RPD3-Type Histone Deacetylases in Maize Embryos[†,‡]

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ABSTRACT: Posttranslational core histone acetylation is established and maintained by histone acetyltransferases and deacetylases. Both have been identified as important transcriptional regulators in various eukaryotic systems. In contrast to nonplant systems where only RPD3-related histone deacetylases (HD) have been characterized so far, maize embryos contain three unrelated families of deacetylases (HD1A, HD1B, and HD2). Purification, cDNA cloning, and immunological studies identified the two maize histone deacetylase HD1B forms as close homologues of the RPD3-type deacetylase HDAC1. Unlike the other maize deacetylases, HD1A and nucleolar HD2, HD1B copurified as a complex with a protein related to the retinoblastoma-associated protein, Rbap46. Two HD1B mRNA species could be detected on RNA blots, encoding proteins of 58 kDa (HD1B-I) and 51 kDa (HD1B-II). HD1B-I (zmRpd3) represents the major enzyme form as judged from RNA and immunoblots. Levels of expression of HD1B-I and -II mRNA differ during early embryo germination; HD1B-I mRNA and protein are present during the entire germination pathway, even in the quiescent embryo, whereas HD1B-II expression starts when meristematic cells enter S-phase of the cell cycle. In line with previous results, HD1B exists as soluble and chromatin-bound enzyme forms. In vivo treatment of meristematic tissue with the deacetylase inhibitor HC toxin does not affect the expression of the three maize histone deacetylases, whereas it causes downregulation of histone acetyltransferase B.

The ϵ-amino group of conserved lysine residues within the N-terminal extensions of core histones can be posttranslationally acetylated (*1*). Twenty-six to twenty-eight lysine sites per nucleosome are subject to this reversible modification, resulting in a remarkable heterogeneity of nucleosomes with respect to the degree and pattern of acetylation (*2, 3*). Histone acetyltransferases introduce acetyl groups from acetyl-CoA onto the ϵ-amino group, with each acetate reducing the net positive charge of the histone. The removal of acetate is catalyzed by the activity of histone deacetylases (HD).[1] The specific acetylation state of a histone molecule in the nucleosome is generated and maintained by these two enzyme activities. The recent discovery that numerous transcriptional regulators associate with or even represent histone acetyltransferases or HDs stimulated general interest in the role of chromatin structure in gene regulation. It has been demonstrated that histone acetyltransferases and deacetylases can interact with specific DNA-binding activator or repressor proteins, thereby modulating transcriptional activity of specific promoters by locally changing chromatin structure (*4*). It is still unclear whether acetylation directly alters nucleosomal structure; the recently published 2.8 Å resolution structure of the nucleosome core particle (*5*) argues for a role of lysine acetylation in destabilization of the nucleosome higher-order structure rather than of the nucleosome itself. However, histone acetylation could as well 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 finding that nonhistone proteins, like p53 or proteins directly involved in transcription, are acetylated by histone acetyltransferases in vivo and in vitro (*6, 7*).

Human histone deacetylase HDAC1 was purified by trapoxin affinity chromatography and identified as a highly conserved homologue of the yeast transcriptional regulator RPD3 (*8*); HDAC1 is associated with the retinoblastoma-associated protein Rbap. Rbap is also bound to the cytoplasmic histone acetyltransferase Hat1 of yeast (*9*), indicating a histone targeting function of Rbap. Human Rbap48 is associated with HDAC1, while human Rbap46 is part of the HAT-B holoenzyme (*10*). Homologues of members of the HDAC/RPD3 protein family have now been identified in many eukaryotes as components of large multiprotein complexes that include DNA binding repressors or corepressors such as SIN3, MAD, UME6, YY1, MAX, SMRT, and NCoR (*11*−*17*). Surprisingly, *RPD3* counteracts ge-

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[‡] The cDNA sequence of histone deacetylase HD1B-II has been deposited in Gen Bank under accession number AF045473.

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[1] Abbreviations: HD, histone deacetylase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DIG digoxygenin.

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nomic silencing in *Drosophila* and yeast (*18*, *19*); mutations in the *Drosophila* gene that is homologous to *RPD3* enhance position-effect variegation, and *RPD3* deletions in yeast show increased repression at telomeric loci. These results were unexpected because, in contrast to the well-accepted correlation between acetylation and gene activation, histone acetylation in these cases is associated with repressed transcriptional activity. *RPD3* deletions in yeast also retard full induction of the *PHO5* promoter (*19*). On the other hand, it has been shown that efficient silencing in yeast requires a so-called heterochromatin acetylation pattern (*20*) with lysine 12 of histone H4 being preferentially acetylated. However, HDAC/Rpd3 preferentially deacetylates lysines 5 and 12 in histone H4 (*8*, *19*). These results might be explained by the interaction of specific proteins with histones/nucleosomes that have distinct acetylation patterns; such highly specific acetylation might be involved in the complex regulation (activation as well as repression) of gene activity, but this might differ among individual genes and be dependent on a variety of other factors.

In nonplant systems, only RPD3-related HDs have been identified and characterized so far. In contrast, plants contain multiple, distinct HD activities (*21*−*23*). In germinating maize embryos, three HDs were purified and characterized: HD1A, HD1B, and HD2. HD2 has been purified to homogeneity (*24*), and cloning of the encoding cDNA revealed HD2 as a nucleolar phosphoprotein (*25*), unrelated to RPD3-type deacetylases. Based on partial amino acid sequences of highly purified HD1A (*26*), this enzyme is also unrelated to RPD3, thus representing another novel type of deacetylase (Pipal, Lusser, and Brosch, unpublished results). These enzymes essentially differ from each other with respect to kinetic and enzymatic properties (*23*), binding to chromatin (*22*), and substrate and lysine site specificity (*27*). HD1B is present as two enzyme forms (HD1B-I and HD1B-II) that can be separated by chromatography and are distinguished by their k_M values for core histones (*23*). In this paper we report the purification of HD1B and the cloning of a cDNA encoding an RPD3 orthologue (here called HD1B-II). While this work was in progress, a different maize cDNA (zmRpd3) encoding a protein related to RPD3 was cloned and shown to complement a yeast *RPD3* null mutant (*28*). We provide evidence that both HD1B forms are RPD3-related HDs. The two cDNAs (HD1B-II, zmRpd3) encode a 51 kDa protein (HD1B-II) and a 58 kDa form (HD1B-I/zmRpd3). HD1B-II is expressed at a considerably lower level than zmRpd3, as judged from mRNA and protein amounts. Using anti-RPD3 antibodies that recognize both forms of maize RPD3-like HDs and DNA probes that discriminate between the two forms of maize RPD3 mRNAs, we report here on the pattern of expression of these two HDs in germinating embryos.

