Distinct Site Specificity of Two Pea Histone Deacetylase Complexes†

Silvia Clemente, Luis Franco, and Gerardo López-Rodas*

Department of Biochemistry and Molecular Biology, University of Valencia, E-46100 Burjassot, Valencia, Spain

Received January 12, 2001; Revised Manuscript Received June 25, 2001

ABSTRACT: We report on the site specificity of two intact pea histone deacetylase complexes. HD1 deacetylates lysines 5 and 16 of H4 in the order K16 > K5, while in the case of H3 the preferred order is K4 ≫ K18 ≈ K9. The specificity of the HD2 complex is markedly different. The preferred residues in H4 are K8 ≈ K5 > K16, while in H3 deacetylation, the complex HD2 prefers sites 4 and 18. To obtain these results, we have used a novel procedure based on the SPOT technique, a method to synthesize peptides on membrane supports. Different sets of membranes with sequentially overlapping histone peptides containing acetylated lysines in the sites corresponding to all in vivo acetylatable residues were incubated with the complexes. The acetyl groups removed by the deacetylase activity were then replaced by radioactive acetate by treating the membranes with labeled acetic anhydride. The subsequent counting of the membranes allows the quantification of the acetate removal in the histone deacetylase reaction in a way that circumvents some of the inconveniences of other available procedures.

The reversible acetylation of the ϵ-amino group of lysyl residues is the best studied posttranslational modification of histones. The acetylation state of a given lysyl residue depends on the relative activity of two sets of enzymes, HATs[1] and HDACs. HATs catalyze the transfer of an acetyl group from acetyl-coenzyme A to the side chain of the selected lysyl residue, while HDACs hydrolytically remove the acetyl group as acetate (*1*). Since its discovery in 1964 (*2*), it has been thought that acetylation of histones is related to transcriptional activation, but the definitive proof only came when the cloning of HATs and HDACs was achieved. In 1996, the group of Allis described the isolation of the gene coding for p55, a catalytically active HAT from the ciliate protozoan *Tetrahymena* (*3*), to find that the sequence derived from the gene showed extensive homology to Gcn5p, a known transcriptional coactivator from *Saccharomyces cerevisiae.* On the other hand, HDAC1, a mammalian deacetylase, was found to be homologous to Rpd3p, a well-known yeast transcriptional repressor (*4*). In this way, direct links between histone acetylation and transcriptional activation and, conversely, between deacetylation and repression were established.

Despite the data on histone acetylation, the molecular reasons by which histone acetylation causes a transcriptionally competent conformation in chromatin are not known. Recent results suggest two potential mechanisms. The first possibility is that histone acetylation would relax chromatin structure in a manner that allows the binding of *trans*-acting factors, which in turn start building up the initiation complex. A second possibility is that histone acetylation acts as a specific signal so that the factors that interact with the histone tails would recognize a given pattern of charged (nonacetylated) and noncharged (acetylated) lysines. The latter possibility was earlier suggested by Loidl (*5*), and it has been recently resumed by Strahl and Allis (*6*), who, with several modifications, extended Loidl's hypothesis for other histone modifications, such as phosphorylation and methylation, which also are candidates for a signaling role. The question as to how many combinatorial sets of histone modifications are functionally operative remains to be solved. It seems likely that different sets of acetylated lysines are associated with different functions, an issue that was initially addressed by using site-specific antibodies (reviewed in ref *7*).

The signaling role of histone acetylation requires that the enzymes involved in this modification are histone- and site-specific. The histone specificity of both the purified or recombinant HATs and their native functional complexes has been ascertained (for reviews, see refs *8* and *9*) by using, in most cases, site-specific antibodies or microsequencing methods. However, the analysis is somewhat more difficult in the case of HDACs, because the reliability of these methods depends on the extensive deacetylation of the acetyllysine substrates. Consequently, our knowledge on the site specificity of HDACs is much more limited, and yet the issue is an interesting one, because the evaluation of the functional significance of histone acetylation as a signaling phenomenon depends on the specificity of both HATs and HDACs.

