Subcellular Location of Enzymes Involved in Core Histone Acetylation†

A. Grabher,‡ G. Brosch,‡ R. Sendra,§ T. Lechner,‡ A. Eberharter,‡ E. I. Georgieva,‡ G. López-Rodas,§ L. Franco,§ H. Dietrich,|| and P. Loidl*,‡

Department of Microbiology and Central Laboratory Animal Facilities, University of Innsbruck, Medical School, Fritz-Preglstr.3, A-6020 Innsbruck, Austria, and Department of Biochemistry and Molecular Biology, University of Valencia, 46100 Burjassot, Valencia, Spain

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ABSTRACT: Multiple enzyme forms of histone deacetylase and histone acetyltransferase exist in germinating maize embryos. We analyzed the association of the different enzymes to chromatin by ion exchange chromatography of subcellular fractions from different time points of embryo germination. The vast majority of histone deacetylase HD-1A was not bound to chromatin, since it was solubilized during chromatin isolation, regardless of its phosphorylation state and the phase of embryo germination. In contrast, HD-2 was chromatin bound during the entire germination pathway. Histone deacetylase HD-1B was present in a chromatin-bound and a soluble form; the ratio between these two forms changed during germination. Both nuclear histone acetyltransferases, HAT-A1 and HAT-A2, were tightly chromatin-bound and could only be released from chromatin by salt extraction. To test whether histone acetyltransferases or deacetylases are associated with the nuclear matrix, we analyzed nuclear matrix preparations from yeast, *Physarum*, and maize step by step for both enzyme activities. This analysis confirmed that part of the activity is chromatin bound, but no significant enzyme activity could be found in the final nuclear matrix, regardless of the preparation protocol. This result was further substantiated by detailed analysis of histone deacetylases and acetyltransferases during cellular fractionation and nuclear matrix preparation of chicken erythrocytes. Altogether our results suggest that the participation of these enzymes in different nuclear processes may partly be regulated by a distinct location to intranuclear components.

Core histones can be reversibly modified by a number of posttranslational reactions, such as phosphorylation, acetylation, ADP-ribosylation, and ubiquitination (Bradbury, 1992). The posttranslational acetylation of the ϵ-amino group of lysine residues within the N-terminal histone domains was first discovered by Allfrey and co-workers (Allfrey et al., 1964) and is assumed to play an important role in the modulation of structural transitions of chromatin during different nuclear processes, although the precise mechanisms are still far from clear (Loidl, 1988, 1994; Turner, 1991; López-Rodas et al., 1993; Tordera et al., 1993).

The dynamic state of histone acetylation is maintained by two enzyme activities, histone acetyltransferase and histone deacetylase. Histone acetyltransferases link the acetyl moiety of acetyl-CoA to the ϵ-amino group of specific lysine residues. This modification can be reversed by the action of deacetylases. There are 26−28 possible acetylation sites within a nucleosome (Doenecke & Gallwitz, 1982). The usage of these lysine residues by histone acetyltransferases has been shown to occur in a nonrandom fashion (Turner & Fellows, 1989; Thorne et al., 1990; Clarke et al., 1993), although recent *in vitro* studies have shown that histone acetyltransferase B from pea does not display a strict order of lysine site usage (Mingarro et al., 1993). Histone acetyltransferases can be classified with respect to their intracellular location and substrate specificity into nuclear A-type and cytoplasmic B-type enzymes. Extensive investigations of the substrate specificity of histone acetyltransferases in yeast, pea, maize, and *Physarum* have revealed that H3 and H4 are the predominant substrate molecules for these enzymes (Sendra et al., 1986; López-Rodas et al., 1989; López-Rodas et al., 1991a,b; López-Rodas et al., 1992), whereas H2A and H2B only represent minor substrates, although multiple enzymes or enzyme forms exist.

Histone deacetylases have been most extensively studied in plant cells (Sendra et al., 1988; López-Rodas et al., 1991b; Brosch et al., 1992). In maize embryos three distinct enzymes are present, HD-1A, HD-1B, and HD-2, which differ in their substrate specificity. The deacetylases follow a distinct individual activity pattern during maize embryo germination, indicating distinct and individual functions during the differentiation program (Georgieva et al., 1991). The substrate specificity of HD-1A is furthermore regulated by reversible phosphorylation (Brosch et al., 1992); the fact that histone deacetylase HD-1A is phosphorylated and as a consequence exhibits a shift in its substrate specificity provide evidence that this enzyme could be involved in cellular signal transduction pathways.

It has been recently claimed that histone deacetylases are specifically associated with the nuclear matrix, representing

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* Author to whom correspondence should be addressed.

‡ Department of Microbiology, University of Innsbruck.

§ Department of Biochemistry and Molecular Biology, University of Valencia.

|| Central Laboratory Animal Facilities, University of Innsbruck.

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matrin proteins (Hendzel & Davie, 1992). Nuclear matrins have been identified as a class of internal matrix proteins, common to different mammalian cell types (Belgrader et al., 1991; Hakes & Berezney, 1991). In contrast to Hendzel and Davie (1992), we could show in maize embryos that histone deacetylase activity of the nuclear matrix only represents a very small residual proportion of the total cellular activity (Brosch et al., 1992). In order to substantiate this finding we prepared nuclear matrices with different preparation procedures from yeast, *Physarum*, maize, and chicken erythrocytes to test for histone deacetylase and histone acetyltransferase activity. In accordance with our previous findings, we did not detect significant enzyme activities of either enzyme in the final nuclear matrix preparations. Using ion exchange chromatography we could show that the different forms of histone deacetylases and histone acetyltransferases differ from each other with respect to their solubility and chromatin association. Moreover, we could demonstrate that the ratio of chromatin bound/soluble deacetylase HD-1B transiently changes during embryo germination; this indicates that location of the enzymes represents an additional level of regulation for changes in the acetylation of core histones.

