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HAT1 and HAT2 Proteins Are Components of a Yeast Nuclear Histone Acetyltransferase Enzyme Specific for Free Histone H4*

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We have analyzed the histone acetyltransferase enzymes obtained from a series of yeast *hat1*, *hat2*, and *gcn5* single mutants and *hat1,hat2* and *hat1,gcn5* double mutants. Extracts prepared from both *hat1* and *hat2* mutant strains specifically lack the following two histone acetyltransferase activities: the well known cytoplasmic type B enzyme and a free histone H4-specific histone acetyltransferase located in the nucleus. The catalytic subunits of both cytoplasmic and nuclear enzymes have identical molecular masses (42 kDa), the same as that of HAT1. However, the cytoplasmic complex has a molecular mass (150 kDa) greater than that of the nuclear complex (110 kDa). The possible functions of HAT1 and HAT2 in the yeast nucleus are discussed. In addition, we have detected a yeast histone acetyltransferase not previously described, designated HAT-A4. This enzyme is located in the nucleus and is able to acetylate free and nucleosome-bound histones H3 and H4. Finally, we show that the *hat1,gcn5* double mutant is viable and does not exhibit a new phenotype, thus suggesting the existence of several histone acetyltransferases with overlapping functions.

Acetylation of lysine residues in nucleosome core histones is a reversible process that occurs in all eukaryotic organisms studied. This post-translational modification is extremely specific since it involves particular lysines in the N-terminal region of the four core histones. Thus, four specific lysine residues in histones H4, H3, and H2B can be acetylated, whereas histone H2A can only be acetylated at one lysine residue (reviewed in Refs. 1 and 2).

Two main types of histone acetyltransferase (HAT)[1] have been characterized according to the criteria of intracellular location and histone specificity (reviewed in Ref. 3). The HAT-A class comprises nuclear enzymes that catalyze the postsynthetic acetylation of all core histones. HAT-B enzymes are cytoplasmic and seem to be responsible for the acetylation of free histones (primarily histone H4) before the process of chromatin assembly.

The link between histone acetylation and some of the most important biological processes of the cell has been under study for years (reviewed in Refs. 1–6). Only recently, however, has evidence been found to link this modification to, at least, chromatin assembly and gene activation (reviewed in Ref. 3). The discovery that the protein encoded by the yeast *GCN5* gene, previously described as a transcriptional adaptor required by a group of transcriptional activators (7), works as the catalytic subunit of a HAT activity (8) provides a direct link between histone acetylation and gene transcription. GCN5 is a nuclear HAT-A enzyme that acetylates H3 and, to a lesser extent, H4 in their free forms but is unable to modify any nucleosome-bound histone (9, 10). However, we have recently demonstrated that GCN5 is a member of a multisubunit complex possessing HAT activity that modifies H3 in nucleosomes (11). Moreover, it has also been reported that GCN5 functions in two multisubunit complexes that are able to acetylate nucleosomal histones. Both complexes contain ADA2 and ADA3, whereas the larger complex also contains SPT3, SPT7, and SPT20/ADA5 (12). The direct link between histone acetylation and gene activation has been confirmed by the finding that mammalian $TAF_{II}250$, which is a subunit of the general transcription factor TFIID, has HAT activity (13). Human $TAF_{II}250$ and its homologues in *Drosophila* and yeast are HAT-A enzymes specific for the free histones H3 and H4 *in vitro*. *Drosophila* $TAF_{II}230$ and human and yeast GCN5 show the same specificities toward lysine 14 of free histone H3 (3, 9). GCN5 is also highly selective for lysines 8 and 16 of free histone H4 (3, 9). Consistent with these results, acetylation of those lysine residues has been correlated with transcriptional activation (reviewed in Ref. 14). Other proteins, which have been identified as HAT-A enzymes and are able to modify histones in nucleosomes, are P/CAF, a human protein related to GCN5 but which does not show homology with any known yeast protein (10), the steroid receptor SRC-1 (15), and ACTR, a nuclear receptor coactivator (16).