EXPERIMENTAL PROCEDURES

Organism and Culture Conditions. Maize seeds (*Zea mays*, strain Cuzco) were germinated in darkness for 72 h on cotton layers soaked with water at 28 °C. Whole seedlings (0−22 h of germination) or meristematic parts of the roots (30−72 h) were harvested into liquid nitrogen for chromatin preparation.

HD Assay. HD activity was determined as described (*21*) using [^{3}H]acetate-labeled chicken reticulocyte histones (*29*) as substrate. Aliquots (25 μL) of samples were mixed with 10 μL of total [^{3}H]acetate-prelabeled histones (1.5 mg/mL). This mixture was incubated at 30 °C for 20 min. The reaction was terminated by the addition of 36 μL of 1 N HCl/0.4 M acetate and 0.8 mL of ethyl acetate. After centrifugation at 8000*g* for 5 min, an aliquot of 600 μL of the upper phase was counted in 3 mL of liquid scintillation cocktail.

Extraction and Purification of HD1B. Frozen seedlings (−80 °C) of maize were ground to powder in an Ika grinding machine (Janke-Kunkel, Staufen, Germany). Frozen tissue powder was suspended in homogenization buffer [4 mL/g; 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, and 40% (v/v) glycerol]. The mixture was stirred until the temperature reached −10 °C and filtered consecutively through 200 and 100 μm (pore size) nylon membranes. After centrifugation at 10000*g* for 15 min at 4 °C, the supernatant was decanted. The pellet was washed twice in homogenization buffer (2 mL/g, with a centrifugation step at 6000*g* for 15 min) and resuspended in 1 mL/g buffer B [15 mM Tris-HCl, pH 8.0, 10 mM NaCl, 0.25 mM EDTA, 10 mM 2-mercaptoethanol, and 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 (chromatin fraction) was the source for histone deacetylase HD1B.

For purification of HD1B, the chromatin fraction of a total of 16 kg (wet weight) of embryo tissue was used. The supernatant was subjected to Q-Sepharose (Pharmacia-Biosystems, Uppsala, Sweden) fast protein liquid chromatography (FPLC) for separation of histone deacetylases HD1B and HD2 (*24*). For processing of 16 kg of starting material, eight Q-Sepharose chromatographic runs were performed. The chromatin portion (2 L) was loaded onto a 200 mL Q-Sepharose fast-flow column equilibrated with buffer B. Proteins were eluted with a linear gradient (1.4 L) of NaCl (0.01−0.5 M) in buffer B. Fractions of 18 mL were collected and assayed for HD activity. Fractions with high HD1B activity were pooled and concentrated by ammonium sulfate precipitation (20−45%). For processing of eight Q-Sepharose chromatographic runs, four ammonium sulfate precipitations were performed. The pellet of each precipitation was resuspended in 50 mL of buffer B and then dialyzed three times against 10 volumes of buffer B. The dialysates were subjected to four poly(lysine)−agarose (high molecular weight) chromatographic runs (Sigma, St. Louis, MO) for further purification of HD1B. Proteins were eluted with a linear gradient (300 mL) of NaCl (0.01−1 M) in buffer B. Fractions of 5 mL were collected and analyzed for HD1B activity. This type of chromatography separated the two HD1B forms, HD1B-I and -II (*23*). For further purification, only peak fractions of HD1B-I of each poly(lysine)−agarose chromatography were pooled, concentrated to 2 mL, and subjected to four Sephacryl Hi-Prep S-200 gel-filtration chromatographic runs (Pharmacia-Biotech, Uppsala, Sweden). Fractions of 1.5 mL were collected and analyzed for HD activity.

Peak fractions of the four Sephacryl S-200 gel-filtration chromatographic runs were pooled, dialyzed against 10 volumes of buffer B, and loaded onto histone−agarose (Pharmacia Biotech, Uppsala, Sweden). Proteins were eluted with a linear gradient (45 mL) of NaCl (0.01−0.8 M) in

buffer B. Fractions of 1.5 mL were collected and assayed for HD activity.

HD1B-I peak fractions were pooled, dialyzed against sodium phosphate buffer [pH 8.0, with 0.1% (v/v) 2-mercaptoethanol] and subjected to hydroxyapatite chromatography (Bio-Rad, Hercules, CA). Proteins were eluted with a linear gradient (40 mL) of sodium phosphate (0.01−0.5 M). Fractions of 1.5 mL were collected and analyzed for HD activity. Fractions containing HD activity were pooled, concentrated to 1 mL, and subjected to Superdex S-75 gel-filtration chromatography (Pharmacia-Biotech, Uppsala, Sweden). For calculation of the molecular weight, marker proteins (bovine serum albumin, RNase A, aldolase, ovalbumin, and chymotrypsin) were used. Fractions of 1.5 mL were collected, assayed for deacetylase activity, and analyzed by SDS−PAGE and silver staining.

Size Exclusion Chromatography (Superdex S-200). Peak fractions of HD1-B after Q-Sepharose chromatography were pooled, concentrated to a final volume of 1 mL by centrifugation in Amicon Centriprep-10 microconcentrators, and applied onto a Superdex S-200 FPLC column (2.5 × 100 cm; 120 mL; Pharmacia). The column was equilibrated with 0.2 M NaCl in buffer B. The flow rate was maintained at 1 mL/min, and fractions of 1.5 mL were collected and assayed for HD activity. Aliquots of fractions 34, 46, and 52, corresponding to the indicated molecular weights of 500 000, 120 000, and 60 000 Da, respectively, were used for immunoblotting, with antibodies against Rpd3 or LeMSI1 (Rbap).

Isolation of a Maize cDNA Encoding an RPD3 Orthologue. Total RNA was purified from maize seedlings after 72 h of germination. cDNA was synthesized with Superscript II reverse transcriptase (Gibco-BRL) according to the manufacturer's instructions. Briefly, 7 μg of total RNA was mixed with $(dT)_{17}$-adapter primer (5′-GACTCGAGTCGACATCGAT$_{17}$-3′), heat-denatured, and after addition of the reverse transcription (RT) mixture, incubated for 50 min at 42 °C. The RT product (2 μL of 20) was used for PCR amplification employing 150 pmol each of degenerate primers AC1 (5′-CARGGNCAYCCNATGAARCC-3′) and AC2R (5′-TTYTTNGCRTGRTGNA-3′). Primer sequences were designed from identical peptide sequences of human and *Drosophila* RPD3/HDAC proteins that correspond to amino acids 26−32 (primer AC1), 139−144 (primer AC2R, reverse primer), and 96−101 (primer AC3R, reverse primer, digoxygenin-labeled), respectively, of human HDAC1 (GenBank accession no. U50079). RT-PCR products were fractionated on a 1% agarose gel, blotted onto Hybond N nylon membrane (Amersham), and hybridized with digoxygenin-labeled primer AC3R (5′-GGRCARTCYTCNCCNAC-3′). Hybridization revealed a 354-bp PCR product, which was purified from the agarose gel and cloned into vector pGEM-T (Promega) for sequencing.