EXPERIMENTAL PROCEDURES

Cultivation of Organisms. Maize seeds (*Zea mays* M320; Institute of Genetics, Bulgarian Academy of Sciences, Sofia, Bulgaria) were germinated in darkness for various periods of time (0, 12, 24, 40, and 72 h) on cotton layers soaked with water at 28 °C, 4 g of whole embryos (0, 12, 24 h) and of meristematic tissue of the root (40, 72 h) were harvested into liquid nitrogen for chromatin preparation (Steinmüller & Apel, 1986) or nuclear isolation (López-Rodas et al., 1991a).

Exponentially growing microplasmodia of the acellular myxomycete *Physarum polycephalum* (strain M_3b, a Wis1 isolate) were cultivated in semidefined nutrient medium at 26 °C under reciprocal shaking as described (Daniel & Baldwin, 1964). Microplasmodia were harvested by centrifugation at 300*g* for 30 s and processed for nuclear isolation (Loidl & Gröbner, 1987) or *in situ* nuclear matrix preparation (Waitz & Loidl, 1988).

Saccharomyces cerevisiae (strain 1383 CECT; ATCC 9763) was cultivated as described (López-Rodas et al., 1985). Spheroplasts were obtained as described (Lohr & Ide, 1978). Cells were incubated for 30 min in 100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 75 mM 2-mercaptoethanol at 4 °C. After centrifugation at 3000*g* for 5 min, the cells were washed with digestion buffer (1.1 M sorbitol, 24 mM sodium phosphate, pH 6.5). The cellular sediment was resuspended in digestion buffer at a density of 0.2 g of cells/mL of buffer and 2 mg of Novozym 234 (Novo BioLabs, Bagsvaerd, Denmark)/g of cells was added. The suspension was kept at 37 °C for 10 min under gentle shaking. The quality of the spheroplasts was checked by light microscopy. Spheroplasts were collected by centrifugation at 4000*g* for 10 min. Novozym was eliminated by three washings with digestion medium. Spheroplasts were used for nuclear matrix preparation as described below.

Preparation of Soluble and Chromatin Fractions. For subcellular localization of histone deacetylases and histone acetyltransferases in maize, frozen embryos [2 g of dry embryos (0 h) or 4 g of 12 and 24 h] and meristematic parts of the root (40, 72 h) were ground to powder in a mortar. Chromatin was prepared following an established procedure (Steinmüller & Apel, 1986). Briefly, frozen tissue powder was suspended in 20 mL of homogenization buffer [20 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 5 mM KCl, 0.25 M sucrose, 0.25% (v/v) Triton-X-100, 0.1% (v/v) 2-mercaptoethanol, 40% (v/v) glycerol] at −20 °C. The mixture was stirred until the temperature reached −10 °C, homogenized in a Potter-Elvehjem homogenizer, and filtered consecutively through 200 and 100 μm nylon membrane. After centrifugation at 10000*g* for 15 min at 0 °C the supernatant was decanted and saved. This supernatant is referred to as the "soluble" fraction and it contains soluble cytoplasmic as well as soluble nuclear proteins. The pellet was washed twice in homogenization buffer (20 mL each, with a centrifugation step at 6000*g* for 15 min at 4 °C) and resuspended in 10 mL of buffer B [15 mM Tris-HCl, pH 7.9, 10 mM NH_4Cl, 0.25 mM EDTA, 10 mM 2-mercaptoethanol, 10% (v/v) glycerol]. The solution was stirred for 2 h at 4 °C and subsequently centrifuged for 15 min at 12000*g*. The resulting supernatant is referred to as "chromatin" fraction. Both supernatants (soluble and chromatin) were then digested with alkaline phosphatase as described (Brosch et al., 1992). For this purpose the solutions were adjusted to 5 mM $MgCl_2$ and kept on ice for 10 min. Alkaline phosphatase (from bovine intestinal mucosa, type VII-N, Sigma, St. Louis, MO) was added (130 IU/mL) and the mixture incubated for 1 h at 28 °C. After phosphatase digestion, samples were subjected to Mono-Q HPLC for separation of histone deacetylases.

Preparation of the Nuclear Matrix. For the analysis of enzyme activities in nuclear matrix preparations, isolated nuclei of maize, microplasmodia, or isolated nuclei of *Physarum* and spheroplasts of yeast were encapsulated into agarose beads as described elsewhere (Waitz & Loidl, 1988; Eberharter et al., 1993). Encapsulated samples were processed in two different ways.

(1) Encapsulated samples (in the case of *Physarum*, whole microplasmodia) were washed with buffer I [20 mM Tris-HCl, pH 7.3, 20 mM KCl, 0.05 mM spermine, 0.125 mM spermidine, 0.5 mM EDTA−KOH, pH 7.3, 0.2 mM PMSF, 1% (v/v) thiodiglycol]. After washing the pellet with buffer I containing 0.75% Triton-X-100, beads were incubated in the same solution for 10 min on ice. After centrifugation both supernatants were combined. The beads were incubated in 1 M NaCl (in buffer I) for 10 min at 4 °C, centrifuged at 1000*g* for 5 min, and then incubated in 2 M NaCl (in buffer I) for 10 min on ice. After centrifugation, the pellet was washed with buffer II (buffer I without EDTA, but containing 50 mM NaCl and 5 mM $MgCl_2$) and incubated with DNase I (50 μg/mL) in buffer II for 20 min at 25 °C. After DNaseI digestion beads were washed with buffer I. The resulting pellet is referred to as "standard nuclear matrix" and was analyzed for histone acetyltransferase and histone deacetylase activity.

(2) Encapsulated samples (in the case of *Physarum*, isolated nuclei) were subjected to a nuclear matrix preparation protocol similar to that described by Hendzel and Davie (1992), except that treatment with DNase I was the last step in the matrix preparation scheme. Nuclei were incubated with buffer 1 (10 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 0.2 mM $MgCl_2$, 0,75% Triton-X-100) for 10 min at 4 °C. After centrifugation the pelleted beads were incubated

consecutively in 0.4 M KCl (in buffer 1) and in 1 M KCl (in buffer 1) each for 10 min on ice. Subsequently the pellet was resuspended in buffer 1 and incubated with DNase I (250 μg/mL) for 1 h at 25 °C. After centrifugation at 1000*g* for 5 min at 4 °C the resulting pellet was analyzed for enzyme activities.

