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Analysis of the histone acetyltransferase B complex of maize embryos

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ABSTRACT

Purified histone acetyltransferase B (HAT-B) from maize consists of two subunits, p50 and p45. Cloning of the cDNA and genomic DNA encoding the catalytic subunit p50 revealed a consensus motif reminiscent of other acetyltransferases. Internal peptide sequences and immunological studies identified p45 as a protein related to the Retinoblastoma associated protein Rbap. Antibodies against recombinant p50 were able to immunoprecipitate the enzymatic activity of p50 as well as p45. Consistent with the idea that HAT-B is involved in acetylation of newly synthesized histone H4 during DNA replication, mRNA and protein levels are correlated with S-phases during embryo germination. Inhibition of histone deacetylases by HC toxin or Trichostatin A caused a decrease of the *in vivo* expression of HAT-B mRNA. Regardless of its predominant cytoplasmic localization, a significant proportion of HAT-B-p50 is present in nuclei, irrespective of the cell cycle stage, suggesting an additional nuclear function.

INTRODUCTION

Acetylation of core histones is a ubiquitous post-synthetic modification found in all animal, plant and fungal species so far examined (1). Acetylation occurs at specific lysine residues in the N-terminal extensions of the core histones. A total of 28 N-terminal lysine sites per nucleosome may become reversibly modified. This results in a remarkable heterogeneity of nucleosomes with respect to the pattern of modification. It is still unclear whether distinct acetylation patterns directly alter nucleosomal structure or act as signals, much like protein phosphorylation, to trigger chromosomal events by changing protein–histone interactions. This view was conceptually supported by the finding that non-histone proteins, like p53 or general transcription factors, are acetylated by histone acetyltransferases (HATs) *in vivo* and *in vitro* (2,3).

Recent research on histone acetylation rapidly increased our knowledge on the involved enzymes, HATs and histone deacetylases (HDs). Multiple nuclear HATs and HDs have been cloned and identified as transcriptional regulators (4). It has been demonstrated that HATs and HDs can interact with specific DNA-binding activator or repressor proteins, thereby modulating transcriptional activity of promoters. Research has therefore been focused on the role of HATs and HDs for gene regulation, whereas little attention has been paid on possible effects on DNA replication or repair.

Evidence has accumulated that aberrant histone acetylation is involved in the pathogenesis of malignant disease. It has been shown that a fusion of CBP and a putative acetyltransferase takes place in patients with acute myeloid leukemia, indicating that disregulation of HAT activity contributes to carcinogenesis (5). The tumor suppressor gene product p53 was shown to be an *in vivo* and *in vitro* substrate for p300/CBP, which also have intrinsic HAT activity (2); p53 is activated through an acetylation–phosphorylation cascade (6).

By means of substrate specificity and intracellular localization, HATs have been grouped into two classes: A-type enzymes, which are localized in the nucleus and acetylate nucleosomal core histones, and B-type enzymes that can be obtained from cytoplasmic extracts with a specificity for free core histones, especially H4 (7). In contrast to HDs, B-type HATs are characterized by only moderate amino acid sequence conservation; however, the identified members of this enzyme class have a molecular mass of 45–50 kDa (7–9) and are associated with the retinoblastoma-associated protein Rbap48 (e.g. 8,9). HAT-B has been shown to be specific mainly for H4, causing diacetylation of H4 at lysines 12 and 5 (e.g. 8–10). Recently, the crystal structures of yeast Hat1p and a Gcn5-related N-acetyltransferase have been solved (11,12). B-type HATs are most likely responsible for the acetylation of newly synthesized histone H4 (in some organisms potentially also H3) and are involved in the maturation of chromatin during DNA replication. Yeast Hat2p (the Rb associated protein, Rbap48) is also a subunit of CAF1 (chromatin assembly factor), which escorts an acetylated H3/H4 tetramer to the replication forks (13), as well as of Rpd3-type deacetylase HDAC1 (14), but not of nucleolar HD2-type deacetylases (15).

Maize is a particularly interesting experimental system with respect to enzymes involved in histone acetylation because at present it represents the only organism where multiple HATs and HDs have been characterized by biochemical and molecular terms (e.g. 7,10,15–25). In particular, maize is the only system where three different HDs have been identified and characterized by substrate and lysine site specificity (22,23). Our laboratory

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has purified the cytoplasmic B-type HAT of germinating maize embryos to homogeneity; the purified enzyme consists of two protein subunits with molecular weights of 45 000 and 50 000 and is highly specific for free histone H4 (7,10). Molecular cloning of the encoding cDNA identified the 50 kDa protein as the catalytic subunit, whereas immunological studies and internal peptide sequences indicated a strong homology between the 45 kDa protein and Rbap46/48. Protein and mRNA levels of both HAT subunits fluctuate during the early germination pathway in clear correlation with S-phase. Maize HAT-B protein is present in the dry embryo as stored protein despite of the absence of the corresponding mRNA which cannot be detected up to 22 h after start of embryo germination. HAT-B is also present in nuclei, indicating additional nuclear functions in the absence of replicative DNA synthesis. Treatment of embryo tissue with the HD inhibitors HC toxin or Trichostatin A causes a decrease of HAT-B-p50 mRNA *in vivo*.

MATERIALS AND METHODS

Extraction, purification and protein microsequencing of HAT-B

Maize seeds (*Zea mays*, strain Cuzco) were germinated in darkness for different time periods (0–72 h) on cotton layers soaked with water at 28°C. Meristematic parts of the roots of embryos were harvested into liquid nitrogen for cellular fractionation (21,26). HAT-B was purified from the soluble supernatant by a series of chromatographic steps (Fig. 1) as described in detail previously (7). Purified HAT-B was subjected to SDS–10%PAGE (27) with subsequent blotting onto Immobilon PSQ membrane (Millipore). Protein bands on the membrane were stained with Coomassie blue for 3 min and destained by diffusion in 40% methanol/10% acetic acid. HAT-B-p50 and HAT-B-p45 were excised from the dry blot, digested with Lys-C endoproteinase and resulting peptides after reversed-phase HPLC were subjected to Edman degradation.