Amplification of the 5′ and 3′ ends of the cDNA was achieved by applying the RACE protocol (*30*). The gene-specific primer C1R (5′-CGCCCAGTTGACGGTGAT-3′) was used for 5′ and the $(dT)_{17}$-adapter primer for 3′cDNA synthesis, respectively. The PCR reaction for 3′RACE contained 2 μL of 3′cDNA as template, and 20 pmol each of gene-specific primer B2 (5′-CATCCGAATGGCGCATTC-3′) and adapter primer (5′-GACTCGAGTCGACATCGA-3′) and was performed for 31 cycles of 1 min at 95 °C, 1 min at 56 °C, and 1 min at 72 °C. Aliquots of PCR products (1 μL) were used for nested PCR with the gene-specific primer B3 (5′-CTCCACCGCCTCCTCGA-3′) and the adapter primer. 5′RACE was performed with poly(dA)-tailed 5′cDNA and 20 pmol each of the gene-specific primer C2R (5′-GCGGTTAAGCTTGACGGC-3′) and the adapter primer. Following second-strand synthesis with $(dT)_{17}$-adapter primer, 31 cycles of amplification were carried out at an annealing temperature of 54 °C. Nested PCR was performed with primer C3R (5′-CTGGCAGAAGGGGAAGAG-3′) and adapter primer. PCR products were fractionated on 1−1.5% agarose gels, and specificity of products was determined by hybridization with a digoxygenin-labeled gene specific DNA fragment. Positive products were excised from the agarose gel, purified, and cloned into pGEM-T for sequencing. The cDNA sequence of HD1B-II was deposited in GenBank (AF045473).

RNA Blot Analysis. HD1B transcript expression during maize embryo germination was determined by RNA gel blot analysis. Total RNA from different germination stages (whole embryos 0, 4, and 15 h; meristematic parts of the roots 22, 30, 46, 60, and 72 h after imbibition) of maize embryos was extracted with TRIzol total RNA isolation reagent (Gibco−BRL). Total RNA (15 μg) for each time point was subjected to electrophoresis in 1.2% agarose/1.1% formaldehyde gels, blotted onto Hybond-N membrane (Amersham), and hybridized with a DIG-labeled DNA fragment covering either the 3′-terminal 642 bp (bp 1214−1856 from transcription start site) of HD1B-I cDNA or the 3′-terminal 546 bp (bp 1195−1741 from transcription start site) of HD1B-II cDNA, in "high SDS" hybridization solution (12 h at 42 °C). No cross-hybridization occurred with the two probes. Washes were carried out twice for 5 min in 1× SSC/0.1% SDS at room temperature and twice for 15 min in 0.1× SSC/0.1% SDS at 55 °C. Blots were exposed to X-ray film. Blots and ethidium bromide-stained gels were analyzed by laser densitometry and evaluated by Molecular Dynamics ImageQuant software. The amount of HD1B mRNA at each germination time point was corrected for small differences in the amount of RNA loaded onto each gel slot.

Extraction of HD1B Protein. Embryos/meristematic parts of the roots at different time points of embryo germination were harvested into liquid nitrogen. After 4 g of each sample was ground in an Ika grinding machine, the powder was suspended in 40 mL of buffer A (0.5 M NH_4Cl, 0.25 mM EDTA, 10 mM 2-mercaptoethanol, and 15 mM Tris-HCl, pH 7.9) by six strokes of a motor-driven (200 rpm) Potter-Elvehjem Teflon homogenizer. The homogenate was stirred on ice for 30 min and centrifuged for 15 min at 27000*g*. The supernatant was saved. The pellet was resuspended in 1 mL of buffer A (0.25 mM EDTA, 10 mM 2-mercaptoethanol, and 15 mM Tris-HCl, pH 7.9). Aliquots of the supernatant and the resuspended pellet were mixed with SDS sample buffer (*31*), analyzed by SDS−PAGE, and blotted onto nitrocellulose membrane. Equal amounts of protein (50 μg) of the different samples were loaded on the gel. Blots and gels were quantitated by laser densitometry.

Immunodetection of HD1B Protein. Purified HD1B-I or II, purified HD1A (*26*), purified HD2 (*24*), and total protein extracts of embryos of different germination stages were subjected to SDS−PAGE (10%) with subsequent blotting onto nitrocellulose membrane. Blots were incubated with

either antibodies against recombinant mouse Rpd3/HDAC1 (*32*; dilution 1:7500) or antibodies against LeMSI1 (*33*; Rbap homologue in tomato; dilution 1:750) for 2 h at room temperature. Secondary antibody−alkaline phosphatase conjugates were used for detection.

HC Toxin Treatment of Zea mays Embryos. Approximately 160 maize seeds were germinated for 72 h, excised from the grain, and split into two halves. One half (approximately 80 embryos) was put into 500 mL of MS medium + 1% sucrose (Sigma Chemical Co.) supplemented with 10 μg/mL HC toxin, and the other one was put into 500 mL of MS medium + 1% sucrose without toxin. Both parts were incubated for 5 h at 28 °C with sufficient aeration. Subsequently the meristematic parts of the roots were harvested into liquid nitrogen. RNA was isolated from HC toxin-treated and control samples, electrophoresed on 1.2% agarose/1.1% formaldehyde gels, and blotted onto Hybond-N membrane. Hybridization was performed with PCR-generated DIG-labeled DNA probes corresponding to the entire coding regions of HD1B-II, histone deacetylase HD2 cDNA (*25*), zmRpd3 cDNA (*28*), LeMSI1 cDNA (*33*), and HAT-B−p50 cDNA (*35*).

RESULTS

Purification of Maize Histone Deacetylase HD1B. Germinating maize embryos contain four HDs (HD1A, two forms of HD1B, and HD2; *23, 29*). To achieve a fractionation of these different enzymes we employed a crude chromatin preparation (*34*). When germinating embryos at 72 h after start of germination were used, the chromatin fraction contained HD1B and the nucleolar enzyme HD2 (*22, 24*). To purify HD1B from this chromatin extract we established a purification protocol of six chromatographic steps (*29*). The chromatin extract was first applied to Q-Sepharose chromatography (*23, 24*); after enzyme activity determination, the peak fractions were pooled and concentrated by ammonium sulfate precipitation (20−45%). The precipitate was dialyzed and applied to a poly(lysine) (high molecular weight)−agarose column. During this chromatography, HD1B splits into two forms, HD1B-I and HD1B-II (*23*). These two forms were not separated either by Q-Sepharose chromatography or by fractionated ammonium sulfate precipitation. Peak fractions of HD1B-I after poly(lysine)−agarose chromatography were then subjected to Sephacryl Hi-Prep S-200 and subsequent histone−agarose chromatography, before they were loaded onto a hydroxyapatite column. Figure 1A shows the elution profile of the enzyme activity after the final purification (Superdex S-75 gel filtration) step. HD1B-I eluted at an apparent molecular weight of ∼60 000. The final peak fraction of a typical purification procedure was subjected to SDS−PAGE, which revealed a prominent band at a molecular weight of ∼58 000 after silver staining (Figure 1B, lane 1).