For subcellular fractionation and nuclear matrix preparation of chicken erythrocytes, male white Leghorn chicken of the obese strain were used (Dietrich, 1989). A 25 mL sample of blood was washed twice with buffer C (10 mM Tris-HCl, pH 7.1, 25 mM sodium citrate, 0.14 M NaCl) with centrifugation at 500*g* at 4 °C for 5 min. The cell sediment was resuspended in 5 vol of lysis buffer [10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 10 mM NaCl, 0.5% (v/v) Triton-X-100] and incubated under gentle shaking for 30 min. After checking the lysis of the cells under phase contrast, nuclei were pelleted at 2000*g* for 10 min in a fixed-angle rotor. Nuclei were resuspended in an excess of wash buffer (lysis buffer without Triton) and centrifuged at 2000*g* for 10 min. This washing was repeated five or six times until hemoglobin was completely removed. The cell lysate and the combined wash supernatants were analyzed for histone deacetylase and histone acetyltransferase activity.

Isolated nuclei were split into two aliquots: aliquot 1 was resuspended in buffer II [20 mM Tris-HCl, pH 7.3, 20 mM KCl, 0.05 mM spermine, 0.125 mM spermidine, pH 7.3, 0.2 mM PMSF, 1% (v/v) thiodiglycol] and digested with DNase I (50 μg/mL) for 20 min at 25 °C. Subsequently nuclei were pelleted at 2000*g* for 5 min and washed once with buffer I [20 mM Tris-HCl, pH 7.3, 20 mM KCl, 0.05 mM spermine, 0.125 mM spermidine, 0.5 mM EDTA−KOH, pH 7.3, 0.2 mM PMSF, 1% (v/v) thiodiglycol]. After centrifugation nuclei were resuspended in 1 M NaCl (in buffer I) for 10 min at 4 °C, again centrifuged at 2000*g* for 5 min, and then incubated in 2 M NaCl (in buffer I) for 10 min on ice. After centrifugation the resulting nuclear matrix was washed with buffer I.

Aliquot 2 was resuspended in buffer 1 (10 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 0.2 mM $MgCl_2$) and incubated with DNaseI (250 μg/mL) for 1 h at 25 °C. After centrifugation at 2000*g* for 5 min nuclei were washed in buffer 1. Nuclei were consecutively incubated in 0.4 M KCl (in buffer 1) and 1 M KCl (in buffer 1) with a centrifugation step in between. The resulting nuclear matrix was finally washed in buffer 1. Sample aliquots of all steps of preparation were analyzed for histone deacetylase and histone acetyltransferase activity.

Chromatographic Procedures. Mono-Q-HPLC. Samples (soluble and chromatin) were filtered through 0.22 μm millipore filters and the whole chromatin sample and half of the soluble fraction (equivalent to 2 g of embryos), respectively, were loaded onto a Mono-Q HR5/5 column (Pharmacia-Biosystems, Uppsala, Sweden). Proteins were eluted with 80 mL of a linear gradient of NH_4Cl (0.01−0.5 M) in buffer B with a flow of 0.8 mL/min. Fractions of 1.2 mL were collected and assayed for histone deacetylase activity.

DEAE Ion Exchange Chromatography. The soluble fraction (equivalent to 4 g of embryos harvested at 72 h of germination) was dialyzed against buffer B, filtered, and applied onto a column of DEAE-Sepharose CL-6B (10 mL; Pharmacia-Biosystems, Uppsala, Sweden). Proteins were eluted with 60 mL of a linear NH_4Cl gradient (0.01−0.5 M) with a flow of 12 mL/h. Fractions of 1.2 mL were collected and assayed for histone acetyltransferase activity.

Resource-Q-FPLC. The first pellet after homogenization of frozen tissue powder (see chromatin preparation) was resuspended in 0.5 M NaCl (in buffer B) and extracted for 30 min on ice under gentle stirring. After centrifugation at 100000*g* for 65 min, the supernatant was dialyzed against buffer B and an amount equivalent to 15 g of embryos (72 h) was applied to a 1 mL Resource-Q FPLC column (Pharmacia-Biosystems, Uppsala, Sweden). Proteins were eluted with 30 mL of a linear NH_4Cl gradient (0.01−0.5 M) at a flow of 1 mL/min. Fractions of 1.2 mL were collected and assayed for histone acetyltransferase activity. In this case the amount of [^{14}C]acetyl-CoA was 5-fold as compared to the standard assay.

Enzyme Activity Assays. Histone Deacetylase Assay. Histone deacetylase activity was determined as described (Sendra et al., 1988) using [^{3}H]acetate-prelabeled chicken erythrocyte histones (Ferenz & Nelson, 1985) as substrate. Samples taken during the sequential steps of the nuclear matrix preparation were dialyzed against buffer B prior to the enzyme assay. Sample aliquots of 100 μL in buffer B were mixed with 10 μL of total [^{3}H]acetate-prelabeled chicken erythrocyte histones (1.5 mg/mL). This mixture was incubated at 37 °C for 1 h. The reaction was stopped by addition of 36 μL of 1 N HCl, 0.4 M acetate and 0.8 mL of ethyl acetate. After centrifugation at 10000*g* for 5 min an aliquot of 600 μL of the upper (organic) phase was counted for radioactivity in 5 mL of liquid scintillation cocktail.

Histone Acetyltransferase Assay. Histone acetyltransferase activity was assayed with chicken erythrocyte whole histones as substrate as described previously (López-Rodas et al., 1985; López-Rodas et al., 1991a). A 100 μL portion of sample was incubated with 120 μg of histone substrate and 0.05 μCi of [1-^{14}C]acetyl-CoA (ICN Biomedicals, Costa Mesa, CA; 60 mCi/mmol) in a final volume of 125 μL for 20 min at 37 °C. An aliquot of 100 μL of the reaction mixture was collected onto a glass fiber filter (Whatman GF/F). Filters were air-dried for 5 min and then submersed in ice-cold 25% (w/v) trichloroacetic acid. After 20 min filters were washed twice with 25% trichloroacetic acid, once with ethanol, once with a mixture of ethanol:ethyl ether (1:1, v/v), and finally with ethyl ether. After drying of the filters for 10 min at 70 °C, radioactivity was measured by liquid scintillation spectrophotometry.