HAT assay

For determination of enzyme activity an established standard assay using purified chicken erythrocyte core histones and [^{14}C]acetylCoA was employed (7,10). Trichostatin (0.4 µM) was added to all reactions to inhibit the action of HDs.

Isolation of a maize cDNA encoding HAT-B-p50

Total RNA was purified from maize seedlings after 72 h of germination. cDNA was synthesized using 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 according to the 3′RACE protocol for amplification of the 3′ends of HAT-B-p50 cDNA (28). Degenerate PCR primers were designed from peptide sequences obtained by microsequencing of Lys-C endoprotease digested HAT-B-p50 (Fig. 2A); primer A1 (5′-CARGARCARCARYTNAAYGA) corresponds to amino acids 432–438, and primer A2 (5′-GAYATHCARATHGARGARAT) corresponds to amino acids 441–447. Amplification of the 3′ end was achieved by two consecutive PCR reactions, the first one employing primers A1 and adapter primer (5′-GACTCGAGTCGACATCGA), and the second one with 1 µl of the preceding PCR as template and primers A2 and $d(T)_4$-adapter (5′-CGAGTCGACATCGATTTT) at an annealing temperature of 48 and 46°C, respectively. The resulting PCR products were analyzed by electrophoresis on agarose gels and blotted onto Hybond N-nylon membrane (Amersham). Hybridization with ^{32}P-labeled oligonucleotide A3 (5′-GARGARATHGCNGGNGT; amino acids 445–450) in standard hybridization solution (5× SSPE, 0.1% lauroyl sarcosine, 0.02% SDS, 1% Boehringer blocking reagent) identified a specific 250 bp PCR product. Another PCR approach using sense primer A4 (5′-AARATHAAYGTNTGGAT) corresponding to amino acids 79–84 of HAT-B-p50 cDNA (Fig. 2A) and reverse primer A2R (5′-ATYTCYTCDATYTGDATRTC; amino acids 441–447) and 2 µl of RT product, yielded a 1106 bp fragment which proved to be specific by hybridization with ^{32}P-labeled oligonucleotide A1. Both specific PCR fragments were purified from the gel, cloned into pGEM-T vector (Promega) and subjected to DNA sequencing. 5′ RACE (28) was performed employing primers B1R (reverse primer; 5′-CAACTCGAACAATCTCAACT) for reverse transcription of mRNA, and primers B2R (reverse primer; 5′-CCTTCGAAAATGTATGCAGGA), $(dT)_{17}$-adapter primer and adapter primer, respectively, for PCR with oligo(dA)-tailed cDNA as template. Thereby, second strand synthesis was carried out with $(dT)_{17}$-adapter primer with 1 cycle of 1 min denaturing, 5 min 40°C and 10 min extension, followed by 31 cycles with primers B2R and adapter primer with an annealing temperature of 56°C. Nested PCR was performed with reverse primer B3R

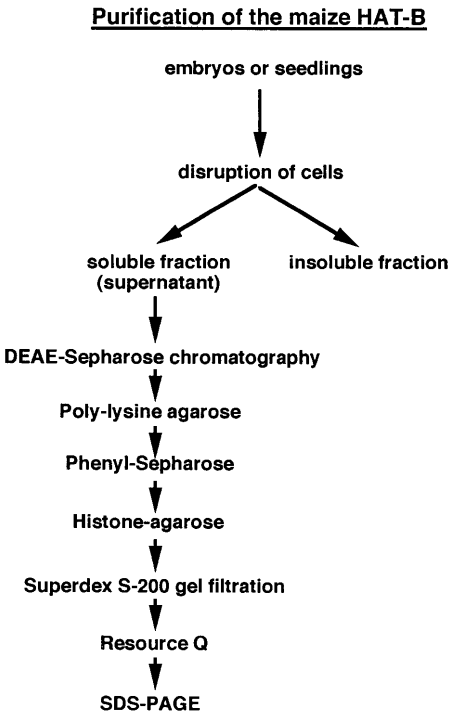

Figure 1. Purification of maize HAT-B by a series of chromatographic steps. HAT-B was purified from total maize seedlings at 72 h after start of embryo germination according to a published procedure (7).