The direct link between histone acetylation and chromatin assembly was recognized following the purification from human nuclear extracts of CAF-1 (chromatin assembly factor 1), a complex of three polypeptides (p46, p48, and p150) that is able to promote *de novo* nucleosome assembly during *in vitro* DNA replication. This factor interacts specifically with post-translationally modified cytoplasmic H3/H4 dimers but does not assemble histones isolated from nuclei (17–19). More recently, another chromatin assembly complex has been found that contains the three subunits of CAF-1 plus H3 and H4 (20). Chromatin assembly complex histone H3 is a mixture of the non-acetylated and monoacetylated isoforms of the histone. Chromatin assembly complex H4 is a mixture of four isoforms,

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[1] The abbreviations used are: HAT, histone acetyltransferase; CAF, chromatin assembly factor; PMSF, phenylmethylsulfonyl fluoride.

ranging from non-acetylated to triacetylated. Lysines 5, 8, and 12 have been identified as the modified residues using site-specific antibodies that recognize H4 molecules modified at each of the four acetylatable lysines (20). It has been demonstrated that in newly synthesized histone H4, lysines 5 and 12 are acetylated (reviewed in Ref. 3), whereas in H3 only lysine 9 is acetylated (9). B-type HATs are responsible for this acetylation of newly synthesized histones and thus the acetylation associated with chromatin assembly. Some of these enzymes have been biochemically characterized and show specificity for histone H4 at the amino acid positions mentioned above (21–24). However, a major advance in this field has been made by the cloning of the first *HAT* gene (25). The protein encoded by the yeast *HAT1* gene is the major cytoplasmic HAT-B activity. This enzyme acetylates lysine 12 of free histone H4 but does not modify this histone when packaged in chromatin (25, 26). HAT1 co-purifies with another protein, namely HAT2, which strongly increases the affinity of the enzyme for histone H4 and is closely related to p48 (26), one of the three subunits of CAF-1 mentioned above.

The studies reported in this paper aim to connect the important advances achieved in cloning genes that encode proteins possessing HAT activity to the biochemical studies of the yeast HAT enzyme types.

In yeast it has been possible to identify the genes for three enzymes with HAT activity, GCN5 and $TAF_{II}130$ (HAT-A type) and HAT1 (HAT-B type). However, using biochemical methods, previous results from our laboratory have been reported showing that the yeast *Saccharomyces cerevisiae* contains at least four HAT activities (27). Three of these enzymes are of nuclear type A, and they differ in their histone specificities. The fourth enzyme represents a canonical type B cytoplasmic H4-specific HAT activity (28). By using *gcn5* mutants we have recently reported that GCN5 is the catalytic subunit of one of the enzymes previously designated HAT-A2 (11).

In this paper we analyze and characterize the HAT activities obtained by biochemical methods from a series of mutants of the *HAT1*, *HAT2*, and *GCN5* genes, and *hat1,hat2* and *hat1,gcn5* double mutants. Comparing these results to those obtained from wild type yeast strains, we demonstrate that the HAT1 and HAT2 proteins are present not only in the yeast cytoplasmic HAT-B enzyme but also in a nuclear HAT enzyme with specificity for free histone H4. In addition, we identify a new yeast nuclear HAT activity, designated HAT-A4, which is specific for histones H3 and H4.

EXPERIMENTAL PROCEDURES

Strains and Media—*S. cerevisiae* strain BMA64-1a (*MATa, ura3-1, trp1-Δ2, leu2-3, 112, his3-11, ade2-1, can1-101*), which has a W303 genetic background, was used for *HAT1* disruption yielding the BQS176 (BMA64-1a plus *hat1::KanMX3*) strain; GMY27 (*MATα, ade2-101, leu2-3, 112, his3-Δ200, lys2-801, ura3-52, gcn5::HisG*) was a gift of Dr. N. Silverman. BQS176 and GMY27 were crossed to give the diploid strain BQS177 which was sporulated and analyzed to obtain the haploid strain BQS177-3d (*MATα, hat1::KanMX3, gcn5::HisG, his3, leu2-3, 112, ura3, ade2-1*). Strain UCC623 (*MATa, ura3-52, lys2-801, ade2-101, trp1-Δ1, his3-Δ200, leu2-Δ1, hat2::TRP1*) was a gift of Dr. D. Gottschling and used for *HAT1* disruption yielding the BQS241 (UCC623 plus *hat1::KanMX3*) strain. Genotypes of the yeast strains were verified by Southern analysis and growth on synthetic media with or without supplements and/or G418 (Geneticin, Life Technologies, Inc.).