RESULTS

Histone deacetylases are nuclear enzymes; in principal these enzymes may exist as soluble, chromatin-bound or nuclear matrix-associated forms. In maize there are three distinct histone deacetylases with different individual patterns of activity during embryo germination (Georgieva et al., 1991; Brosch et al., 1992). Preliminary experiments, using Mg^{2+} precipitation of chromatin from crude maize extracts, indicated differences among these enzymes with respect to their association to chromatin (results not shown). We therefore started out by investigating the intranuclear location of histone deacetylases HD-1A, HD-1B, and HD-2, analyzing a soluble fraction, isolated chromatin, and nuclear matrix preparations. For a clearcut separation of HD-1A from HD-1B, it is necessary to treat samples with alkaline phosphatase in order to shift HD-1A into its dephosphorylated form,

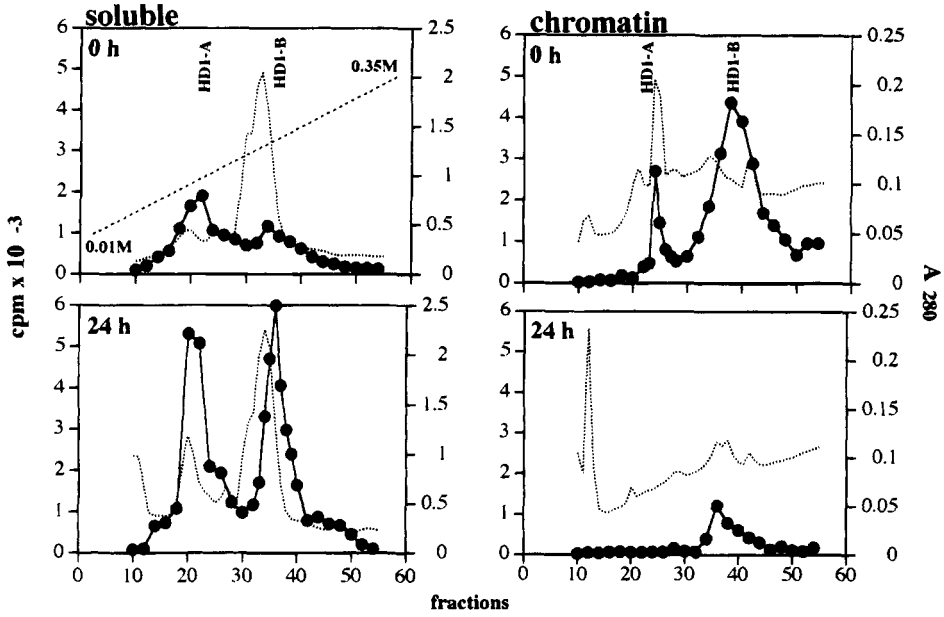

FIGURE 1: Distribution of soluble and chromatin-bound histone deacetylases HD-1A and HD-1B in maize embryos. Embryos at 0 and 24 h of germination were harvested and a soluble and chromatin-bound fraction were prepared. After phosphatase digestion samples were subjected to Mono-Q HPLC and proteins were eluted with a linear salt gradient (shown from 0.01−0.35 M). Histone deacetylase HD-1A eluted at ~170 mM NH_4Cl and HD-1B at 290 mM NH_4Cl. Enzyme activity is expressed as cpm; protein concentration was monitored by A_{280} measurement. The positions of HD-1A and HD-1B are indicated. Note that the position of HD-2 elution (~0.4 M) is not included in the graphs. ●, histone deacetylase activity (cpm); •••, A_{280}.

which then separates well from the HD-1B peak during anion exchange chromatography (Brosch et al., 1992).

Chromatin Association of Histone Deacetylases HD-1A, HD-1B, and HD-2 during Maize Embryo Germination. When embryos at 24 h of germination were separated into a soluble and chromatin fraction, Mono-Q HPLC revealed that HD-1A was exclusively present in the soluble fraction, whereas HD-1B was also bound to chromatin, although the proportion of soluble HD-1B was predominant (Figure 1). In contrast, at the start of embryo germination (0 h), HD-1A was present as a soluble and chromatin-bound form and HD-1B was predominant in chromatin. Therefore, a pronounced difference exists between HD-1A and HD-1B with respect to chromatin association at these time points of germination.

To elucidate the changes of distribution of HD-1A and HD-1B during the embryo differentiation pathway, we analyzed the chromatin-bound and soluble fraction at 0, 12, 24, 40 and 72 h after start of seed imbibition. The quantitative evaluation of all chromatographic profiles is shown in Figure 2. Total HD-1A activity showed little variation during embryo germination. Total HD-1B activity decreased to about 30% of the initial activity during progression from 0 to 72 h (Figure 2A). At the start of germination more than 60% of the total HD-1 activity was chromatin-bound. This value steadily decreased during germination; at 72 h only ~30% of the HD-1 activity was still associated with chromatin (Figure 2B). Interestingly, the soluble form of HD-1 sharply increased during the time course from 0 to 24 h of germination (more than a 2-fold increase), but returned to the initial activity at 40 and 72 h (Figure 2B). Figure 2C shows that HD-1A and HD-1B contribute to the soluble enzyme activity with different proportions during germination. At the start of germination the main soluble HD-1 activity was due to HD-1A. At 12 h this ratio completely changed to the opposite. During the further time course of germination the proportion of HD-1B in the soluble fraction steadily decreased. At 72 h only ~15% of the soluble enzyme activity was due to HD-1B; the vast majority of soluble HD-1 comprised of HD-1A (Figure 2C).

As a control we performed chromatographies with samples that had not been treated with phosphatase; except the weak separation of HD-1A and HD-1B in those cases, where both enzymes were present in roughly equal amounts, omission of phosphatase digestion did not change the results.