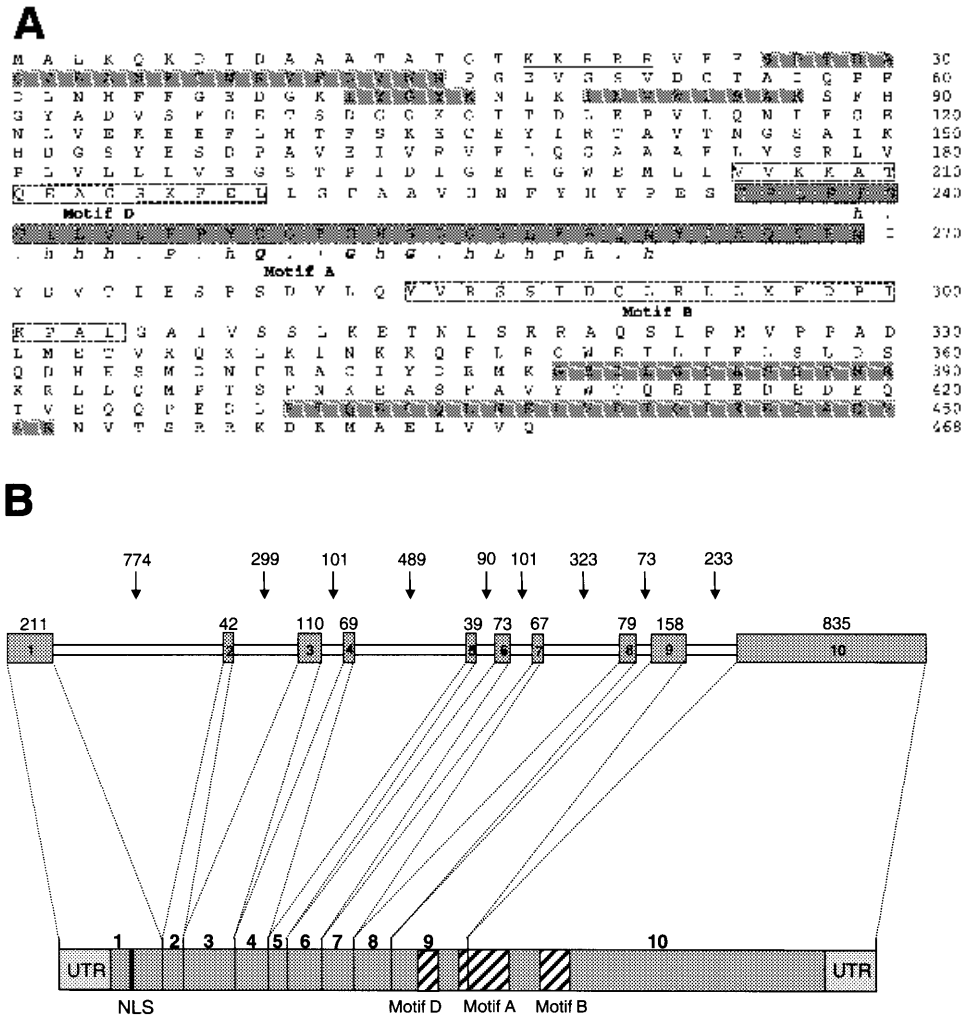

Figure 2. Amino acid sequence deduced from the cDNA sequence and genomic organization of the maize HAT-B-p50 gene. (**A**) Total maize embryo RNA was reverse transcribed. Based on amino acid sequences of HAT-B-p50 peptides, degenerate oligonucleotide primers were designed and used for PCR amplification of cDNA fragments. PCR yielded several products that were analyzed by hybridization with ^{32}P-labeled nested primers. Specific PCR fragments were cloned into pGEM-T vector (Promega) and subjected to DNA sequencing. Subsequently, the 3′ and 5′ ends of the cDNA were amplified, subcloned and sequenced. Peptide sequences derived from protein microsequencing are shaded. Sequence domains (motifs D, A and B) which are conserved among HATs from various organisms and other protein acetylases (35) are boxed; the previously defined acetyltransferase signature motif (34) is boxed and shaded. Invariant glutamine and glycine residues are marked bold. A putative nuclear localization signal is underlined. (**B**) The genomic structure of HAT-B-p50 is shown with exons 1 to 10 (shaded boxes with numbers) and introns (double lines). The lengths are shown above exons and introns. The structure of the HAT-B-p50 cDNA is shown at the bottom. The conserved HAT sequence motifs D, A and B are indicated. UTR, untranslated regions; NLS, putative nuclear localization signal.

(5′-ATTTCGCACTTATCCACACA) and the resulting specific 430 bp product was cloned into pGEM-T vector and sequenced. The cDNA sequence of HAT-B-p50 was deposited in GenBank (U90274).

Cloning of the HATB1 genomic sequence

The genomic sequence of the HAT-B1 gene was identified by a PCR approach using primers corresponding to the very 5′ and 3′ terminal end, respectively, of the cDNA. Two PCRs were carried out to amplify two overlapping fragments covering the 5′ and the 3′ part of the genomic sequence. Therefore the 5′ primer HAT1 (5′-CGCCCGAACGCAGCCATC; nucleotides 1–17 of HAT-B-p50 cDNA) was combined with an internal antisense primer H3R (5′-TACTACCAGAAGCATTTCCC; nucleotides 725–741), whereas the 3′ primer HAT3R (5′-GTG-CCTATGCTTGTGTTG; nucleotides 1664–1683) was used together with primer HAT2 (5′-GGTTGAAGGTTCCACGC) corresponding to nucleotides 684–701 of the HAT-B-p50 cDNA. The PCRs were carried out according to the manufacturer's instructions using the ExpandTM Long Template PCR System (Boehringer Mannheim) and 100 ng of maize genomic DNA as template at an annealing temperature of 55°C. Extension was allowed for 2 min during the first 5 cycles and 3 min for the following 21 cycles, while times for denaturing and annealing remained constant at 20 and 45 s, respectively. The resulting 3 kb fragment covering the 5′ terminal part of the sequence and

the 1.2 kb fragment corresponding to the 3′ terminal part were cloned into pGEM-T vector (Promega) and sequences of the inserts were determined. Sequences were compared to the HAT-B-p50 cDNA and intron borders were defined. Thereby it became obvious that primer HAT2 is spanning an intron/exon boundary. Nevertheless could it be employed successfully for the amplification of the 3′ end of the genomic HAT-B-p50 sequence, most probably due to the relatively high GC content at its 3′ end. A PCR performed with primers HAT1 and HAT3R in order to amplify the whole genomic sequence resulted in amplification of a single 4.2 kb fragment. The genomic sequence of HAT-B-p50 was deposited in GenBank (AF171927).

Overexpression of 6× His-tagged HAT-B-p50 in *Escherichia coli* and production of polyclonal antibodies

Maize HAT-B-p50 coding sequence from amino acid 41–469 was amplified with primer UE5′ (5′-ACAGGATCCTTTCTA-GTATGGAATCCAGGC) and primer UE3′ (5′-AAGCTTTC-ATTGAACCACCAGCTCTGC), both of them carrying 'add-on' restriction sites for *Bam*HI (UE5′) and *Hin*dIII (UE3′), respectively. Using these sites, HAT-B-p50 encoding sequence was cloned into pQE9 vector (Qiagen), thus creating an in-frame fusion with a vector sequence encoding a 6-histidine tag. The recombinant plasmid was transformed into *E.coli* strain M15[pREP4] and protein expression was induced upon addition of 1 mM IPTG. More than 90% of 6× His tagged HAT-B-p50 turned out to be located in the insoluble fraction after bacterial cell lysis and was purified by affinity chromatography on Ni-NTA (nitrilo-tri-acetic acid) resin (Qiagen), according to the manufacturer's instructions using 8 M urea as denaturing agent. Recombinant protein was dialyzed against PBS. Approximately 300 µg of purified protein supplemented with an equal volume of complete Freund's adjuvant was used for immunization of a rabbit. Booster injections were administered once after 3 weeks and twice in 7 day intervals. Bleeding was performed 7 days after the final boost.