To address this question, we describe an approach based on the SPOT technique, a method to synthesize sets of histone peptides on membrane supports (*10*) first used to map antibody epitopes (*11*). This strategy has allowed us to study the site specificity of two pea HDAC complexes first

† This work was supported in part by Grant PB97-1368 from DGICYT (Spain) and Programa d'Accions Especials AE97-16 from Generalitat Valenciana. S.C. is a recipient of a predoctoral fellowship from Conselleria de Cultura, Educaciò i Ciencia (Generalitat Valenciana).

* Author for correspondence. Phone: +34-963864385 ext 34. Fax: +34-963864635. E-mail: Gerardo.lopez@uv.es.

[1] Abbreviations: FPLC, fast-performance liquid chromatography; HAT, histone acetyltransferase; HDAC, histone deacetylase; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis.

10.1021/bi0100844 CCC: $20.00
Published on Web 08/10/2001

isolated in our laboratory, namely, HD1 and HD2 (*12*). These complexes can be isolated by a two-step chromatographic procedure, and they can be considered as intact complexes due to the minimal manipulation required. The specific deacetylation of H3 and H4 occurs in a precise and distinct manner by both complexes, and therefore, our results show that native HDAC complexes are able to distinguish among the different acetyllysines in both histones.

EXPERIMENTAL PROCEDURES

Isolation of Pea HDAC Complexes. Pea crude HDACs were prepared by a method based on that of Sendra et al. (*12*). Pea seeds were imbibed for 18 h at 0−4 °C to avoid germination, and the embryonic axes (48 g) were excised from the cotyledons, frozen in liquid nitrogen, and ground in a mortar. The dry powder was suspended in a buffer containing 5 mM $MgCl_2$, 5 mM KCl, 0.25 M sucrose, 40% (v/v) glycerol, 0.25% (v/v) Triton X-100, 15 mM β-mercaptoethanol, and 20 mM Tris-HCl, pH 7.8 (buffer A), at a ratio of 4 mL of buffer/g of dry powder. The slurry was stirred on ice until the temperature rose to −10 °C, and it was further dispersed in a Potter−Elvehjem homogenizer with two strokes of the pestle. The suspension was filtered through two layers of cheesecloth and centrifuged at 10000*g* for 15 min. Then 160 mL of the supernatant was loaded onto a 40 mL Q-Sepharose Big Beads column, equilibrated in a buffer containing 10 mM NaCl, 0.25 mM EDTA, 10 mM β-mercaptoethanol, 10% (v/v) glycerol, 0.05% Tween, and 15 mM Tris-HCl, pH 7.9 (buffer B). The column was washed with 5 bed volumes of buffer B and eluted with 400 mL of a lineal gradient of NaCl (10−500 mM) in buffer B. Fractions of 7.2 mL were collected, and the HDAC activity was determined by a previously reported procedure (*12*). Briefly, histones labeled in vivo with [^{3}H]acetate were incubated with the fractions for 60 min at 37 °C. The released acetate was converted into acetic acid by adding HCl, extracted with ethyl acetate, and counted. The fractions showing enzymatic activity were pooled, concentrated to 25 mL, clarified by filtration, and loaded onto a 6 mL Q-Resource column, equilibrated with buffer B. The column was washed in an FPLC system (Pharmacia) with 5 mL of buffer B and eluted with 60 mL of a linear gradient of NaCl (10−500 mM) in buffer B. Fractions of 1.3 mL were recovered and analyzed for HDAC activity (*12*). The fractions corresponding to the peaks of HDAC activity were pooled and used without any further treatment.

Determination of Site Specificity of HDACs. Libraries of membrane-bound overlapping peptides containing the acetylated lysine residues of H3 and H4 in vivo were custom-synthesized by Genosys. The H3 peptides were as follows: (αAc-A)RT(ϵAc-K)QTAR, (αAc-A)RT(ϵAc-K)QTAR(ϵAc-K)STG, (αAc-T)GG(ϵAc-K)APR(ϵAc-K)QLAT, (αAc-A)-PR(ϵAc-K)QLAT(ϵAc-K)AAR, (αAc-Q)LAT(ϵAc-K)AAR-(ϵAc-K)SAP, and (αAc-A)AR(ϵAc-K)SAPSTGGV, where Ac stands for an acetyl group. The following H4 peptides were used: (αAc-S)GRG(ϵAc-K)GG, (αAc-G)RG(ϵAc-K)-GG(ϵAc-K)GLG, (αAc-G)G(ϵAc-K)GLG(ϵAc-K)GGA, (αAc-G)LG(ϵAc-K)GGA(ϵAc-K)RH, (αAc-G)GA(ϵAc-K)RHR-(ϵAc-K)IL, and (αAc-R)HR(ϵAc-K)ILRDNI.