HD-2, which elutes at an ionic strength of ~0.4 M salt during Q-Sepharose chromatography, was always found in the chromatin-bound fraction, regardless of the stage of embryo germination; no HD-2 activity could be detected in the soluble fraction of either germination stage (results not shown).

Figure 2D shows that 70% of the total cellular histone deacetylase activity (HD-1A, HD-1B, HD-2) is chromatin-bound at the start of embryo germination. This value decreases transiently to ~25% at 24 h to stay constant at a level of 50% during later stages of germination. The percentage of total soluble deacetylase activity exhibits a corresponding increase until 24 h after start of seed imbibition (Figure 2D).

We would like to point out that we checked each of the washing steps during cellular fractionation for a possible loss of enzyme activity. However, none of the washings contained enzyme activity; therefore the recovered activity in the soluble and chromatin-bound fraction represents the total cellular histone deacetylase activity.

Location of Maize Histone Acetyltransferases. DEAE-Sepharose chromatography of the soluble fraction (whole seedlings at 72 h of germination) revealed only one histone acetyltransferase peak (Figure 3); this peak corresponds to the cytoplasmic HAT-B enzyme (López-Rodas et al., 1991a). When chromatin preparations were applied to DEAE-

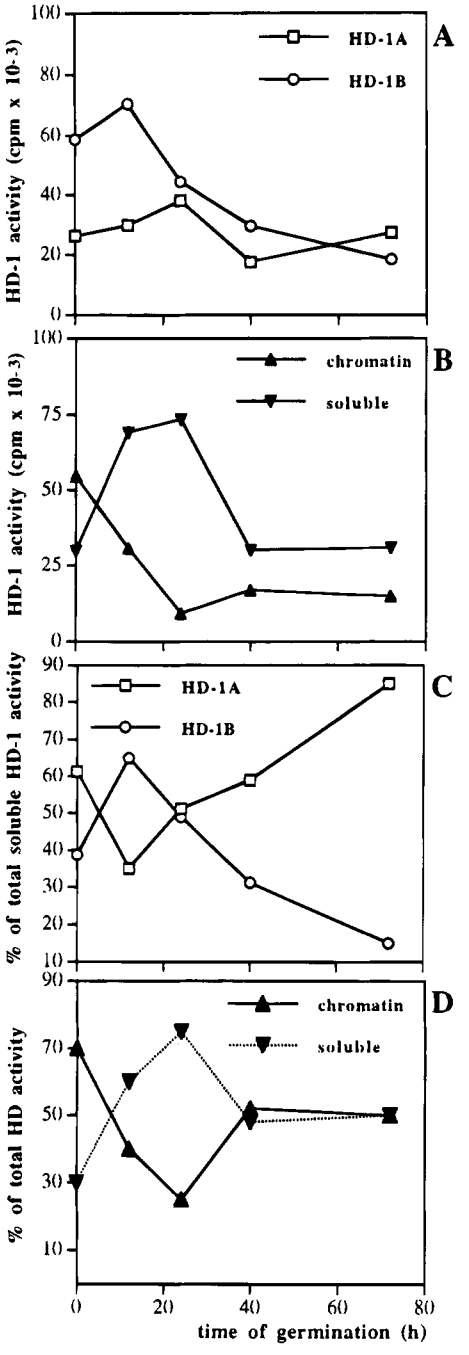

FIGURE 2: Pattern of histone deacetylase activity during maize embryo germination. (A) total enzyme activity of histone deacetylases HD-1A and HD-1B; cpm were calculated from chromatographic profiles of samples from 0, 12, 24, 40, and 72 h of embryo germination; (B) chromatin-bound and soluble histone deacetylase HD-1 activity; cpm were calculated from chromatographic profiles; (C) percentage of the total soluble HD-1 activity of histone deacetylases HD-1A and HD-1B; (D) percentage of total histone deacetylase activity (HD-1A, HD-1B, HD-2) in chromatin-bound and soluble form.

Sepharose or Resource-Q columns, enzyme activity could be hardly detected in the chromatographic fractions (results not shown). This indicated a tight association of the nuclear histone acetyltransferases to chromatin. We then extracted the crude chromatin pellet with 0.5 M NaCl and subjected the extract to Q-Resource-FPLC. Two peaks of enzyme activity were detected (Figure 3), which in terms of elution characteristics correspond to the two nuclear enzymes HAT-A1 and HAT-A2 (López-Rodas et al., 1991a). We could not get evidence that the tight chromatin association of HAT-A1 and HAT-A2 changes during the different stages of germination.

Histone Acetyltransferases and Histone Deacetylases Are Not Specifically Associated with the Nuclear Matrix. Apart from soluble and chromatin-bound forms, nuclear enzymes may also be part of the nuclear matrix. It has been reported that histone deacetylase represents a constituent of the internal nuclear matrix (Hendzel & Davie, 1992; Davie & Hendzel, 1994). However, previous results from our laboratory argued against this claim (Brosch et al., 1992). We therefore analyzed the different steps of nuclear matrix preparation for histone acetyltransferase and histone deacetylase activity in three different organisms, *Z. mays*, *Physarum polycephalum*, and *Saccharomyces cerevisiae*. Two different matrix preparation procedures were compared to exclude the possibility of artefacts due to a specific preparation protocol. Isolated nuclei of *Physarum* and maize and spheroplasts of yeast were encapsulated in agarose beads and the nuclear matrix was prepared according to the method of Hendzel and Davie (1992). Apart from a minor activity which was liberated by washing the samples and lysing them with Triton-X-100, the vast majority of the enzyme activity (histone acetyltransferase and histone deacetylase) was solubilized by sequential extraction with 0.4 and 1.0 M KCl (more than 65%). A small amount of activity was solubilized during DNase I treatment (5−15%), whereas the final nuclear matrix only contained trace amounts of enzyme activity, below 5% in all cases (Figure 4, left panels). The same results were obtained when we encapsulated yeast spheroplasts, whole *Physarum* microplasmodia, and isolated maize nuclei and prepared the nuclear matrix by a different procedure (Waitz & Loidl, 1988), using high NaCl concentrations for extraction of proteins (standard matrix). Again the predominant proportion solubilized during salt extraction and only 2−4% of the activity could be detected in the final nuclear matrix (Figure 4, right panels). The results did not differ among the three organisms. It should be noted that changes in the preparation protocol (presence/absence of mercaptoethanol or dithiothreitol, different sequence of extraction steps), omission of agarose encapsulation, and the cell cycle (*Physarum*) or germination stage (maize) did not influence the results (data not shown).