To check the recombinant protein for HAT activity, bacteria were lysed under non-denaturing conditions, centrifuged to remove debris, and the supernatant (containing ~10% of total HAT-B-p50) was assayed for enzymatic activity.

RNA blot analysis

HAT-B-p50 transcript expression during maize embryo germination was determined by RNA gel blot analysis. Total RNA from whole embryos (0–22 h after seed imbibition) or meristematic parts of the roots of embryos (30–72 h after seed imbibition) of maize embryos was extracted using TRIzol total RNA isolation reagent (Gibco BRL). Total RNA (15 µg) from 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 PCR fragment generated with primers A4 and A2R in 'High SDS' hybridization solution. Blots were exposed to X-ray film. Blots and ethidium bromide stained gels were analyzed by laser densitometry and evaluated by Molecular Dynamics Image Quant software. The amount of HAT-B-p50 mRNA at each germination time point was corrected for small differences in the amount of RNA loaded onto each gel slot.

Poly(A)$^+$ RNA was isolated from total RNA of embryos germinated for 0, 30 and 72 h using Dynabeads Oligo $(dT)_{25}$ (Dynal) according to the manufacturer's instructions. Poly(A)$^+$ RNA purified from 150 µg of total RNA was subjected to electrophoresis on 1.2%/1.1% formaldehyde gels with subsequent blotting onto Hybond-N-membrane (Amersham).

Immunoblotting of HAT-B during embryo germination

Embryos (0–22 h) or meristematic parts of the roots (30–72 h) at different time points of embryo germination were harvested into liquid nitrogen. After grinding 4 g of each sample 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, 15 mM Tris–HCl, pH 7.9) by six strokes of a motor-driven (200 r.p.m.) Potter–Elvehjem Teflon homogenizer. The homogenate was stirred on ice for 30 min and centrifuged for 15 min at 27 000 *g*. The supernatant was saved. The pellet was resuspended in 1 ml of buffer A (0.25 mM EDTA, 10 mM 2-mercaptoethanol, 15 mM Tris–HCl, pH 7.9). Aliquots of the supernatant and the resuspended pellet were mixed with SDS-sample buffer (27), 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 were incubated with antibodies against recombinant maize HAT-B-p50 (dilution 1:10 000) or antibodies against LeMSI1 (Rbap46 homolog in tomato, dilution 1:10 000) (29) for 2 h at room temperature. Secondary antibody-alkaline phosphatase conjugate or the ECL-system (Amersham) was used for detection. Blots and Coomassie blue stained gels were quantitated by laser densitometry.

Immunoprecipitation of HAT-p50 and p45

Antibodies against recombinant HAT-B-p50 were purified by Protein G-Sepharose affinity chromatography (Hi-trap affinity columns, Pharmacia-Biotech). Purified antibodies were coupled to Protein A-Sepharose according to (30). Antibodies (2 mg) were incubated with 1 ml of wet beads under gentle shaking for 1 h. Beads were then washed twice with 10 vol of 0.2 M sodium borate (pH 9.0), centrifuged at 3000 *g* for 5 min and subsequently suspended in 10 vol of 0.2 M sodium borate. Solid dimethylpimelimidate was added to a final concentration of 20 mM and beads were incubated for 30 min at room temperature on a head over head shaker. The reaction was terminated by washing the beads once in 0.2 M ethanolamine (pH 8.0) for 2 h under gentle shaking. After centrifugation (3000 *g*), antibody coupled Protein A-Sepharose was extensively washed with 0.1 M glycine (pH 3.0) to remove residual non-covalently bound antibodies. Finally, beads were resuspended in buffer B [15 mM Tris–HCl, pH 8.5, 10 mM NaCl, 0.25 mM EDTA, 10 mM 2-mercaptoethanol, 10% (v/v) glycerol] and aliquots were used for immunoprecipitation experiments.

For immunoprecipitation, purified HAT-B was mixed with antibody-coupled Protein A-Sepharose and incubated overnight under head over head shaking at 4°C. Beads were then sedimented, washed twice with an excess of buffer B and resuspended in buffer B for enzymatic assay. In addition, the supernatant after immunoprecipitation as well as the immunocomplexes were subjected to SDS–10%PAGE with subsequent western blotting. Precipitation was immunodetected with anti-6× His-HAT-B-p50 antibody, and co-immunoprecipitation of the 45 kDa HAT-B subunit was shown by immunodetection with an antibody against tomato LeMSI1.

Indirect immunofluorescence microscopy

Nuclei were isolated from whole seedlings at 60 h after start of embryo germination according to a published procedure (31). Isolated nuclei were applied onto poly-lysine coated microscopy slides. After equilibration in PBS and blocking in 2% milk proteins, antibodies against recombinant HAT-B-p50 were applied (2.5 mg IgG/ml) for 3 h at room temperature. Second antibody-FITC conjugate (Dako, 1:50) was used for visualization of antigen–antibody complexes. Nuclei treated with secondary antibody alone (without specific antibodies against HAT-B-p50) served as negative controls.

HC-toxin treatment of *Z.mays* embryos

Approximately 160 maize seeds were germinated for 72 h, excised from the grain, and split into two halves. One half (~80 embryos) was put into 500 ml MS-medium + 1% sucrose (Sigma Chemical Company) supplemented with 10 µg/ml HC-toxin, and the other one was put into 500 ml 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 plant tissue and controls, electrophoresed on 1.2% agarose/1.1% formaldehyde gels, and blotted onto Hybond-N membrane. Hybridization was performed using PCR generated DIG-labeled DNA probes corresponding to the entire coding region of HAT-B-p50 cDNA, histone deacetylase HD2 cDNA (15) and zmRpd3 cDNA (24).