Disruption of HAT1 Gene—We followed the method of Wach *et al.* (29) as follows: the oligonucleotides HAT1S1, 5′GTCTTTGGTCTACTGCATCGCCTCAAAAGGTTATAGCATCCTCTCTGCTGCCATG3′, and HAT1S2, 5′TTAACCTTGAGATTTATTTATCGACTCTATAATACTCCGGCAAAGACGCGAGCGA3′, were used to amplify a polymerase chain reaction fragment from linearized pFA6-KanMX3 as template. 2 μg of polymerase chain reaction fragment were used to transform yeast strains. This disruption deletes the complete *HAT1* open reading frame.

Subcellular Fractionation and Preparation of Extracts—Yeast strains were grown at 28 °C to exponential phase in liquid YPD medium (1% yeast extract, 2% peptone, 2% glucose). Cells were harvested by centrifugation at 1500 × *g* for 5 min and washed twice in distilled water at room temperature. After centrifugation, the cell pellet was weighed and resuspended in 1 ml/g pretreatment buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 M sorbitol, 60 mM 2-mercaptoethanol), incubated with gentle agitation at room temperature for 15 min, collected by centrifugation (1500 × *g*, 5 min), and resuspended in 3 ml/g digestion buffer (pretreatment buffer containing only 5 mM 2-mercaptoethanol). Yeast spheroplasts were produced by incubation with Zymolyase 20T (Bayer, 2 mg/g cells) at 37 °C for 20–45 min with gentle agitation. Spheroplasts were collected at 4000 × *g* for 10 min and gently washed three times in digestion buffer at 4 °C.

For yeast nuclei purification, a modification of the procedure of Amati and Gasser (30) was used. Spheroplasts were resuspended in digestion buffer (0.5 ml/g cells) and dropped on 20 volumes of cold lysis buffer (18% Ficoll (Amersham Pharmacia Biotech), 10 mM Tris-HCl, pH 7.5, 20 mM KCl, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM PMSF, 2 μM *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64), and 1 μg/ml chymostatin) with continuous agitation. Cellular debris and unlysed cells were removed by centrifuging twice at 3000 × *g* for 5 min. Nuclei were pelleted by centrifugation at 20,000 × *g* for 20 min. The post-nuclear supernatant was discarded, and the pellet containing crude nuclei was resuspended in 10 volumes of purification buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 10%(v/v) glycerol, 1 mM PMSF, 2 μM E64, and 1 μg/ml chymostatin). The suspension was mixed with an equal volume of 66% Percoll (Amersham Pharmacia Biotech) in the same solution and centrifuged at 30,000 × *g* for 35 min. A band of clean nuclei was collected from the upper part of the Percoll gradient, diluted at least three times with recovery buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, 1 mM PMSF, 2 μM E64, and 1 μg/ml chymostatin), and centrifuged (20,000 × *g*, 15 min). The supernatant was removed by aspiration, and nuclei were washed with the same solution and recovered by centrifugation at 15,000 × *g* for 10 min. The final nuclear pellet was used for enzyme extraction as described below.

Whole cell extracts were obtained by the salt dissociation/ultracentrifugation method previously described (11). After dialysis against buffer B (15 mM Tris-HCl, pH 7.9, 0.25 mM EDTA, 5 mM 2-mercaptoethanol, 0.05% (v/v) Tween 20, 10% (v/v) glycerol, 10 mM NaCl), cell extracts were made to 80 mM NaCl by addition of solid NaCl, centrifuged at 27,000 × *g* for 10 min and used for chromatographic fractionation.