To substantiate these findings in vertebrate cells we carefully analyzed enzyme activities at each step of subcellular fractionation and nuclear matrix preparation in chicken erythrocytes. When chicken erythrocytes were lysed in detergent-containing buffer and washed repeatedly, more than 80% of the total histone deacetylase activity were released (Figure 5). The remaining activity in the isolated nuclei (less than 20% of the total activity) could not be solubilized by further low salt washing steps. When the nuclei were then digested with DNase I, part of this nuclear activity (35−40%) was released. Deproteinization with consecutive high salt extraction steps (either using 1 and 2 M NaCl (Figure 5, upper part) or 0.4 and 1 M KCl (Figure 5, lower part) caused solubilization of 40−50% of the nuclear histone deacetylase activity. Only ~3% was found in the nuclear matrix. If one considers that less than 20% of the total activity was retained in the isolated nuclei, the percentage of matrix-associated deacetylase only amounts to ~0.5% of the total enzyme activity. Essentially the same results were obtained when using rat liver tissue as the experimental system (data not shown).

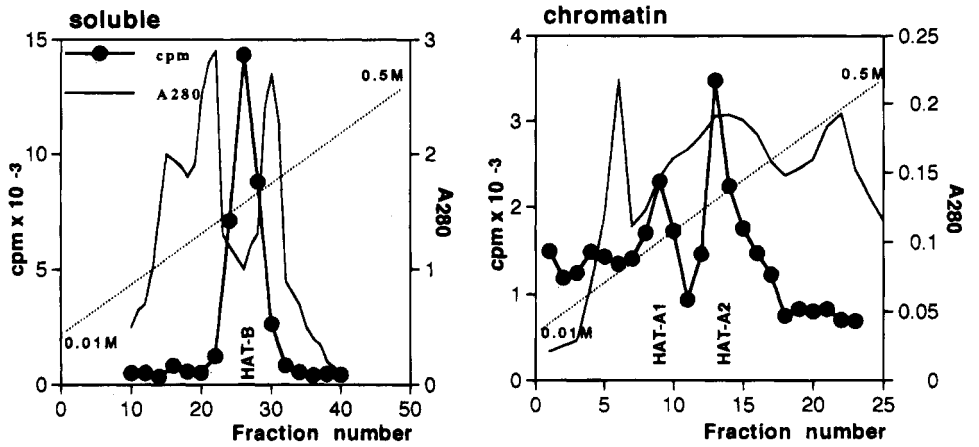

FIGURE 3: Analysis of soluble and chromatin-bound histone acetyltransferase activity at 72 h of maize embryo germination by ion exchange chromatography. The soluble fraction was applied to DEAE-Sepharose CL-6B chromatography. The salt-extract of the chromatin was subjected to Resource-Q FPLC with elution of proteins by a linear salt gradient (0.01−0.5 M). Fractions of 1.2 mL were collected. Enzyme activities are expressed as cpm. Protein was monitored by A_{280} measurements. Positions of histone acetyltransferases HAT-B (soluble), HAT-A1 (chromatin), and HAT-A2 (chromatin) are indicated.

We also checked for histone acetyltransferase activity during chicken erythrocyte nuclear matrix isolation, but only found negligible amounts of activity in the final nuclear matrix (results not shown), thus confirming our results with yeast, *Physarum*, and maize.

DISCUSSION

The very low level of association with the nuclear matrix of histone deacetylases was not unexpected, since preliminary data of maize embryos indicated that the vast majority of the enzyme activity is readily eluted by moderate concentrations of salt (Brosch et al., 1992). The data presented in this manuscript show that this finding is indeed independent of the species, since plants, yeast, *Physarum*, chicken erythrocytes, and rat liver yield identical results. It is furthermore independent of the matrix preparation procedure, which is in line with a recent study on nuclear matrix isolation procedures, where it was shown that different preparation protocols yield almost identical results in *Physarum* and mammalian cells (Eberharter et al., 1993). However, the results are contradictory to the data of Hendzel and Davie (1992), who claimed histone deacetylase to be a marker protein for the internal nuclear matrix. The discrepancy cannot be attributed to species differences, since all species investigated in this report yield identical results; moreover, it cannot be explained as a result of the presence or absence of reducing agents during the preparation, as suggested by Davie and Hendzel (1994). Our data demonstrate the very lose association of histone deacetylase activity to nuclear substructures in chicken erythrocytes, since ~80% of the activity is already released during nuclear isolation. One possible explanation for the apparent discrepancy with previous results (Hendzel and Davie, 1992) could be an extraordinarily high loss of activity during nuclear isolation, because Hendzel and Davie (1992) did not check the total enzyme activity in cellular homogenates.

Our data are in line with the steady state acetylation of histone H4 of the nuclear matrix of *Physarum polycephalum*; recently we could show that the nuclear matrix of *Physarum* is distinguished from bulk chromatin by an extraordinarily high acetylation state of H4 (Lang et al., 1993). Whereas H4 in bulk nuclear chromatin has an average acetate content (N) of around 1.1 during the entire cell cycle, the nuclear matrix bound H4 was distinguished by an acetate content of around 1.75, regardless of the cell cycle stage (Lang et al., 1993). For this reason we suspected that nuclear histone acetyltransferase activity could be at least partly bound to the nuclear matrix and hence explain this elevated acetylation state of matrix bound core histones. Our results, however, clearly show that neither histone acetyltransferase nor histone deacetylase is associated with the nuclear matrix. The lack of association cannot be due to a transient association during the cell cycle, as found for other nuclear proteins, e.g. *c-myc* protein (Waitz & Loidl, 1991), since we compared the S-phase and G_2-period of the *Physarum* cell cycle, but did not find differences with respect to matrix association of the enzymes. One could argue that the very small proportion of enzyme activity (~0.5−3% of the total activity) could be specifically associated to transcriptionally active chromatin domains; however, the fact that no differences in the amount of matrix-bound enzyme could be observed during the synchronous *Physarum* cell cycle argues against this possibility; in *Physarum*, 80% of transcription takes place in the G_2-period. The same results were obtained for early and late stages of maize embryo germination. Other mechanisms may exist that determine the extremly high acetylation state of matrix-bound core histones.