RESULTS

Cloning of HAT-B-p50 encoding cDNA and genomic organization of the HAT-B-p50 gene

A series of chromatographic steps (Fig. 1) was used to purify maize HAT-B to homogeneity. The purified protein consists of two subunits (7), p50 and p45 (Fig. 3, lane 3). Internal peptide sequences of HAT-B-p50 were used to design degenerate oligonucleotide primers for cloning the encoding cDNA by a RT–PCR approach. The final PCR product (1684 bp) was cloned and sequenced; it contained a putative full-length open reading frame of 1407 bp encoding a protein of 469 amino acids with a calculated molecular mass of 52.7 kDa (Fig. 2A). Despite weak overall sequence homology throughout the entire open reading frame to N-acetyltransferases and HATs, the region from amino acids 235–269 of HAT-B-p50 matches the previously defined acetyltransferase signature motif A, which is found in A-type and B-type HATs of different origin (5,32–35) and which contains invariant glutamine and glycine residues (Fig. 2A). Due to the presence of this motif in a variety of enzymes that acetylate diverse substrates, it is likely to participate in acetyl-CoA binding or catalytic activity itself. Moreover, sequence similarities to the other conserved domains (motif B and D) could be observed.

The sequence of the gene encoding the catalytic subunit p50 of the HATB complex was determined by a PCR approach using primers corresponding to the 5′- and 3′-termini of the respective cDNA. Comparison of the obtained sequence of 4176 bp with the previously identified cDNA sequence revealed 10 exons interrupted by 9 introns (Fig. 2B). The size of the exons ranged between 39 (exon 5) and 835 bp (exon 10), whereas the introns had an average length of 200 bp except for intron 1, which was 774 bp long. All exon/intron boundaries conform to the consensus splicing signal (GT..AG). Exons 1–9 encoding the N-terminal half of HATB-p50 show relatively small sizes (39–211 bp) and appear to be clustered in four groups interrupted by relatively long introns (233–774 bp) corresponding to exon 1, which contains the 5′ untranslated region and the putative nuclear localization signal, exons 2–4, exons 5–7 and exons 8 and 9 (Fig. 2B). Exon 9 contains the conserved motif D and exon 8 corresponds to a sequence of yeast Hat1 that is involved in acetylCoA binding as determined by crystal structure analysis (11). Furthermore, this region has been suggested to be involved in histone binding (11) since it contains amino acid residues that are highly conserved within either the Hat1 family or the Gcn5 family of HATs, but not between these. This may be the reason for the different substrate specificities of the two HAT families (36). The other two sequence domains (motif A and the less conserved motif B) that are highly homologous among not only histone- and N-acetyltransferases but also other metabolic enzymes, drug resistance enzymes and several proteins with unknown functions (35), are positioned in one large exon (exon 10) which also comprises the rest of the C-terminal sequence of HAT-B-p50.

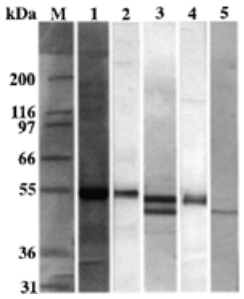

Figure 3. Immunoblot detection of purified maize HAT-B polypeptides (p50, p45) by antibodies against recombinant HAT-B-p50 and antibodies against tomato LeMSI1 (Rbap homolog). HAT-B-p50 was expressed as a 6His-fusion protein in *E.coli*. Lane 1, Coomassie blue stained SDS–PAGE of purified recombinant p50; lane 2, immunoblot of lane 1 with anti-rHAT-B-p50 antibodies (purified IgG); lane 3, Coomassie blue stained SDS–PAGE of highly purified maize embryo HAT-B (p50, p45); lane 4, immunoblot of lane 3 with anti-rHAT-B-p50 antibodies; lane 5, immunoblot of lane 3 with antibodies against LeMSI1 (a tomato homolog of Rbap). Molecular mass marker proteins (kDa) are shown (M). Due to the histidine-tag, rHAT-B-p50 migrates slightly slower than the native protein (at an apparent molecular mass of 53 kDa). Secondary antibody alkaline phosphatase conjugates were used for immunodetection.

Immunological identification of p50 as the catalytic subunit of HAT-B and p45 as a protein related to the retinoblastoma associated protein

HAT-B-p50 encoding cDNA was expressed as a histidine-tagged protein in *E.coli*. The recombinant protein was purified by Ni-NTA affinity chromatography and administered to rabbits for raising an antiserum against HAT-B-p50. The recombinant protein itself was enzymatically inactive. Figure 3 shows that the antibodies against p50 specifically detect the recombinant protein as well as the 50 kDa subunit of purified maize HAT-B. In immunoprecipitation experiments the antibodies were