The membranes were incubated with 50 mM citraconic anhydride in 100 mM borate buffer, pH 9.0, for 15 min on ice. An equal amount of fresh citraconic anhydride was then added, and the incubation was allowed to proceed for another 15 min. This step is necessary to block the otherwise reactive groups in the subsequent reaction with acetic anhydride. Omission of the citraconic anhydride blocking results in a high background on the finally processed membrane, which makes the detection of labeled spots more difficult. The membranes were then rinsed three times with buffer B and incubated for 40 min at 30 °C in small size cuvettes with 2 mL of the desired HDAC complex. A total of 400 μg of H1/H5 histones and 10 μL of a protease inhibitor cocktail (Sigma) were included. After incubation, the membranes were rinsed three times with buffer B and once with 100 mM borate buffer, pH 9.0. After incubation with the HDAC complexes, it is expected that the acetyl groups were removed from the acetyllysines in proportion to the substrate specificity of the enzyme. To quantify the acetate removal, membranes were then incubated on ice with 8 μCi/mL [^{14}C]acetic anhydride. After 30 min, a further amount (8 μCi/mL) of [^{14}C]acetic anhydride was added, and the incubation was extended for another 15 min. The membranes were then rinsed five times with borate buffer and dried for 5 min over Whatman paper at 70 °C, and the radioactivity was detected by autoradiography and quantified with a Packard Instant Imager. Our experimental protocol, therefore, results in the quantitative incorporation of labeled acetate in the sites where the HDAC activity has removed the acetyl groups.

Other Methods. Polyacrylamide gel electrophoresis was carried out according to Laemmli (*13*). For Western blot analysis (*14*) commercial antibodies against human HDAC1 and HDAC2 (Santa Cruz, C19) were used at a dilution 1:2000. These antibodies recognize epitopes corresponding to specific amino acid sequences located at the carboxyl terminus of each enzyme, which are specific to multicellular eukaryotic organisms, including plants and mammals (*15*). No cross-reactivity was detected between both HDACs.

RESULTS

The possibility of obtaining HDAC complexes with a minimal manipulation (*12*) prompted us to select *Pisum sativum* as the starting material. The HDAC activity of pea embryos is resolved into a major and a minor peak (Figure 1A), which correspond, respectively, to the previously reported HD1 and HD2 complexes (*12*). Pea HD2 activity is almost insensitive to ionic strength changes in the salt concentration range of the gradient used (*12*). Therefore, no corrections for the inhibitory effect of increasing ionic strength (*16*) have to be done, and the low activity of HD2 seems to indicate that this complex is less abundant than HD1.

Pooled fractions from peaks HD1 and HD2 were analyzed by SDS−PAGE. Figure 1B shows that both peaks contain a complex composition as expected from crude complexes. Western blot analyses demonstrate that catalytic subunits homologous to HDAC1 and HDAC2 are present in both complexes. Actually bands cross-reacting with antibodies against human HDAC1 and HDAC2 are present in both peaks (Figure 1B). The immuno-cross-reactivity between mammalian and plant Rpd3-like deacetylases was first observed by Loidl and co-workers (*17*). Nucleotide sequences of HD1 homologues are highly conserved with 55−96%