The central point of this report is that histone acetyltransferases and deacetylases differ with respect to their association to chromatin; nuclear histone acetyltransferases, in general, are much more tightly bound to chromatin than deacetylases, since 0.5 M salt is necessary to elute them from purified chromatin, whereas histone deacetylases are solubilized from isolated chromatin by low ionic strength buffers. The striking finding was that the distribution between soluble and chromatin bound activity of the different histone deacetylases fluctuated during the embryo differentiation pathway. This indicates a further level of regulation of biological functions of these enzymes in the sense that participation of one of the enzyme forms in a biological process can be determined by its transient association to distinct chromatin domains. This idea is attractive in the light of recent cytological data linking a site-specific acetylation pattern of H4 in defined chromosome regions to certain

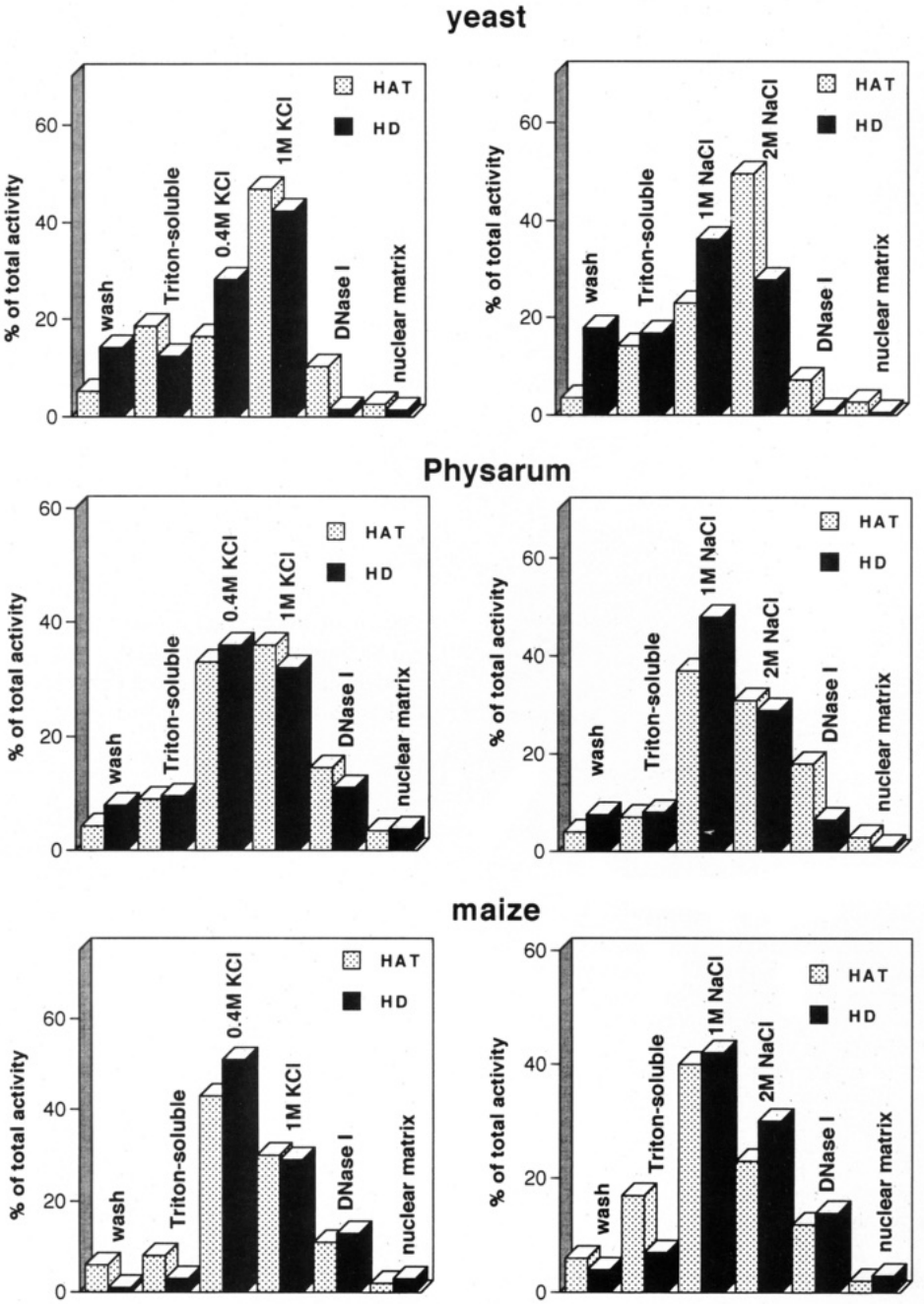

FIGURE 4: Presence of histone acetyltransferase and histone deacetylase activity in different steps of nuclear matrix preparations from yeast, *Physarum*, and maize. Enzyme activities are expressed as percentage of total activity. (right panels) Nuclear matrix was prepared following procedure 1 (Waitz & Loidl, 1988), (left panels) nuclear matrix was prepared following procedure 2 (Hendzel & Davie, 1992; see Experimental Procedures).