When crude salt extracts of total maize seedlings after 72 h of germination were subjected to SDS−PAGE with subsequent blotting onto nitrocellulose membranes, an antibody against recombinant mouse RPD3 detected two protein bands. The most abundant immunosignal was observed at a molecular weight of 58 000, whereas a second minor band appeared at 51 000 (Figure 1B, lane 2). When the peak fraction of the purified HD1B-I after the final gel-filtration chromatography (Figure 1A; fraction 39) was probed with the anti-RPD3 antibody, a single band appeared at a molecular weight of 58 000 (Figure 1B, lane 3). This pure HD1B-I preparation contained a prominent band of 58 kDa and a few faint contaminating protein bands, as judged from silver-stained SDS−polyacrylamide gels (Figure 1B, lane 1). The blot of a partially purified preparation [peak fraction of HD1B-I after poly(lysine)−agarose chromatography] was then incubated with antibodies against recombinant LeMSI1 (a tomato homologue of the mammalian retinoblastoma binding protein Rbap; *33*). Figure 1B (lane 4) shows that the antibody against tomato LeMSI1 decorated a protein of 45 kDa in the partially purified HD1B-I preparation. The same protein was also detected as a subunit of the cytoplasmic maize histone acetyltransferase B (*35*) and identified as an Rbap-related protein by protein microsequencing (Lusser and Loidl, unpublished results). The association between HD1B and p45 (Rbap) is not tight, since highly purified HD1B eluted as a monomer in gel-filtration chromatography at a molecular mass of ∼60 000 Da; such highly purified HD1B did not yield an immunosignal on Western blots when probed with the antibody against tomato LeMSI1 (results not shown).

When partially purified HD1B-II [pooled peak fractions after poly(lysine)−agarose chromatography] was used for immunoblotting, the antibody against mouse RPD3 yielded an immunosignal at 51 kDa (Figure 1B; lane 5). This corresponds to the second minor HD1B band after immunoblotting of crude cellular extracts (Figure 1B, lane 2). The antibody against LeMSI1 reacted with a protein of 45 kDa (lane 6). For comparison we also blotted chromatographic peak fractions of partially purified maize HD1A (*26*) and HD2 (*24, 25*). Neither the anti-RPD3 antibody nor the anti-LeMSI1 antibody recognized proteins in the HD1A or HD2 preparations (Figure 1B, lanes 7−10). The same results were obtained when highly pure HD1A or HD2 were used for immunoblotting (results not shown). These data clearly identify HD1B-I and -II as proteins related to RPD3. HD1B-I represents a 58 kDa protein, whereas HD1B-II has a lower molecular mass of 51 kDa. From immunoblotting of crude cellular extracts (Figure 1B, lane 2) or partially purified HD1B-II preparations (Figure 1B, lanes 5 and 6), as well as from enzyme activity determinations during enzyme purification, it was evident that HD1B-I represents the more abundant enzyme form than HD1B-II.

To substantiate the finding that HD1B is part of a higher molecular weight complex with an Rbap-related protein, we subjected pooled HD1B peak fractions after Q-Sepharose chromatography to Superdex S-200 gel-filtration chromatography. HD1B eluted in three enzyme activity peaks of approximately 60, 120, or 500 kDa; in each of these peaks the maize Rbap protein was present, as revealed by immunoblotting with an LeMSI1 antibody (Figure 1C). This result confirmed our previous observation that the HD1B complex is unstable during the chromatographic purification procedure.

While this work was in progress, a cDNA sequence of a maize RPD3 homologue (zmRpd3) became available in the database. This cDNA encoded an RPD3-related protein of a calculated molecular mass of 58 kDa (*28*); however, the authors could not attribute this sequence to one of the biochemically well-characterized HDs of maize. The pre-