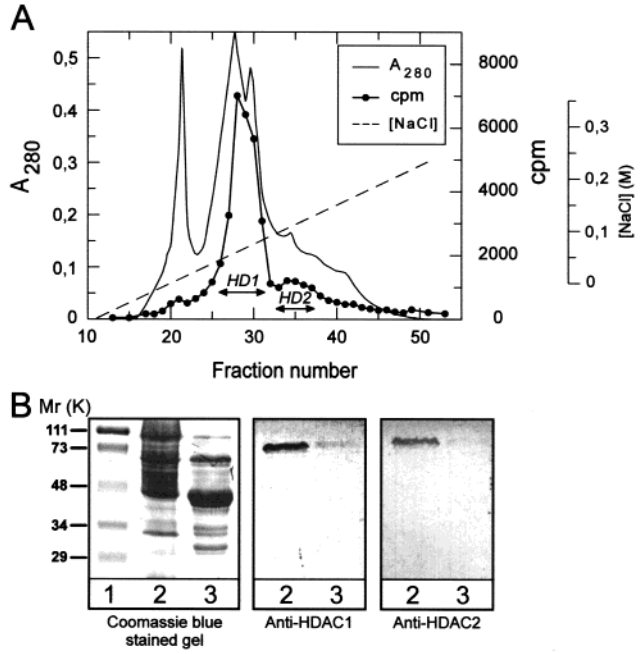

FIGURE 1: Ion-exchange chromatography on Q-Resource FPLC of pea histone deacetylase activity. Pea embryo histone deacetylase activity, prepared as described in Sendra et al. (*12*), was purified by Q-Sepharose Big Beads chromatography and loaded onto a 6 mL Q-Resource FPLC column. The pea HDAC complexes were eluted from the column by a lineal gradient of NaCl (10−500 mM). (A) Elution profile of proteins and HDAC activity. (B) SDS−PAGE and Western blots of the pooled fraction from peaks HD1 (lane 2) and HD2 (lane 3). Molecular weight markers were loaded on lane 1.

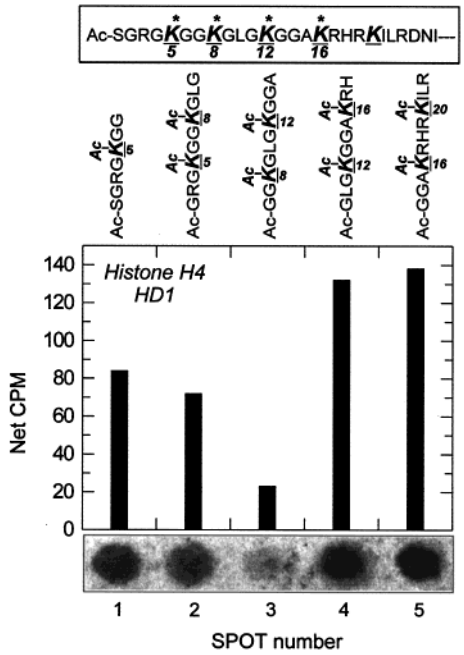

FIGURE 2: Site specificity of pea histone deacetylase complex HD1 with histone H4. The in vivo acetylatable lysines in histone H4 are marked in the upper panel. The sequences of acetylated histone H4 peptides coupled to SPOT's membranes are also shown. The lower panel corresponds to the evaluation of the level of HD1 enzymatic deacetylation of each histone H4 peptide and the fluorography of the membranes. The experimental protocol, as described under Experimental Procedures, results in the quantitative incorporation of labeled acetate in the sites where the HDAC activity has removed the acetyl groups.

overall identity among *Arabidopsis*, yeast, *Drosophila*, maize, and humans (*15*). Figure 1B shows that the pea enzymes which cross-react with HDAC1 and HDAC2 antibodies have an apparent molecular weight of about 70 000, i.e., somewhat higher than that of Rpd3-like mammalian and plant enzymes, which lie in the range 51 000−63 000 (*15*, *17*).

Peaks HD1 and HD2 have been used to address the question of site specificity. It should be pointed out that proteolysis during the HDAC assay was effectively inhibited. Histones H1/H5, which are very susceptible to proteolytic attack, were included in the assay both as a bait for proteases and as an indicator of proteolysis. No degradation of linker histones was detected after incubation (data not shown), suggesting that neither of the components of HDAC complexes is substantially degraded. The novel experimental procedure employed to study site specificity is described in detail under Experimental Procedures. The experiments were carried out in duplicate by using two different sets of membrane-bound peptides.