Nuclear extracts were obtained by applying the salt dissociation/ultracentrifugation method to purified nuclei. Yeast nuclei were lysed in extraction buffer (75 mM Tris-HCl, pH 7.9, 0.25 mM EDTA, 5 mM 2-mercaptoethanol, 0.05% (v/v) Tween 20, 1 mM PMSF, 2 μM E64, and 1 μg/ml chymostatin), and the lysate was made 0.5 M with NaCl and centrifuged at 100,000 × *g* for 1 h. The supernatant was used as the source of nuclear histone acetyltransferases.

Cytoplasmic extract was prepared by lysis of spheroplasts according to the procedure of Parthun *et al.* (26). After centrifugation (10,000 × *g* for 10 min) to remove nuclei and cell debris, the supernatant, containing the cytoplasmic fraction, was dialyzed against buffer B and used for chromatographic fractionation.

Fractionation of Enzymes—Crude enzyme extracts, prepared as described above, were fractionated by Q-Sepharose chromatography as previously reported (11), except that a 80–380 mM NaCl gradient (instead of 10–500) was used in order to obtain better resolution of the enzymes. To compare the content of histone acetyltransferase activities in wild type and several mutant yeast strains, identical Q-Sepharose FF columns were loaded with crude enzyme extracts containing similar amount of proteins. No qualitative differences in HAT activities were observed between the different genetic backgrounds of the yeast strains.

The molecular masses of native HAT complexes were estimated by sucrose gradient ultracentrifugation as described previously (11). After centrifugation, gradients were fractionated and aliquots assayed for histone acetyltransferase activity. The position of each enzyme complex in the gradients was determined by its histone specificity (monitored by fluorography, see below).

Histone Acetyltransferase Assays—Liquid histone acetyltransferase activity assays were performed with chicken erythrocyte-free histones or oligonucleosomes and [1-^{14}C]acetyl-CoA (Amersham Pharmacia Biotech, 52 mCi/mmol) as substrates exactly as described previously (11). Chicken whole histones were purified from erythrocyte nuclei (31) by