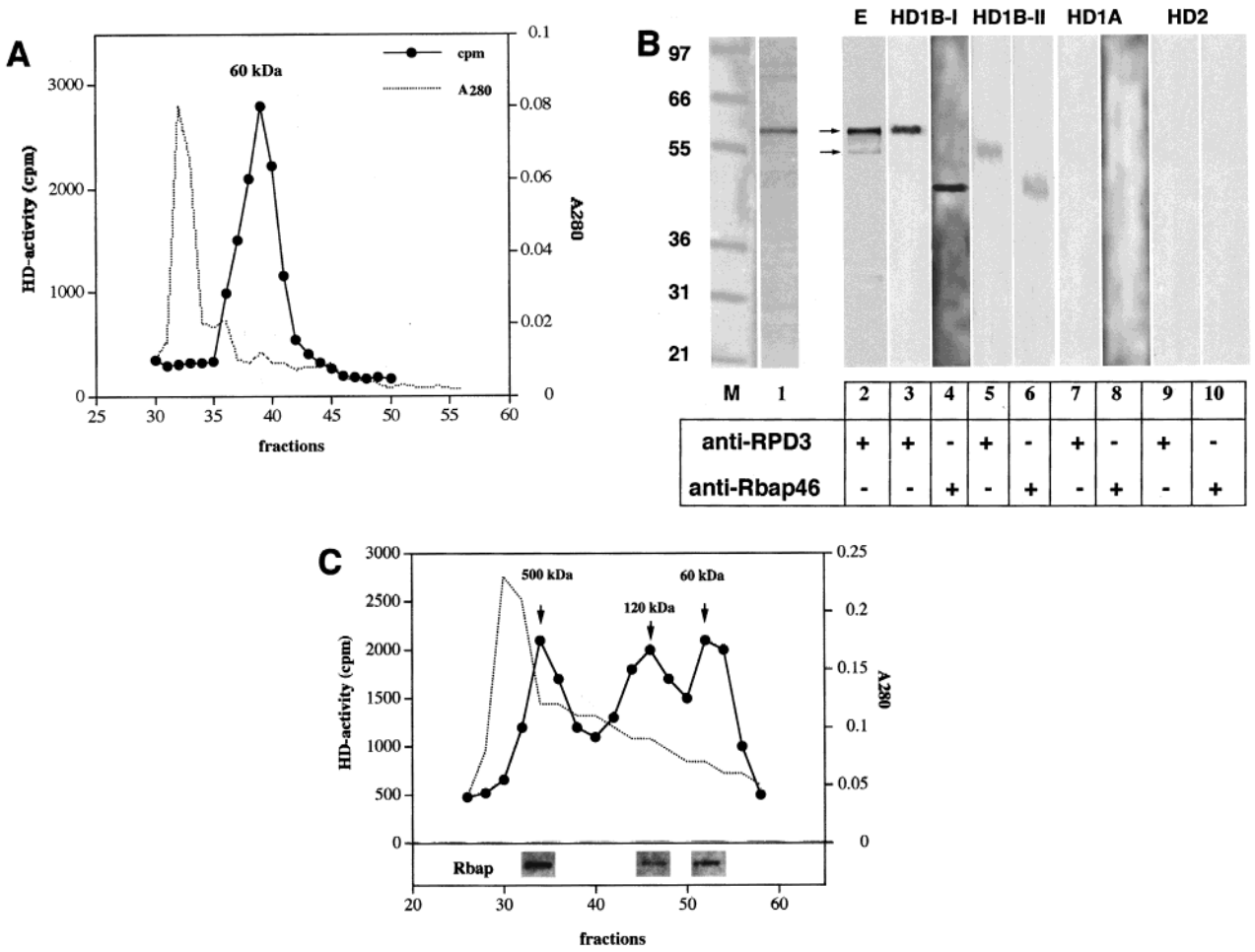

FIGURE 1: Identification of maize histone deacetylase HD1B-I as an RPD3-related protein copurifying with Rbap-related protein. (A) Final Superdex S-75 gel-filtration chromatography of highly purified HD1B-I. A chromatin extract was subjected to a five-step chromatographic purification protocol. The peak fractions of the hydroxyapatite chromatography were concentrated and applied to the final gel-filtration column. HD activity (counts per minute) and protein content (absorbance at 280 nm) were determined in each chromatographic fraction. The maximum of activity elutes in a volume corresponding to a molecular mass of 60 000 Da. (B) Immunological identification of RPD3- and Rbap46-related maize proteins. A peak fraction (Superdex S-75 gel-filtration chromatography) of a highly purified HD1B was subjected to SDS−PAGE with subsequent silver staining (lane 1). A crude cellular extract (lane 2), highly purified HD1B-I (lane 3; fraction 39 of the gel-filtration chromatography of panel A), partially purified HD1B-I [lane 4; peak fraction of HD1B-I after poly(lysine)−agarose chromatography], partially purified HD1B-II [lanes 5 and 6; peak fraction of HD1B-II after poly(lysine)−agarose chromatography], partially purified HD1A (lanes 7 and 8), or partially purified HD2 (lanes 9 and 10) were subjected to SDS−10% PAGE with subsequent blotting onto nitrocellulose membranes. Antibodies against recombinant mouse Rpd3 or against LeMSI1 (tomato Rbap46 homologue) were used for immunodetection. Molecular weight markers were included (lane M). Arrows mark the positions of HD1B-I (MW 58 000) and HD1B-II (MW 51 000). (C) Pooled HD1B fractions after Q-Sepharose chromatography were subjected to Superdex S-200 gel-filtration chromatography. Fractions were assayed for HD activity (●) and analyzed by SDS−PAGE with subsequent blotting onto nitrocellulose membranes and immunodetection with LeMSI1 antibodies; detection was performed by the ECL detection system (Pharmacia). Blot lanes of peak fractions (60, 120, and 500 kDa) are depicted. Protein content was monitored by measuring absorption at 280 nm (⋯).

dicted molecular weight of zmRpd3 (58 000) indicated that it corresponds to HD1B-I.

The fact that anti-RPD3 antibodies did not react with purified HD1A is consistent with the fact that internal peptide sequences (a total of 70 amino acids) from purified HD1A (*26*) do not display any homologies with RPD3-related sequences (Pipal and Brosch, unpublished). It is now clear that maize embryos contain three distinct families of HDs: nucleolar phosphoprotein HD2 (*25*); HD1B, which is related to RPD3 and exists in two forms; and another enzyme, HD1A, whose gene is still uncharacterized. All three enzymes are inhibited by HC toxin (*36*).

Isolation of the cDNA Encoding an RPD3-like Protein from Maize. To identify cDNAs encoding maize homologues of yeast *RPD3*, we used degenerate oligonucleotides corresponding to regions conserved between *Drosophila* Rpd3 and human HDAC1 (*8*, *18*). Reverse transcription PCR with RNA from maize seedlings at 72 h after start of embryo germination resulted in amplification of a 387-bp fragment. Amplification of 5′ and 3′ ends of the cDNA was performed by the RACE protocol (*30*). Sequence analysis of the complete cDNA revealed an open reading frame of 1377 bp encoding a protein of 459 amino acids with a calculated molecular weight of 50 900. The protein displays 58% amino acid sequence identity to human HDAC1, and 59% identity to the recently published maize Rpd3 orthologue (zmRpd3; Figure 2). Sequence alignment of maize HD1B-I (zmRpd3) and HD1B-II with other Rpd3 orthologues showed a high degree of evolutionary conservation among the members of this HD family (Figure 2). One significant difference is that the product of zmRpd3 has an extended carboxy-terminal region of 54 amino acids compared to HD1B-II, resulting