Figures 2 and 3 show the results obtained with HD1. In the case of H4 peptides (Figure 2), the low incorporation of acetic anhydride in spot 3 indicates that the acetyl groups in both lysines 8 and 12 are very resistant to removal by HD1. The high incorporation of acetic anhydride in spot 4 must then mean that lysine 16 is readily deacetylated by HD1. The validity of this result is further checked by the fact that the deacetylation of peptide in spot 5 is roughly similar to that of peptide in spot 4. Actually, the peptide in spot 5 contains an acetylated lysine in position 20, which is not a natural substrate for HDACs (see below). The similar incorporation of acetic anhydride in spots 1 and 2, together with the fact that acetyllysine 8 is a poor substrate (see above), allows us to conclude that HD1 can also deacetylate lysine 5, though to a lesser extent than lysine 16. To summarize the results of Figure 2, the deacetylation of H4 by HD1 is specific, with a strong preference for lysine 16 followed by lysine 5, while lysines 8 and 12 are only poorly deacetylated. The specificity for H3 (Figure 3) is not so strict. By applying a similar line of reasoning, it can be concluded that lysine 4 is readily deacetylated and that HD1 also accepts acetylated lysines 18 and 9 as substrates.

The specificity of complex HD2 (Figures 4 and 5) is markedly different. For instance, the preferred residues in H4 are lysines 8 and 5, followed by lysine 16, while the deacetylation of lysine 12, if any, is very limited (Figure 4). Complex HD2 also shows specificity in H3 deacetylation, sites 4 and 18 being preferred (Figure 5). To validate the method used to study HDAC specificity, negative controls were incorporated in the analysis. Examples of these controls are shown in Figures 4 and 5, where the membranes include peptides with nonacetylatable lysines, namely, lysine 20 of H4 and lysine 27 of H3. In both instances, the extent of deacetylation of chemically acetylated lysines is negligible.

DISCUSSION

The site specificity of HDACs has been much less explored than that of the HATs. From a technical point of view, the study of the site specificity of HATs can be performed by means of microsequencing, or by using site-specific antibodies, provided the latter do not exhibit any undesirable cross-reactivity. Conducting a similar study with HDACs either by Edman degradation or with specific

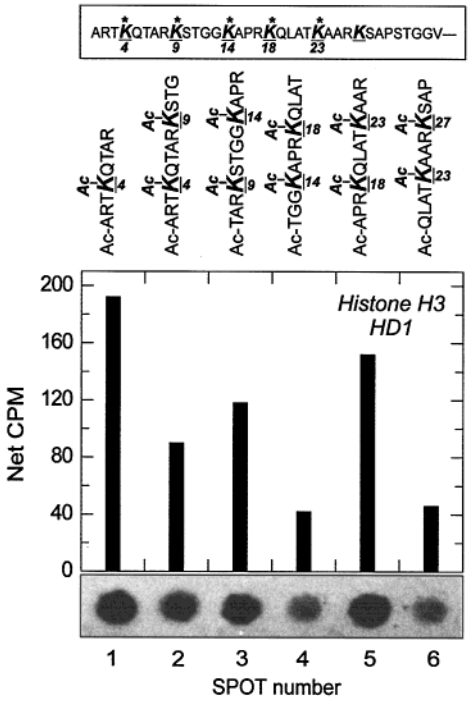

FIGURE 3: Site specificity of pea histone deacetylase complex HD1 with histone H3. The in vivo acetylatable lysines in histone H3 are marked in the upper panel. The sequences of acetylated histone H3 peptides coupled to SPOT's membranes are also shown. The lower panel corresponds to the evaluation of the level of HD1 enzymatic deacetylation of each histone H3 peptide and the fluorography of the membranes.