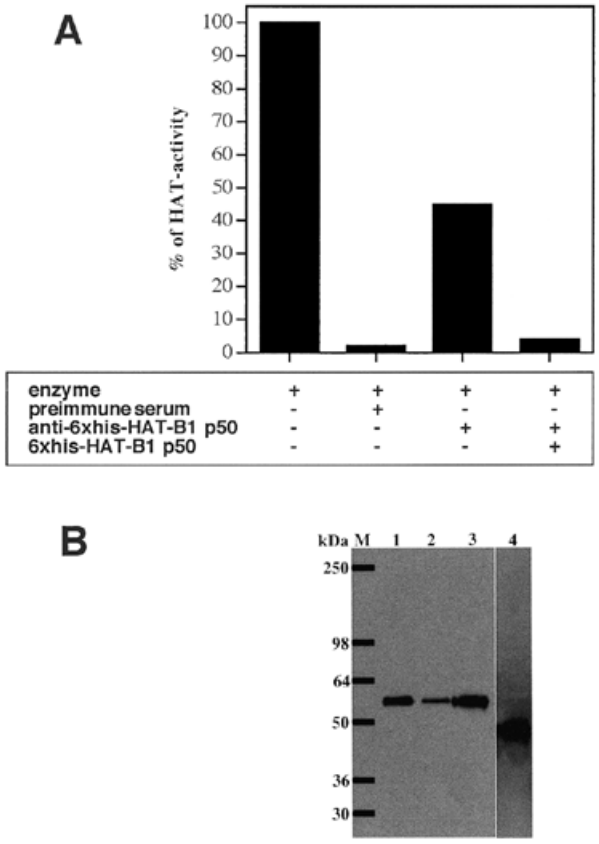

Figure 4. Immunoprecipitation of maize HAT-B by antibodies against recombinant p50. (**A**) Highly purified HAT-B from maize embryos at 60 h of embryo germination was mixed with anti-rHAT-B-p50 antibodies or preimmune serum coupled to Protein A-Sepharose and incubated overnight under head over head shaking at 4°C. Beads were then sedimented, washed and assayed for enzymatic activity. As a control, an excess of enzymatically inactive recombinant p50 was added to the precipitation reaction. (**B**) Purified maize HAT-B (lane 1), the supernatant after immunoprecipitation (lane 2) as well as the immunoprecipitate (lanes 3 and 4) were subjected to SDS–10%PAGE with subsequent western blotting. Loading amounts of lanes 1 and 2 can be compared directly. Immunodetection was performed with anti-rHAT-B-p50 antibodies (lanes 1–3). Co-immunoprecipitation of the 45 kDa HAT-B subunit was verified by immunodetection with an antibody against LeMSI1, an Rbap homolog of tomato (lane 4). Positions of molecular mass markers (M) are indicated (kDa). Immunodetection was done with the ECL detection system (Amersham).

able to immunodeplete a highly purified HAT-B preparation of HAT activity; almost 50% of the enzyme activity could be recovered from the immunoprecipitate (Fig. 4A). The corresponding pre-immune serum was unable to precipitate HAT activity (Fig. 4A). Immunoprecipitation of the native enzyme could be impeded by inclusion of an excess of exogenous, enzymatically inactive, recombinant p50 in the precipitation reaction. Immunoblotting of the supernatant and immunoprecipitate with anti-HAT-B-p50 antibodies showed that the majority of HAT-B-p50 was present in the precipitate (Fig. 4B). These results were also obtained when a crude HAT-B preparation (only a Q-Sepharose chromatography step for separation) was used instead of a highly purified sample (result not shown).

Highly purified HAT-B-p45 was digested with Lys-C endoproteinase and resulting peptides were microsequenced after HPLC separation. Two longer peptide sequences were obtained: peptide 1, KLMMWDLRTNKPEQSALAHRK; and peptide 2, KISELSWNPSEK. When using BLAST database search program for comparison with sequences in available databases, these peptides were found to be highly homologous to retinoblastoma associated proteins and related WD-repeat proteins (e.g. GenBank accession nos O22469, Q60972, Q09028, AF016845, Q60973 and Q16576). We therefore tested a purified HAT-B preparation for reactivity with an antibody against LeMSI1; a tomato WD repeat homolog to retinoblastoma associated protein (29). Figure 3 shows that this antibody specifically recognized the 45 kDa subunit of purified HAT-B. When purified HAT-B was immunoprecipitated with antibodies against HAT-B-p50, the 45 kDa subunit was co-immunoprecipitated as shown in the immunoblot of Figure 4B. Together with the fact that the corresponding yeast enzyme Hat1p is associated with an Rb binding protein, these data strongly suggest maize HAT-B-p45 to be an Rbap-related protein.

Levels of HAT-B expression during maize embryo germination

We extracted total RNA of embryos or meristematic parts of the roots at different time points of embryo germination and probed RNA blots with a DIG-labeled HAT-B-p50 cDNA fragment. The mRNA migrated at the position of the 18S rRNA (Fig. 5A and B). HAT-B-p50 mRNA was not detectable until 15 h of germination when it appeared as a faint signal. At 22 h after start of seed imbibition, the maximum level was reached. The level then decreased up to 60 h before a second small increase at 72 h (Fig. 5A and C). This pattern of expression correlates with the proportion of cells in S-phase, as measured by DNA flow cytometry and S-phase associated markers (37) as well as by activity levels of thymidine kinase (results not shown). We also isolated mRNA at 0, 30 and 72 h of germination (Fig. 5D); the blot revealed complete absence of HAT-B-p50 mRNA in the dry embryo (0 h), a high level of expression at 30 h and a decreased expression at 72 h.

We solubilized total HAT-B protein by salt extraction of cellular homogenates of embryos or meristematic root tissue at different time points of germination. Immunodetection of the corresponding protein blots with antibodies against HAT-B-p50 revealed a first maximum of p50 at 22 h and a second increase at 46 h and later time points during germination (Fig. 6). Immunoblotting with an antibody against tomato LeMSI1 (a protein related to the retinoblastoma associated protein) revealed that this protein has a maximum at 30 h, a decrease at 46 h and a second increase at 60 h of germination (Fig. 6). Strikingly, p50 and p45 proteins were present at early time points of germination, even at 0 h in the dry embryo when mRNA was not yet detectable. Obviously, preformed HAT-B protein is stored during embryogenesis in order to be available for specific functions at the very early germination period.