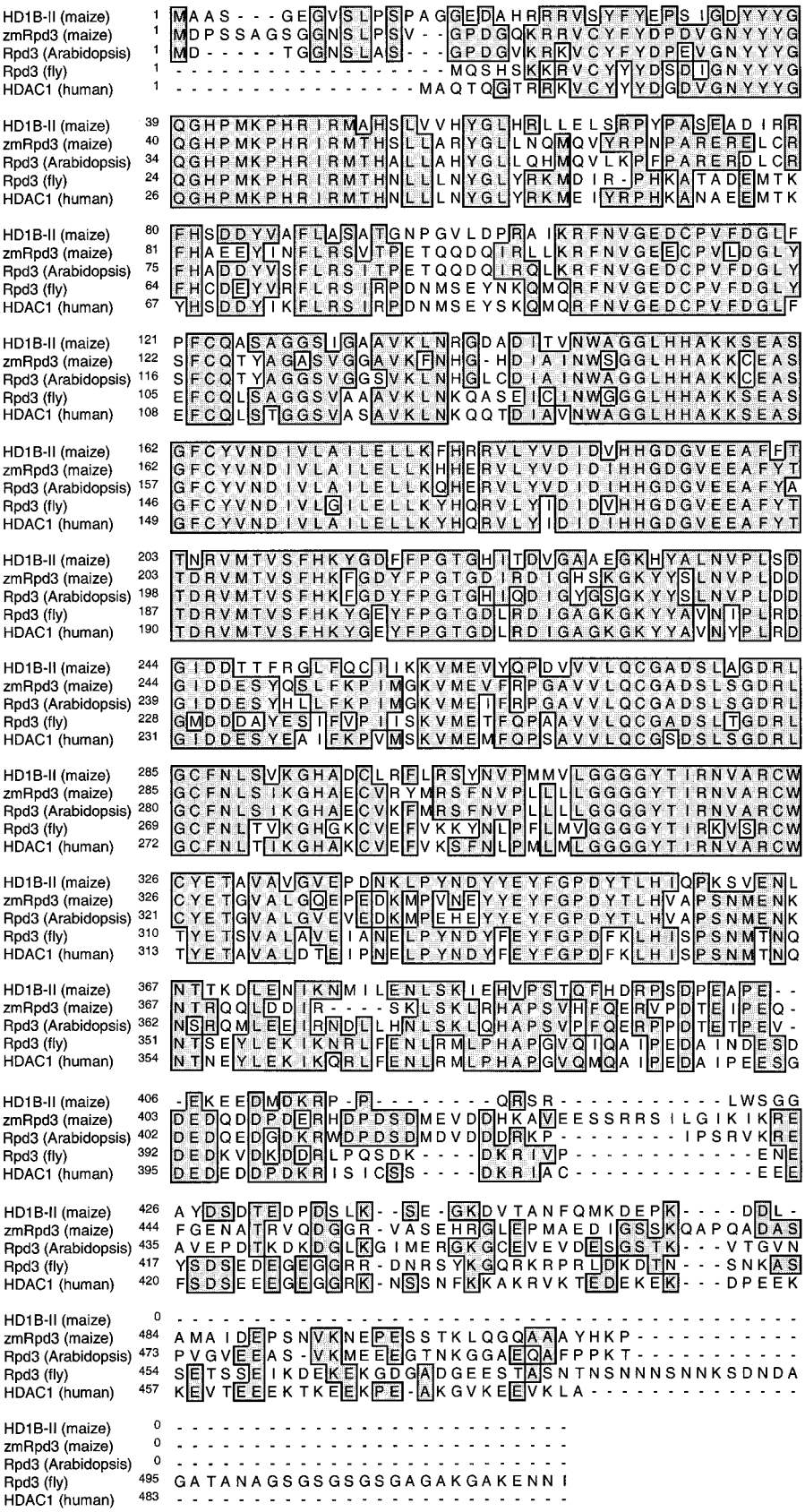

FIGURE 2: Deduced maize HD1B-II sequence aligned with those of maize HD1B-I (zmRpd3) and other RPD3-type deacetylases. Total RNA of maize seedlings (72 h after start of embryo germination) was reverse-transcribed. Oligonucleotide primer sequences were designed from peptide sequences identical in human and *Drosophila* RPD3/HDAC proteins and correspond to amino acids 26−32 (primer AC1), amino acids 139−144 (primer AC2R, reverse primer), and amino acids 96−101 (primer AC3R, reverse primer, DIG-labeled) of human HD1 (GenBank accession no. U50079) and used for PCR amplification of cDNA fragments. A specific 354-bp fragment was detected, cloned into pGEM-T vector and sequenced. The 3′ and 5′ ends of the cDNA were amplified, subcloned, and sequenced. The deduced amino acid sequence was aligned with amino acid sequences of other RPD3-homologous deacetylases of maize, *Arabidopsis*, *Drosophila*, and human. Identical amino acids are shaded and boxed.

in a predicted molecular weight of 57 600; this molecular weight is consistent with the apparent molecular weight of 58 000 for HD1B-I after SDS−PAGE as well as the molecular weight of ∼60 000 after gel-filtration chromatography. The predicted molecular weight of the product of the HD1B-II gene of 51 000 is in line with the apparent molecular weight of 51 000 after SDS−PAGE (Figure 1B). We therefore conclude that zmRpd3 encodes HD1B-I, whereas the cDNA reported in this paper encodes HD1B-II.

HD1B-I and -II mRNA and Protein Levels during Maize Embryo Germination. Hybridization of RNA blots of total RNA from embryos 72 h after start of germination with specific probes for HD1B-I (zmRpd3) or HD1B-II confirmed the difference in their mRNA size (Figure 3A). The mRNAs for HD1B-I and -II differ in abundance, with HD1B-I showing higher expression levels. This is consistent with the abundance of HD1B-I and -II proteins in crude cellular extracts (Figure 1B).

We then analyzed different time points of embryo germination for the amount of HD1B-I or -II mRNA. Figure 3B shows that in all stages investigated HD1B-I mRNA was present during the entire germination period, even in the quiescent embryo. HD1B-II mRNA was undetectable until 15 h after start of germination. Both mRNAs significantly increased at 22 and 30 h of germination, then decreased at 46 h, and again increased with further progression of germination. Except for the early period of germination (0−15 h) the patterns of expression, though quantitatively different, were similar (Figure 3B,C). Hybridization probes have been tested for equal labeling intensities.

To correlate the mRNA levels with the amounts of HD1B proteins, we analyzed extracts and insoluble chromatin sediments at different times of germination with anti-RPD3 antibody on immunoblots. Figure 4 shows that the changes in protein levels in general correlate with the changes in mRNA levels. Whereas HD1B-I protein was present during all stages of germination, HD1B-II protein appeared at 22 h. A small but significant percentage of both enzyme forms was obviously tightly chromatin-bound, in line with earlier observations (*22*), since it was resistant against extraction by 0.5 M NaCl. Whereas the levels of soluble HD1B-I and -II fluctuated during germination, the chromatin-bound proportion only exhibited minor changes (Figure 4).

Effect of HC Toxin on the Expression of Histone Deacetylases. Results on autoregulation of HDAC1 in mammalian cells (*53*) prompted us to address this question in maize embryos. Incubation of meristematic root tissue at 72 h after start of embryo germination with the cyclic tetrapeptide HD inhibitor HC toxin (*36*) did not affect the mRNA expression of maize HDs (HD1B-I, HD1B-II, HD2; Figure 5), but had a pronounced downregulating effect on histone acetyltransferase B (HAT-B−p50) and a slight repressive effect on Rbap. The same effect was observed when trichostatin A was used (results not shown).