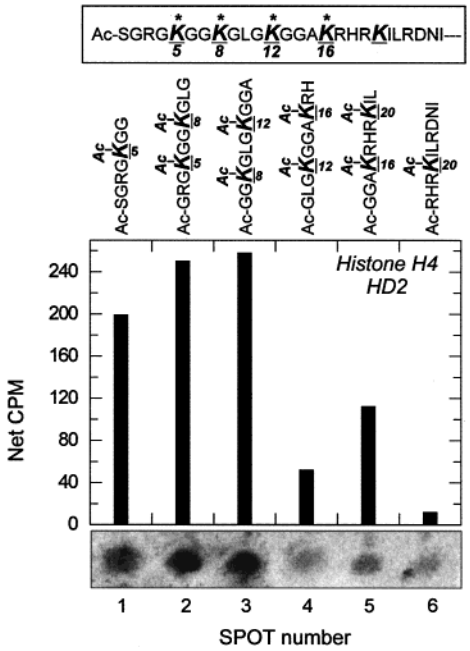

FIGURE 4: Site specificity of pea histone deacetylase complex HD2 with histone H4. The experiment was carried out as described in Figure 2 except that the HD2 complex was used in the SPOT's assay method.

antibodies poses several specific difficulties. The substrates used for the deacetylase assay contain one or more acetyllysines, and unless the reaction has occurred almost to completion, the interpretation of results would require a quantitative, differential estimation of the content of the acetyl groups before and after the reaction. Therefore, the

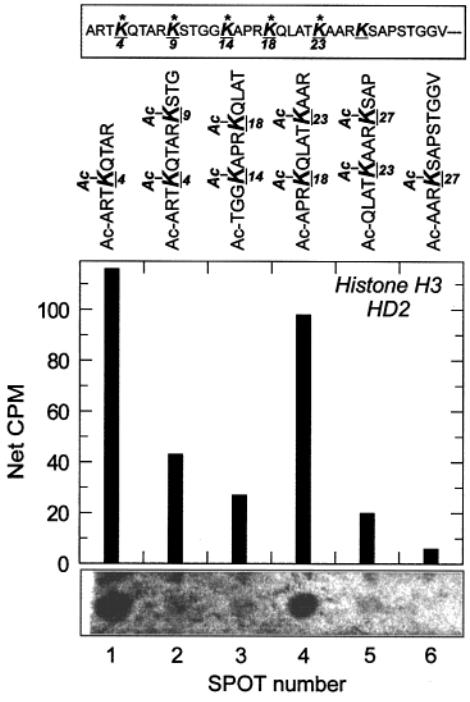

FIGURE 5: Site specificity of pea histone deacetylase complex HD2 with histone H3. The experiment was carried out as described in Figure 3 except that the HD2 complex was used in the SPOT's assay method.

obtention of results relies on the measurement of these differences, which, in turn, requires an accurate measurement of the residual acetyl groups. This is not an easy task when microsequencing methods are used, and the difficulties are even more formidable when using specific antibodies. On the other hand, the use of reaction conditions in which deacetylation proceeds to completion has the obvious disadvantage that the differences among the deacetylation rate of the diverse residues may be obscured. The recourse to site-specific antibodies in combination with acetic acid−urea−Triton gels (*18*) partially circumvents the latter inconveniences, but additional difficulties arise in the interpretation of the results.

Other methods have been recently described in which the deacetylated lysines were quantitatively determined either by using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (*19*) or by a combination of Edman degradation and HPLC (*20*). In this way, deacetylation of lysines in yields as low as 10−20% can be readily quantified, but these procedures either are laborious and time-consuming or require the use of specialized instruments.

The procedure described here allows a quick biochemical determination of the site specificity of histone deacetylation, which circumvents the inconveniences inherent in the use of site-specific antibodies. The selected peptides are long enough to include the sequence motifs presumably recognized by the enzyme. Although no data on the specificity determinants of HDACs are available, it has been reported that the sequence specificity of the yeast HAT Gcn5 requires five residues at each side of lysine 14, the enzyme target in the histone H3 (*21*). This consideration prompted us to usually select peptides 11 residues long. This length usually implies that most of the peptides contain two acetyllysines,

but this overlapping does not normally result in ambiguities. On the contrary, it often provides clearer conclusions. For instance, the resistance of lysines 8 and 12 of H4 to be deacetylated by HD1 is obvious from both the low amount of radioactivity in the peptide containing both residues (Figure 2) and the roughly equivalent amount of radioactivity in peptides 1−2 and 4−5.