Subcellular localization of HAT-B p50

By definition, the B-type HATs are cytoplasmic enzymes, responsible for the acetylation of newly synthesized histones (predominantly H4) during DNA replication. This is in line with the fact that maize HAT-B can be recovered from the

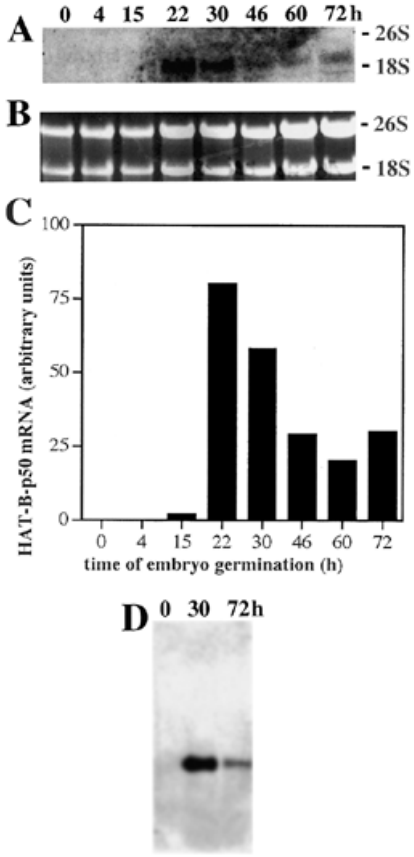

Figure 5. Expression of HAT-B-p50 mRNA during maize embryo germination. Total RNA (B) was isolated from embryos at different times of germination, electrophoresed in 1.2% agarose/1.1% formaldehyde gels, blotted, and hybridized with a DIG-labeled fragment of HAT-B-p50 cDNA (A). The positions of the 18S and 26S rRNA are indicated. The amount of HAT-B-p50 mRNA (**A**) was related to the amount of input RNA (**B**) in order to correct for small differences in the amount of RNA loaded to each gel slot. (**C**) This ratio (labeling intensity:amount of RNA) is expressed in arbitrary units. (**D**) mRNA was isolated from embryos at 0, 30 or 72 h of germination, electrophoresed in 1.2% agarose/1.1% formaldehyde gels, blotted, and hybridized with a DIG-labeled fragment of HAT-B-p50 cDNA.

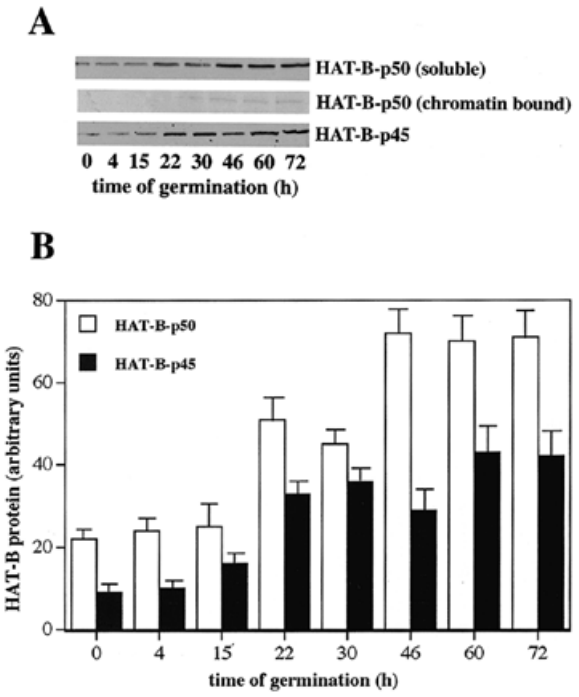

Figure 6. Levels of expression of HAT-B protein during maize embryo germination. HAT-B protein was extracted from embryos at different times of germination. Equal amounts of protein (50 µg) were subjected to SDS–PAGE with subsequent blotting onto nitrocellulose membrane. (**A**) Immunodetection of HAT-B-p50 was done with an anti rHAT-B-p50 antibody in the soluble salt extract as well as in the insoluble chromatin fraction, detection of HAT-B-p45 with an antibody against LeMSI1 only in the soluble fraction. Alkaline-phosphatase conjugated secondary antibodies were used for visualization of bands. (**B**) Immunoblots (HAT-B-p50 and p45, soluble) were analyzed by laser densitometry and evaluated quantitatively using Molecular Dynamics Image Quant software. Protein amounts are expressed as arbitrary units. Data are shown as the mean ± SD of four independent experiments.

soluble cytoplasmic supernatant after homogenization (Fig. 6A) (7,21). However, a small percentage of HAT-B-p50 was also present in the chromatin pellet, resistant to salt extraction, at later germination stages (Fig. 6A). We therefore performed immunocytochemical staining of thin-cuts of embryo tissue with anti-HAT-B-p50 antibodies; these experiments indicated the presence of a minor part of the enzyme in the nuclei (result not shown). To clarify this point, we isolated nuclei according to a published procedure (31) and analyzed them by immunofluorescence microscopy. Figure 7 shows that nuclei are indeed decorated by the antibody. Since we used whole seedlings (at 72 h after start of germination) for this experiment, nuclei from all stages of the cell cycle are present in our preparation. The anti-HAT-B-p50 antibody stained the nuclei, irrespective of the cell cycle stage (Fig. 7).

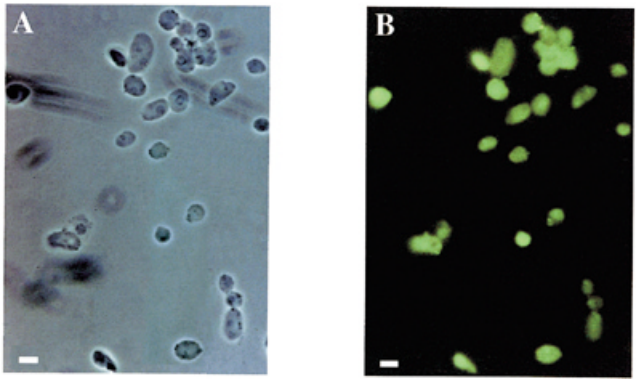

Figure 7. Maize HAT-B-p50 in isolated nuclei. Nuclei were isolated from whole embryos at 60 h of embryo germination. (**A**) Phase contrast microscopy of isolated nuclei. (**B**) Indirect immunofluorescence labeling (FITC) of the corresponding area of (A) with anti-rHAT-B-p50 antibodies (2.5 mg IgG/ml, dilution 1:1000). Bar, 5 µm.