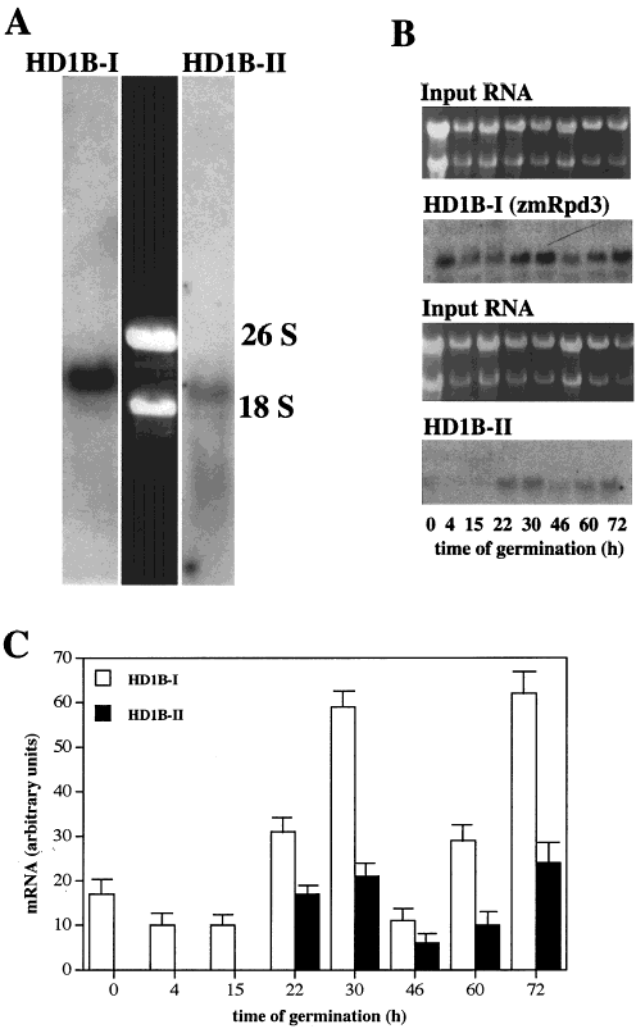

FIGURE 3: Expression of HD1B-I and -II mRNAs during maize embryo germination. (A) Total RNA was subjected to electrophoresis in 1.2% agarose/1.1% formaldehyde gels, blotted onto Hybond-N membrane, and hybridized with a DIG-labeled DNA fragment covering either the 3′-terminal 642 bp of HD1B-I cDNA (lane 1) or the 3′-terminal 545 bp of HD1B-II (zmRpd3) cDNA (lane 3). Lane 2: corresponding lane of the ethidium bromide-stained gel showing the 18S and 26S rRNA. (B) Total RNA was isolated at the indicated time points of embryo germination (from whole embryos at 0, 4, 15, and 22 h; from meristematic parts of the roots at 30, 46, 60, and 72 h), subjected to electrophoresis in 1.2% agarose/1.1% formaldehyde gels, and blotted onto Hybond-N membrane. Blots were probed with the same DIG-labeled DNA fragments as in panel A. (C) The ethidium bromide-stained gel and the corresponding blot were analyzed by densitometry and evaluated quantitatively. The amount of HD1B-I and -II mRNA was related to the amount of rRNA loaded onto each gel slot. This ratio (labeling intensity:amount of RNA) was expressed in arbitrary units. Data are shown as the mean ± SD of four independent experiments.

DISCUSSION

More than 30 years ago Allfrey et al. (*1*) discovered the posttranslational acetylation of core histones, which subsequently was correlated with structural transitions of chromatin during DNA replication (*10, 37−41*) and transcriptional regulation (*4, 15, 42, 43*). During the past few years a major breakthrough in the understanding of the functional relation between histone acetylation and transcriptional control has been achieved by the identification of proteins with intrinsic histone acetyltransferase or HD activity (*4*). A large number of rather diverse transcription regulatory proteins have been shown to possess histone acetyltransferase activity (*4, 44*). Despite a weak overall homology of amino acid sequences, the secondary structure elements of the catalytic domains are conserved among diverse acetyltransferases. Recently, the structure at 2.3 Å resolution of the yeast histone acetyltransferase Hat1−acetyl coenzyme A complex was reported (*45*).

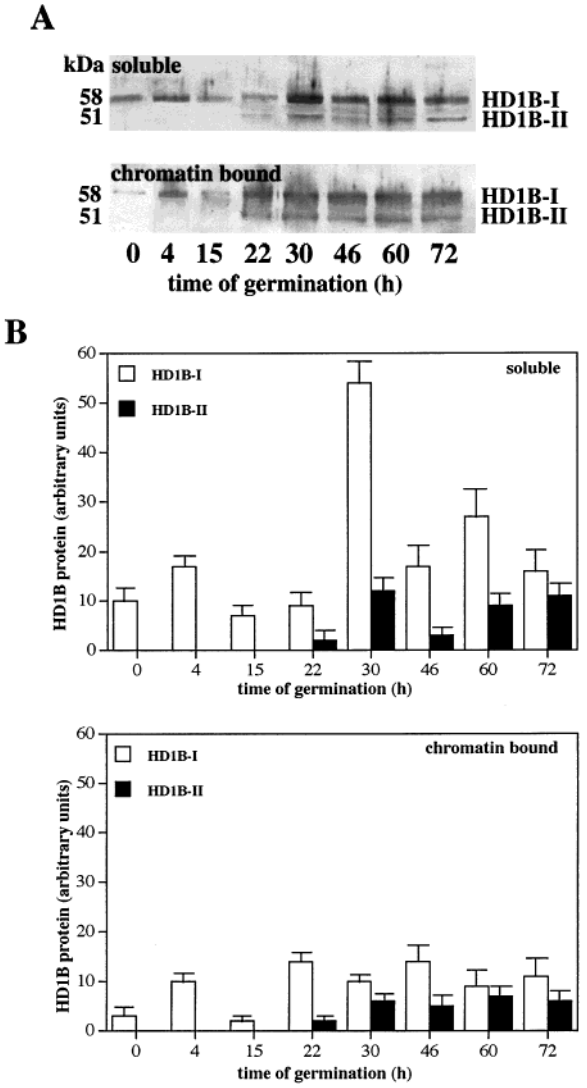

FIGURE 4: Levels of HD1B-I and -II proteins during maize embryo germination. (A) HD1B was extracted from embryos (whole embryos at 0, 4, 15, and 22 h; meristematic parts of the roots at 30, 46, 60, and 72 h) at different times of germination (soluble fraction). Equal amounts of protein (50 μg) from these extracts as well as from the insoluble chromatin sediments were subjected to SDS−PAGE with subsequent blotting onto nitrocellulose membrane. Immunodetection of HD1B was done with an antibody against recombinant mouse HDAC1/Rpd3. The antibody reacted with two proteins of apparent molecular weights of 58 000 (HD1B-I) and 51 000 (HD1B-II). (B) Blots were analyzed by laser densitometry and evaluated quantitatively with Molecular Dynamics Image Quant software. Protein amounts are expressed as arbitrary units. Data are shown as the mean ± SD of four independent experiments.

