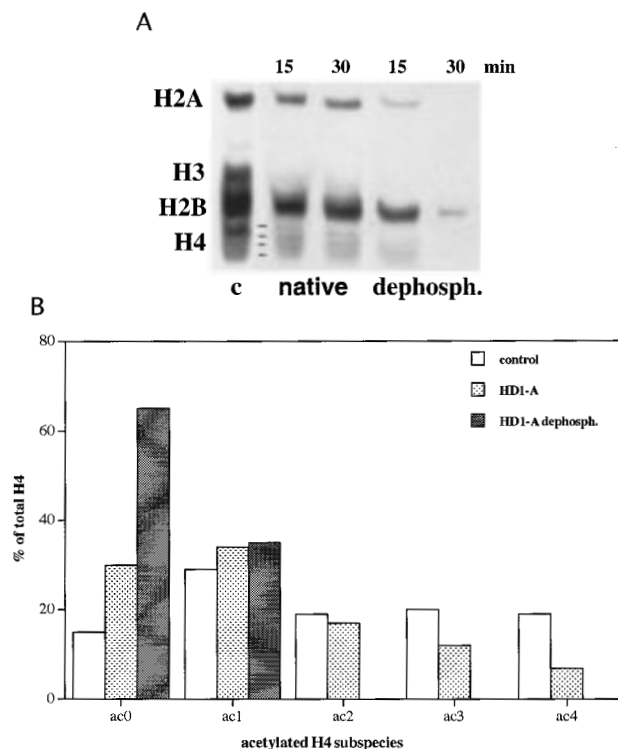


FIGURE 4: Dephosphorylation of HD1-A changes enzyme activity and specificity. (A) An aliquot of purified HD1-A was split; one-half of the enzyme sample was digested with alkaline phosphatase (100 U/mL) for 60 min at 28 °C while the other half was incubated under the same conditions without phosphatase; after incubation the digested and nondigested samples were applied to UNO-Q anion exchange columns (Biorad). HD1-A was eluted by a linear salt gradient (10 mM–500 mM NaCl). The peak fractions of the dephosphorylated and native (undigested) enzyme forms were incubated with [ $^3$ H]-acetate prelabeled histones (lane 1, c) for 15 (lanes 2 and 4) or 30 (lanes 3 and 5) min. After incubation the assay mixtures were subjected to AUT-PAGE with subsequent fluorography. Positions of histones and acetylated H4 subspecies are indicated. (B) Peak fractions of native or dephosphorylated HD1-A after UNO-Q anion exchange chromatography were incubated with hyperacetylated MELC histones. After incubation, assay mixtures were subjected to AUT-PAGE and Coomassie-blue staining. The H4 region of the stained gel was quantitated by laser densitometry using Molecular Dynamics Image Quant software. The amounts of acetylated H4 subspecies are expressed in percent of total H4. (c, control) MELC histones before enzymatic incubation.

## DISCUSSION

The present report analyzed the specificity of maize HDs for free core histones. We used histones from chicken or MELC because it is easily possible to isolate big amounts of histones from these sources and also sufficient amounts of radioactively pre-labeled histones. This is not possible for maize embryos, especially for prelabeled histones. However, the amino acid sequence of maize, chicken or MELC histones is highly homologous, especially for histone H4; chicken H4 differs from maize H4 in 5 amino acids (position 14, 15, 58, 60, and 77). Within the H4 N-terminal extension only maize alanine-14 is replaced by glycine in chicken, and maize arginine-15 is replaced by alanine in chicken; the acetyltable lysines at position 5, 8, 12, and 16 are identical in both species. Since nucleosomes and nucleosomal arrays in chromatin are the physiological substrate for these enzymes, it may well be that the substrate specificity is different when nucleosomes or oligonucleosomes are used as substrate. It

may also be that the observed difference in specificity of the enzymes for distinct lysine positions in H4 is partly determined by other posttranslational modifications of H4; these modifications may be different between chicken reticulocyte H4 and MELC H4. On the other hand, the fact that only HD1-B is able to deacetylate an H4 subspecies diacetylated in positions 5 and 12 (Figure 3) indicates that the enzymes are highly specific for a certain acetylation pattern. We will also test the specificities of the enzymes with nucleosomes reconstituted with purified acetylated core histone subspecies. It has recently been reported that purified recombinant HDAC1 deacetylated core histones and nucleosomes *in vitro* but not intact oligonucleosomes (19); however, this report did not address the question whether certain lysine positions or acetylated histone subspecies are preferred by *RPD3*-related deacetylases. In yeast it was demonstrated that deletion of *RPD3* or its associated partner *SIN3* results in increased acetylation of lysine-5 of H4 in the promoters of UME6-regulated genes (25). This finding is in line with the pronounced specificity of maize HD1-B for acetylated lysine-5 and -12, specifically introduced by cytoplasmic HAT B. Interestingly, the retinoblastoma associated protein *Rbap48* is also associated with the cytoplasmic B-type acetyltransferase (26) and the *RPD3* homologous HDAC1 (5) but not with another human *RPD3* ortholog, HDAC3 (27). In maize, too, *Rbap46* is associated with the cytoplasmic acetyltransferase B and the *RPD3* homologous deacetylase HD1-B (Lechner et al., manuscript submitted). Obviously, *Rbap48* has a regulatory or targeting function for acetylation and deacetylation of the specific replication-associated acetylation pattern of H4. The fact that neither maize HD1-A nor the nucleolar HD2 are able to deacetylate this specific acetylation pattern on lysine-5 and -12 demonstrates that HDs are highly specific for distinct acetylation patterns. The specificity of deacetylases is not restricted to a certain level of acetylation or just distinct acetylated lysine positions but rather deacetylases recognize and interact with a specific acetylation pattern on histone molecules.

The recent progress in histone acetylation research has revealed that acetylation and deacetylation are essentially involved in functional transitions of eukaryotic chromatin. With respect to transcriptional regulation, available data show the important role of histone deacetylation in transcriptional repression (1) and the tight association with other chromatin repressing activities, as was recently reported for DNA-methylation (28, 29). It is now clear that HATs and HDs are regulated by protein phosphorylation, as shown for maize deacetylases (13, 20, 24) and for *GCN5* (30). Moreover, evidence has been presented for the involvement of a protein phosphatase in the inhibition of histone deacetylation by HD inhibitors (31). The facts that histone acetylation enzymes exert their functions as protein complexes of different composition, are associated with protein phosphorylation pathways, and have a very pronounced specificity for a distinct acetylation pattern suggest a highly complex role in the modulation of gene activity, rather than a unique structural effect on nucleosomes by simple changes of histone octamer charge.

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