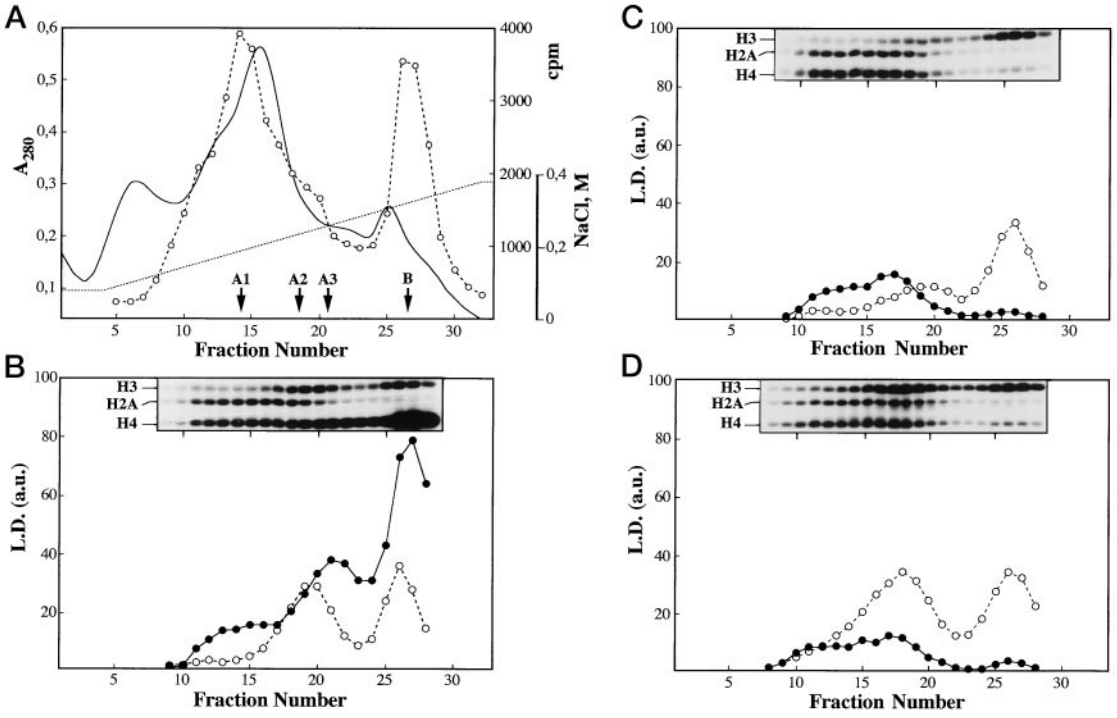

FIG. 1. **Analysis of histone acetyltransferase activities in wild type, *hat1* mutant, and *hat2* mutant yeast strains.** Cell extracts of the different yeast strains were applied to Q-Sepharose FF columns (4 × 1 cm) and eluted with 50 ml of a linear NaCl gradient (80–380 mM). Fractions of 1.8 ml were collected, and the activities of histone acetyltransferases were determined over the whole chromatographic eluates using chicken erythrocyte histones. *A*, chromatographic profile of yeast histone acetyltransferase activities from wild type. Protein concentration was estimated by monitoring A_{280} in the eluate (*solid line*). Enzymatic activity is expressed as cpm (○ and *dashed line*). The *dotted line* represents the NaCl gradient. *A1, A2, A3,* and *B* mark the positions of elution of enzymes in the chromatographic profile. *B*, histone specificity analysis of yeast histone acetyltransferases. After incubation of aliquots from the chromatographic elution shown in *A* with chicken erythrocyte histones in the presence of [^{14}C]acetyl-CoA, histones were recovered and separated by SDS-polyacrylamide gel electrophoresis and subsequently fluorographed (*insert*). Radiolabel incorporated into each histone was determined by densitometry of fluorograms using a 2202 Ultroscan (LKB) densitometer. The labeling density (*L.D.*) values, expressed in arbitrary units (*a.u.*), are plotted against fraction number. ○, labeling density for H3; ●, 0.5 × labeling density for H4. *C* and *D*, specificity analyses of histone acetyltransferases from *hat1* and *hat2* mutant strains, respectively. Symbols are as in *B*. Labeling density values for histone H2A are not significantly different in the various strains used and are not shown.

acid extraction (32). Chicken erythrocyte oligonucleosomes were prepared as described previously (11).

The histone acetyltransferase activity gel assay (in-gel assay) was carried out essentially as described Brownell and Allis (33) using 10% polyacrylamide gels containing 1 mg/ml chicken histones. The SDS concentration in the resolving gel was increased to 0.8% to improve histone solubilization.

Analysis of histone acetyltransferase specificity was performed as follows: 30 μg of free histones or oligonucleosomes were incubated together with chromatographic fractions and 0.01 μCi of ^{14}C-labeled acetyl-CoA at 37 °C for 15 min. The reaction was stopped by adding 100% trichloroacetic acid to a final concentration of 20%. The resulting precipitate was spun down, washed twice with acetone/HCl (100:1, v/v) and twice with acetone, and dried under vacuum. Histones were subjected to SDS-polyacrylamide gel electrophoresis, and the gels were Coomassie Blue-stained, destained, and incubated in Amplify (Amersham Pharmacia Biotech) prior to drying and fluorographed (32). The incorporation of radiolabel into each histone fraction was determined by densitometry of the corresponding band in the fluorogram.