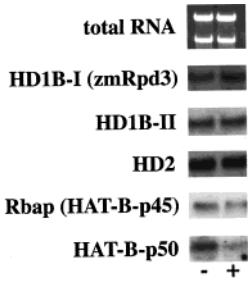

FIGURE 5: Effect of HC toxin on the expression of HD1B-I, HD1B-II, nucleolar HD2, zmRbap, and HAT-B−p50. Maize seeds were germinated for 72 h and excised from the grain. One half was put into 500 mL of MS medium + 1% sucrose (Sigma) supplemented with 10 μg/mL HC toxin; the other half was put into 500 mL of MS medium + 1% sucrose without toxin. Both were incubated for 5 h at 28 °C with good aeration. Subsequently the distal 2−4 mm of the root tips was harvested into liquid nitrogen. RNA was isolated from HC toxin-treated and control material, electrophoresed on 1.2% agarose, 1.1% formaldehyde gels, and blotted onto Hybond-N membrane. Hybridization was performed with PCR-generated DIG-labeled DNA probes corresponding to the entire coding regions of HD1B-II cDNA, zmRpd3 cDNA, deacetylase HD2 cDNA, HATB-p50 cDNA, and Rbap cDNA.

In contrast, only one group of HDs has so far been identified in vertebrates, the members of the HDAC family, which are homologues or highly related proteins of the yeast transcriptional regulator *RPD3* (*8, 15*). HDAC/RPD3 family members have been found as components of large multiprotein complexes containing DNA-binding repressors and corepressors, such as SIN3, MAD, YY1, UME6, SMRT, and NCoR (*11*−*14, 16, 46, 47*). In a few cases enzymatic activity has been demonstrated (*11, 12, 13, 16*).

From plants it was well-known that HDs exist as multiple enzyme forms. The best-characterized system so far is maize, where four HDs (HD1A, HD1B-I, HD1B-II, and HD2) can be discriminated in terms of biochemical and enzymatic properties, subcellular/subnuclear localization, and distinct substrate and lysine-site specificity (*22*−*24, 26, 27, 29*). Whereas HD2 is an acidic nucleolar phosphoprotein, thus representing a novel type of HD (*25*), it remained unclear whether HD1A or HD1B belong to the HDAC/RPD3-type deacetylase family. The present work identifies the two HD1B forms as close homologues of HDAC/RPD3. Since we have purified the fourth maize enzyme HD1A to homogeneity (*26*), we can rule out the possibility that HD1A represents a further RPD3 homologue of maize; biochemical properties, lack of immunoreactivity with anti-RPD3 antibodies, distinct substrate specificity (*27*), and a complete lack of homology of internal HD1A peptide sequences (a total of 70 amino acids; Pipal, Lusser and Brosch, unpublished results) with HDAC/RPD3 sequences clearly argue for a third class of HDs in plants. Therefore maize represents the system in which the complex network of histone deacetylating enzymes is ready for further functional analysis.

With respect to the multiplicity of HDAC/RPD3-type deacetylases in mammalian cells, it was not surprising to find two RPD3-related proteins in maize embryos. In human cells three direct orthologues of yeast *RPD3* have been identified (*8, 47*−*49*). However, the functional significance of the individual HDAC forms is still unclear. In maize there is a clear difference between HD1B-I and -II proteins with respect to the pattern of expression during embryo germination, the abundance, and the solubility from chromatin. Obviously, HD1B-I plays a functional role during the initial stages of germination, since we detected mRNA and protein even in the quiescent embryo; the embryo may depend on a sufficient amount of HD1B-I during the initial hours of germination for DNA repair, a process that is active at the beginning of embryo germination; at that stage HD1B-II is dispensable. It has been shown that within the first hours of embryo germination the DNA damage which is introduced by the dehydration/rehydration process is repaired (for review see ref *50*). It may be assumed that the extensive DNA repair that is occurring at the start of embryo germination (due to DNA damage by dehydration and rehydration) is dependent on specific transitions in chromatin structure that are

facilitated by histone deacetylation. However, when meristematic cells enter S-phase of the cell cycle (*51*), both enzymes are induced, which is reflected in the significant increase in mRNA and protein levels. In murine T-cells, HD1/RPD3 increases during the transition of cells from G_0 to the G_1/S boundary (*32*).

Our finding of Rbap-related protein as a complex partner of HD1B is in line with earlier work that demonstrated Rbap to have a targeting function for enzymes involved in histone acetylation (*8*−*10*). As shown for other systems (*11*−*14*, *46*, *47*), maize HD1B is also associated with Rbap-related protein in two high molecular weight complexes of ∼120 000 and ∼500 000. The complex is unstable and does not resist extensive chromatographic purification. The cytoplasmic histone acetyltransferase B (HAT-B−p50) of maize (*35*) is also tightly associated with an Rbap-related protein (*41*), as was also shown for the cytoplasmic HAT1 of yeast (*9*). The expression pattern during maize embryo germination of the Rbap-related protein (HAT-B−p45) closely resembles the pattern of the catalytic HAT-B subunit (p50) as well as of HD1B-I (*41*). All three proteins have a peak at the time when maximum DNA synthesis occurs in germinating embryos (*51*). We have recently observed an interesting link between the cytoplasmic HAT-B and HD1B (*27*): HAT-B indroduces a characteristic diacetylation pattern on lysine 12 and 5 of newly synthesized H4 (*52*); this specific pattern of H4 acetylation can only be deacetylated by HD1B, but not at all by HD1A or nucleolar HD2 (*27*). Deletion of RPD3 in yeast had a greater impact on lysine 5 and 12 acetylation than on acetylation of other lysines in H3 and H4 (*19*). These results, in particular the HD1B mRNA expression during germination, its association with Rbap-related protein, and the site-specific deacetylation of a replication-linked acetylation pattern on histone H4, argue for a role of HD1B/RPD3 in DNA synthesis, apart from its well-documented role in transcriptional repression.

It has recently been reported that treatment of mammalian cells with trichostatin A resulted in upregulation of HDAC1, whereas other genes were downregulated (*53*). Our results are in clear contradiction, since neither HD1B nor HD2 was affected by HC toxin or trichostatin treatment; only the cytoplasmic histone acetyltransferase HAT-B−p50 was significantly downregulated by deacetylase inhibitor treatment.

The recently presented 2.3 Å resolution structure of yeast histone acetyltransferase Hat1 (*45*) indicated that the specificity of the enzyme for certain histones or histone subspecies may arise not only from the surface structure of the enzyme but also from modification of lysine residues within the recognition site in the histone. The ability of Hat1 to acetylate certain lysine sites could therefore be modulated by the action of other histone acetyltransferases but equally well by deacetylases. The concerted action of multiple acetyltransferases and HDs may therefore create highly specific acetylation patterns on nucleosomes with distinct functional consequences. Maize represents an attractive model system, since at present it is the unique system where different classes of HDs have been characterized and identified.

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