functional properties of those domains (Turner et al., 1992; Jeppesen & Turner, 1993); similar data were recently obtained in yeast (Braunstein et al., 1993), where an immunological approach also revealed a specific acetylation of large chromatin domains (mating type cassettes, telomers) during transcriptional silencing. Such cytogenetic data indicate that acetylation of a distinct lysine residue of H4, detected with strictly site-specific anti-acetyllysine antibodies, mediates unique and specific effects in chromatin of certain chromosomal regions. However, it is not at all clear from these studies whether the acetylation state of a distinct lysine residue is predominantly determined by acetyltransferases or the coordinated interplay of both acetyltransferases and deacetylases. It has been suggested that probably both enzymes, acetyltransferases and deacetylases, are specific for particular acetylatable lysine residues (Turner et al., 1992). One mode of regulation could therefore involve site-specific histone acetyltransferases and histone deacetylases that are specifically, but transiently, bound to certain regions of the chromatin. A subset of enzymes and their transient ability to bind to chromatin could therefore determine the acetylation state of chromatin domains and hence influence certain structural and functional properties. Our result of a variable ratio of soluble/chromatin bound histone deacetylases during maize embryo differentiation suggests that the action of one and the same enzyme in different nuclear processes may be modulated by an alteration of its solubility properties. In this context it is interesting that yeast histone deacetylase exists in two forms, a low and high molecular weight form, which differ considerably in their properties (Sanchez del

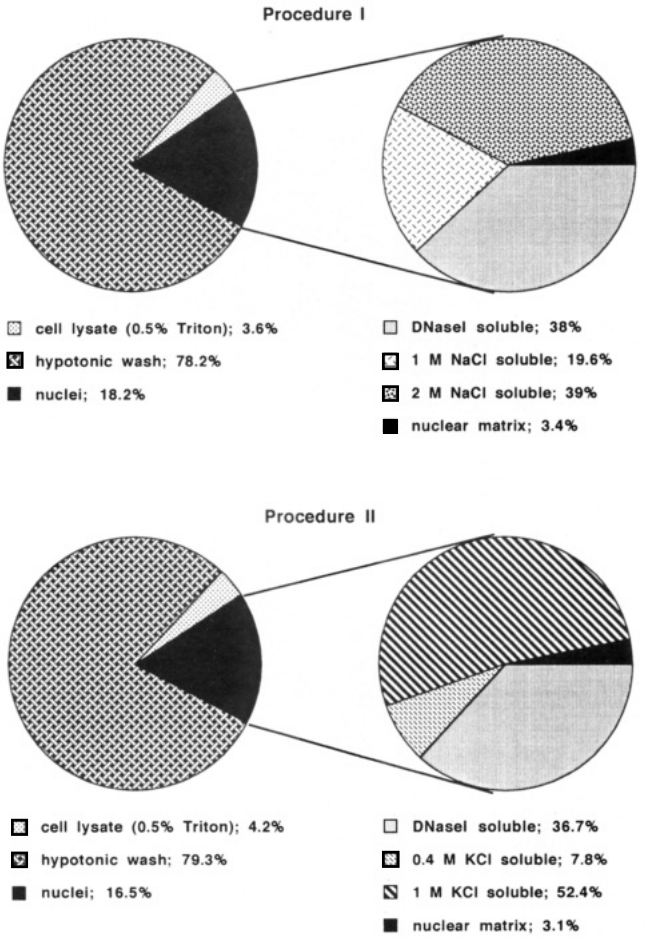

FIGURE 5: Proportion of histone deacetylase activity during cellular fractionation and nuclear matrix preparation of chicken erythrocytes. Enzyme activities were determined during nuclear isolation (lysate, low salt wash, nuclei) and during nuclear matrix preparation (DNase I digestion, high salt extraction, nuclear matrix). Total enzyme activity was calculated from activity in lysate, wash, and isolated nuclei (left diagrams). The activity retained in isolated nuclei was taken as 100% and the proportion of enzyme activity during matrix isolation steps was expressed as percentage of this nuclear activity (right diagrams). Two different matrix isolation procedures were performed. The lower diagrams (KCl method) represent the methodology of Hendzel and Davie (1992).

Pino et al., 1994); the high molecular weight complex was only stable at low ionic strength, was only poorly inhibited by Trichostatin A, and deacetylated oligonucleosomes more efficiently than the low molecular weight form. It may represent the proportion of deacetylase active *in vivo* (Sanchez del Pino et al., 1994).

We would like to point out that the "soluble" fraction contains cytoplasmic proteins (like histone acetyltransferase HAT-B) as well as nuclear proteins that are readily solubilized by low ionic strength buffers (like histone deacetylase HD-1A). The chromatin-bound fraction, however, does not contain cytoplasmic proteins, which is reflected by the complete lack of histone acetyltransferase HAT-B.

Interestingly, histone deacetylase HD-2 does not change its location, since we found this enzyme exclusively bound to chromatin, regardless of the differentiation stage; this is probably due to the fact that this enzyme has a rather specific function, which is reflected by its abundancy in cells in the G_1-period of the cell cycle; in meristematic cells of the dry embryo, where 80% of the cells are in the G_1-period, this enzyme is the predominant histone deacetylase (Georgieva et al., 1991; Georgieva et al., 1994). When the G_1-cell population of meristematic tissue decreases during embryo germination, HD-2 activity decreases in parallel; in whole embryos at 72 h of germination, however, where the percentage of G_1-cells is still more than 65%, this enzyme is present in high amounts. In accord with previous results (Georgieva et al., 1991; Georgieva et al., 1994) we propose that HD-2 has a unique and specific function in the chromatin of G_1-cells.

We postulated that histone deacetylases *per se* have an essential function for binding of specific proteins (containing anionic regions) to chromatin (López-Rodas et al., 1993). Binding of structural or regulatory anionic proteins to certain chromatin domains occurs during various nuclear processes, e.g. also during early transcriptional activation. A recent report demonstrated that administration of trichostatin A, a specific inhibitor of histone deacetylases, leading to hyperacetylated histones *in vivo*, inhibited the development of the starfish embryo during the early gastrula stage (Ikegami et al., 1993). The importance of histone deacetylases for fundamental processes occurring on chromatin is underlined by our finding of a further level of regulation involving the intranuclear location of these enzymes during differentiation of the maize embryo.

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