RESULTS

hat1 and hat2 Mutations Affect Two HAT Enzymes—In order to obtain a better separation of HAT enzymes, we have developed a new system of gradient elution for the Q-Sepharose fractionation of yeast crude extracts (see "Experimental Procedures"). Fig. 1*A* shows the HAT activity profile obtained from wild type yeast extract using chicken-free histones as substrate. *Arrows* mark the elution positions of previously described HAT activities in our laboratory (27, 28). The complexity of the HAT activities eluted in this experiment can be examined by analyzing the incorporation of acetyl groups into free histones. This complexity is shown in the fluorograms and the densitometric radioactivity profiles of individual histones (Fig. 1*B*). In order of elution, one HAT activity can be observed, previously described as HAT-A1, which is defined by the incorporation of radioactive label into chicken histone H2A. Besides H2A, this enzyme acetylates free H4 and, to a lesser extent, H3. Overlapping with HAT-A1, a peak of incorporation into chicken H3 is seen centered on fractions 18–20. This HAT activity, previously named HAT-A2, contains GCN5 protein as the catalytic subunit and is specific for free or nucleosome-bound histone H3 (11). A peak of label incorporation into histone H4 was also observed. As this activity, previously named HAT-A3, was not affected by the mutation *gcn5* (11), it can be considered independent of HAT-A2. In the final fractions, label incorporation can be seen simultaneously in histones H3 and H4. This enzymatic activity was previously attributed to one HAT-B-type enzyme (27, 28, 32).

In order to determine the effects of the *hat1* and *hat2* mutations on the HAT enzymatic activities recovered from yeast, we performed a similar experiment using these mutant strains to obtain the crude enzymatic extracts. Fig. 1*C* shows the fluorogram and densitometric profiles of radiolabeled histones H3 and H4, obtained from *hat1* extracts. Comparison of the densitometric profile of H4 obtained for wild type (Fig. 1*B*) with that of the *hat1* mutant allowed us to detect a strong decrease of incorporation into H4 in the last fractions of the gradient. This result confirms that HAT1 is the catalytic subunit of the major HAT-B type present in yeast, as recently described (25, 26). Moreover, when a similar experiment is performed with yeast *hat2* extracts (Fig. 1*D*), the incorporation of radioactivity into histones follows patterns similar to those obtained with the *hat1* mutant. The only difference between the two gradi-

ents is the activity of HAT-A2. We have observed a slight variability in the extracted activity of this enzyme. In contrast, we have not detected variability in the activity profiles on histone H4 in different extractions. The absence of the B enzyme in both *hat1* and *hat2* mutants demonstrates that enzyme B obtained following our method is formed, at least, by HAT1 and HAT2. It has been reported recently that the specific activity of HAT1 isolated from a *hat2* mutant is 10-fold lower than that of wild type (26). Under the conditions of our experiment, we were not able to detect the presence of HAT1 activity in the gradient, probably due to a large decrease in its enzymatic activity.

The analyses of radioactivity incorporated into histone H4 in Fig. 1, *C* and *D*, also show that the HAT-A3 enzyme is not present in either *hat1* or *hat2* mutants. In these mutants, the peak responsible for the incorporation of label into H4, which elutes just after that yielding incorporation into H3 in wild type (see Fig. 1*B*), is not present (see Fig. 1, *C* and *D*). We have also assayed the enzymatic activities recovered from a *hat1,hat2* double mutant, and the results are similar to those obtained independently with *hat1* or *hat2* single mutants (results not shown).

hat1 and hat2 Mutants Allow the Detection of a New HAT Enzyme—The loss of HAT-B activity in the *hat1* and *hat2* mutants (Fig. 1, *C* and *D*) reveals the presence of another yeast HAT enzyme not previously described. This activity, which in the wild type strain overlaps in the gradient with the HAT-B enzyme, preferentially acetylates free histone chicken H3 and to a lesser extent histone H4. When using yeast histones as substrate, the enzyme acetylates the same histones (results not shown). We call this enzyme HAT-A4 because of its nuclear location (see below).

HAT1 and HAT2 Are Involved in Both Cytoplasmic and Nuclear Histone Acetyltransferases—HAT-A3 is an enzymatic activity previously described as nuclear HAT in our laboratory (28). To confirm that HAT1 and HAT2 are involved in this nuclear enzyme, we isolated the nuclear and cytoplasmic fractions from wild type yeast and the nuclear fraction from *hat1* and *hat2* mutants and analyzed the presence or absence of HAT-A3 enzyme in these extracts using the same chromatographic system as in the previous experiments. Fig. 2, *A* and *B*, shows the fluorograms and densitometric profiles of radioactive incorporation into H3 and H4 obtained with cytoplasmic and nuclear wild type yeast extracts, respectively. The results of these experiments support earlier data demonstrating the nuclear location of HAT-A1, HAT-A2, and HAT-A3 (28) and moreover show that the new HAT-A4 enzyme described here is also a nucleus-located activity. HAT-B enzyme is present in the cytoplasmic elution profile, whereas HAT-A1 is present in both subcellular fractions. Fig. 2*C* shows the results obtained with the nuclear *hat2* mutant extract. After the elution of HAT-A1 enzyme, corresponding to the first peak of incorporation of label into H4, the HAT-A3 activity is absent. The results obtained with the nuclear *hat1* mutant extract are not represented because they are similar to those of *hat2* (results not shown). The above experiments demonstrate that the HAT-A3 enzyme is not an artifact obtained from HAT-B activity in yeast crude extracts.