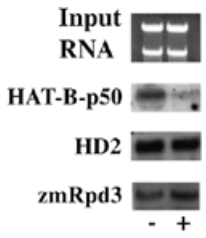

Figure 8. Effect of HC toxin on the expression of HAT-B-p50, nucleolar HD2 and zmRpd3. Maize seeds were germinated for 72 h and excised from the grain. One half was put into 500 ml MS medium+1% sucrose (Sigma) supplemented with 10 μg/ml HC-toxin. The other half was put into 500 ml MS-medium+1% sucrose without toxin. Both were incubated for 5 h at 28°C with good aeration. Subsequently the distal 2–4 mm of the root tips were 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 using PCR generated DIG-labeled DNA probes corresponding to the entire coding regions of HATB-p50 cDNA, histone deacetylase HD2 cDNA and zmRpd3 cDNA.

Effect of HC toxin on the expression of HAT-B-p50

Incubation of meristematic root tissue at 72 h after start of embryo germination with the cyclic tetrapeptide HD inhibitor HC toxin (25) resulted in a pronounced decrease of the HAT-B-p50 mRNA expression level (Fig. 8). The same effect was observed when Trichostatin A was used (results not shown). In contrast, the expression of two maize HDs, zmRpd3 (24) and HD2 (15), was not at all affected by HC toxin treatment (Fig. 8).

DISCUSSION

In this report we show that plant cells contain a cytoplasmic HAT of the B-type that is composed of two subunits. The catalytic subunit of 50 kDa is homologous to yeast Hat1p (8,33), and the second 45 kDa subunit is a member of a conserved family of WD-repeat proteins which are related to the retinoblastoma associated proteins and associated to numerous proteins and enzymes that bind or modify core histones (8,9,14,29,32,38). The HAT-B holoenzyme, previously purified from maize (7) and identified here, has the same characteristics as B-type enzymes characterized in other systems. It has a pronounced *in vivo* and *in vitro* substrate specificity for histone H4, in particular for the non-acetylated isoform of H4 (7,10,32), a similar native molecular mass of 90 kDa (7), and an enzymatically active subunit of almost identical molecular mass of 52.7 kDa. The high specificity of B-type HATs for histone H4, which is acetylated on lysine 12 and 5 with a strict order of site usage (10), and the fact that the expression pattern and enzyme activity pattern is correlated with S-phase (37,38) suggests that HAT-B has a specific function in DNA replication. The same has been demonstrated during the naturally synchronous cell cycle of *Physarum polycephalum* (39). However, the finding that HAT-B protein is present in dry seeds and during the initial period of embryo germination when no replicative DNA synthesis takes place, argues for an additional, yet unknown role of this enzyme in chromatin structure and function. Obviously, HAT-B is synthesized during embryogenesis and stored for the early embryo germination process. It is suggestive that the embryo depends on a sufficient amount of HAT-B during the initial hours of germination. Expression of new HAT-B-p50 mRNA does not start before 15 h of germination, but then sharply increases to reach a maximum at 22 h. 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 (40). It may be assumed that the extensive DNA repair that is occurring at the start of embryo germination is dependent on specific transitions in chromatin structure for which B-type acetyltransferase is required. It is interesting in this context that a proportion of the enzyme is not solubilized by cellular homogenization and salt extraction and can be detected in the nuclei, even in nuclei after isolation; this fact argues for tight binding of a proportion of the enzyme to chromatin. The enzyme is certainly most abundant in the cytoplasm, as judged from numerous biochemical and immunocytochemical investigations (7,8,16,21,39), but a significant amount is present in nuclei, irrespective of the cell cycle stage. It has recently been shown in human cells that FLAG-epitope-tagged Hat1 polypeptide accumulated predominantly in the nuclei of S-phase cells (32). With respect to the recently reported acetylation of non-histone regulatory proteins (2,3), it may well be that HAT-B has additional functions in such acetylation pathways. It is interesting in this context that computer analysis for putative intracellular localization of HAT-B-p50 resulted in predominant cytoplasmic localization, but with a probability of 41% for nuclear location. However, when the N-terminal amino acids (a total of 25 amino acids) were used for the analysis, the localization of this short peptide was designated predominantly nuclear. Apart from the internal peptide sequences which finally led to the present cDNA sequence, we initially also obtained a minor N-terminal peptide sequence in our first protein sequencing approach which matched a peptide starting at amino acid 26. It may well be therefore that two populations of the B-type enzyme are present in maize, a shorter, cytoplasmic enzyme and a longer, preferably nuclear counterpart.

The cytoplasmic portion of HAT-B-p50 is stably associated with p45. At present we cannot decide whether the small nuclear portion of HAT-B-p50 also exists as a complex with p45 because the antibody against LeMSI1 does not give clear-cut results in immunofluorescence microscopy. Even if the antibody would be applicable in the immunofluorescence technique, one would not be able to decide whether the immunosignal is due to specific reaction with p45 or possibly with some other member of the Rbap protein family. It is possible that nuclear HAT-B-p50 has different nuclear protein partners which could be the reason for a distinct, yet unknown functional significance. This will be the subject of more detailed analysis.

It was striking to find a pronounced down-regulation of HAT-B-p50 mRNA as a result of *in vivo* treatment with HD inhibitors; since such a treatment leads to core histone hyperacetylation (25), one would not necessarily expect down-regulation of an acetylating enzyme. This regulation could be due to several reasons; it may be that Trichostatin A slows down the cell cycle, decreasing the proportion of cells in S-phase, therefore also leading to a decrease of histone synthesis and consequently to a decrease of the enzyme responsible for acetylation of newly synthesized H4. Alternatively, it could well be that the expression of the HAT-B gene is itself affected by acetylation, regardless of cell cycle perturbations, so that a change of the acetylation pattern within the HAT-B gene leads to repression of its expression.

It is remarkable that B-type HATs are functionally and structurally conserved from plants and fungi to mammalian cells, yet the overall sequence homology is rather low. Besides a consensus signature motif characteristic of a variety of acetylCoA binding enzymes (35), there are no conserved elements among B-type HATs. However, the crystal structure of the yeast Hat1-acetylCoA complex at 2.3 Å resolution has revealed structural surface motifs which are conserved among the whole protein superfamily, including Gcn5 and p300/CBP (11).

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