The above-discussed novel procedure has been applied to the study of site specificity of HDAC complexes. Both HAT and HDAC catalytically active subunits are complexed with several other proteins that fulfill either regulatory or targeting functions (*8*, *9*, *22*). In studying the specificity of these enzymes, special attention should be paid to prevent their excessive manipulation, because the loss of nonenzymatic components of a complex may change its histone and/or the site specificity. It has been shown, for instance, that the yeast complexes ADA and SAGA, which share the same HAT activity, namely, Gcn5p, exhibit distinct site specificity in H3. ADA acetylates lysines 14 and 18, while SAGA also acetylates lysines 9 and 23 (*23*). This specific behavior contrasts with that of recombinant Gcn5 alone, which only acetylates lysine 14 of free H3 and is not able to accept nucleosomes as substrate (*24*). Therefore, the noncatalytic partners of these HAT complexes not only permit them to acetylate nucleosomes but also extend in a specific way the usage of lysyl residues. In this way, the less a complex is altered during its preparation the more reliable the information on its specificity. Assuming that the above circumstances are also valid for HDACs, we conducted our study with HD1 and HD2 complexes from ungerminated pea embryonic axes (*12*). There are two reasons for this choice. First, a two-step chromatographic procedure allows the separation of both complexes. Second, in the ungerminated embryonic axes, the proteolytic activity in the conditions used for HDAC assay is almost negligible, as we have ascertained in the present work. Both circumstances imply that the enzymatic assay is carried out with intact complexes. An additional, minor HDAC complex is present in germinating pea embryos, which possess a high activity toward acetylated H4 (*12*). This complex, which accumulates when DNA replication starts (24−30 h after the onset of germination), probably is involved in the deacetylation of H4 after chromatin assembly, but the concomitant increase in proteolytic activity makes it difficult to handle it without loss of its components.

It has been recently pointed out that the two major HDAC complexes in higher eukaryotes, namely, NURD and SIN3, are involved in specific functions in development rather than in maintaining a given homeostasis of histone acetyl groups (for a review, see ref *24*). Pea HD1, which is also the major complex not only in the ungerminated embryo (Figure 1) but also after the onset of germination (*12*), contains HDAC activities homologous to HDAC1 and HDAC2 (Figure 1B), the catalytic subunits of both NURD and SIN3. It is tempting to speculate that HD1 is involved in the processes of embryo development and hence its abundance at this stage of the plant life cycle. For instance, this behavior seems to be the case of maize histone deacetylase HD1, whose activity is low at the initial stage of differentiation and becomes the predominant enzyme at later stages (*25*). Components similar to those of NURD and SIN3 have been found as part of plant HDACs. For instance, a protein related to the retinoblastoma-associated protein, Rbap46, has been described associated to a maize Rpd3-type deacetylase (*17*). On the other hand, *PKL*, a gene sharing homology to the component of NURD Mi-2, has been implicated in the transition from embryonic to vegetative development in *Arabidopsis* (*26*).

The available data on plant deacetylase complexes have been recently reviewed (*27*). Rpd3-type complexes, similar to that found in other eukaryotes, are well characterized, but there are, in addition, some peculiar complexes, such as the complex containing the nucleolar deacetylase HD2 (*28*). Plant HDAC complexes obviously should be studied in more detail.

Finally, the present results show that intact HDAC complexes are site-specific, as are some recombinant enzymes (*19*, *20*). Our results add to those of the Loidl's group, who had previously examined the specificity of maize HDAC complexes toward positions 5, 8, and 12 of H4 (*18*). Therefore, it seems obvious that the specificity of both HAT and HDAC complexes has to be studied to understand the significance of a map of posttranslational signals in the N-terminal histone tails. In this context, as the acetylation state of a given lysine results from the relative activities of both HATs and HDACs, the analysis of the regulatory mechanisms of both HATs and HDACs also seems extremely important.

ACKNOWLEDGMENT

The authors thank Drs. Peter Loidl and Elena I. Georgieva for advice and valuable comments on the manuscript.

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BI0100844