The Catalytic Subunits of HAT-B and HAT-A3 Have the Same Molecular Masses but the HAT-B Complex Is Greater Than the HAT-A3 Complex—We determined the molecular masses of the catalytic subunits of the HAT-B and HAT-A3 enzymes by means of HAT activity gel assays (33). To perform these assays we used the cytoplasmic HAT-B enzyme (*fraction 26*, Fig. 2*A*), while the HAT-A3 activity was assayed in two different fractions, one was a nuclear fraction (*fraction 20* represented in Fig. 2*B*) in which the HAT-B enzyme is absent, and the other was obtained from a *gcn5* mutant (*fraction 21* represented in Fig. 5*A*) thus ensuring the absence of GCN5. As can be seen in Fig. 3, the catalytic subunits of the HAT-B and HAT-A3 enzymes showed identical molecular masses of about 42 kDa, the same as that described for HAT1 protein (25, 26). This confirms that HAT-B and HAT-A3 share the same catalytic subunit, namely HAT1.

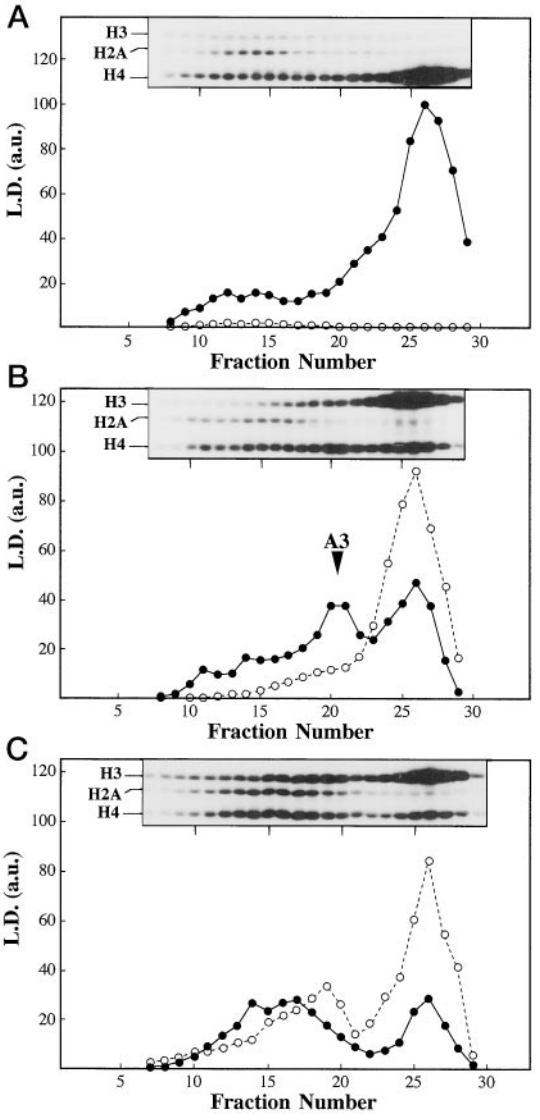

FIG. 2. **Distribution of yeast histone acetylating activities between nucleus and cytoplasm.** A cytoplasmic extract from the wild type strain (*A*), and nuclear extracts from the wild type (*B*) and *hat2* mutant strains (*C*) were prepared as described under "Experimental Procedures" and resolved by Q-Sepharose chromatography by using the conditions indicated in Fig. 1*A*. After incubation of selected chromatographic fractions with chicken histones and labeled acetyl-CoA, the reaction products were analyzed by SDS-polyacrylamide gel electrophoresis and subsequent fluorography (*insets*). Labeling density (*L.D.*) values for H3 (○) and H4 (●) are plotted against the fraction number. The position of HAT A3 in the elution gradient is indicated in *B*. The migration of H3, H2A, and H4 is indicated in the fluorograms.

In order to determine the molecular sizes of the HAT-B and HAT-A3 enzymatic activities, fractions containing these enzymes were pooled (*fractions 25–28* in Fig. 2*A* for HAT-B and *fractions 18–21* in Fig. 5*A* for HAT-A3) and analyzed by ultracentrifugation in sucrose gradients (data not shown). The enzymes were identified by their specificity toward free H4 (11). The maximum HAT activity obtained with the HAT-B enzyme

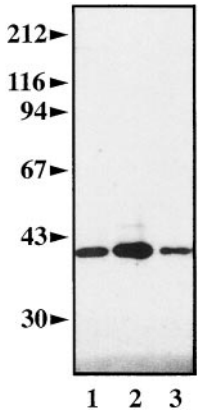

FIG. 3. **Determination of the molecular mass of the catalytic subunit of the HAT-A3 and HAT-B enzymes by activity gel assay.** Samples corresponding to HAT-A3 and HAT-B enzymes were electrophoresed in a 10% SDS-polyacrylamide gel containing 1 mg/ml chicken histones and assayed for HAT activity using the activity gel assay. Fluorograph of ^{14}C-labeled acetate incorporation into histones is as follows: *lane 1*, HAT-A3 from a *gcn5* mutant (*fraction 21* in Fig. 5*A*); *lane 2*, HAT-B from a wild type cytoplasmic extraction (*fraction 26* in Fig. 2*A*); *lane 3*, HAT-A3 from a wild type nuclear extraction (*fraction 20* in Fig. 2*B*). The mobilities of molecular mass standards are indicated in kDa.

was found in fractions corresponding to a molecular mass of around 150 kDa, whereas that for HAT-A3 was 110 kDa. As the molecular masses of both complexes are notably greater than the molecular mass of the HAT1 protein (42 kDa), these complexes must contain several subunits, among which are the HAT1 and HAT2 proteins.

HAT1 and HAT2 Proteins Are Not Involved, in Vitro, in HAT Activities That Acetylate Nucleosome-bound Histones—The fact that HAT-A3 is a nuclear enzyme would suggest, but not prove, that it is able to acetylate nucleosome-bound histones. To investigate this, we performed a similar experiment to that presented in Fig. 2, *B* and *C*, but using chicken nucleosomes as substrate. Fig. 4 shows the HAT activity profile obtained from wild type and *hat1* mutant yeast nuclear extracts. Data obtained with *hat2* extracts are not shown because they are similar to those of the *hat1* mutant. As shown in Fig. 4, the incorporation of radioactivity into individual histones obtained from both extracts follows the same patterns. These results indicate that HAT1 or HAT2 proteins are dispensable for the acetylation of nucleosomes under our experimental conditions. It is important to point out that the HAT-A4 enzyme described here is able to acetylate nucleosome-bound histones. It acetylates H3 and to a lesser extent H4, although its overall level of activity is low.

The hat1,gcn5 Double Mutant Does Not Exhibit Any Synthetic Phenotype Effect—The *gcn5* mutation exhibits only slight phenotypic effects, such as slow growth on minimal medium (7), and there are no obvious growth rate phenotypic consequences of deleting the *HAT1* gene from yeast (25). To see if it were possible to detect a more severe phenotype than either of the single mutants, we constructed a *hat1,gcn5* double mutant as detailed under "Experimental Procedures." This double mutant behaved similarly to the *gcn5* single mutant in that it grew slowly on minimal medium. We also analyzed the HAT activities recovered from this double mutant by means of the incorporation of radioactivity into individual histones. Fig. 5 shows a comparison of the results obtained with the *gcn5* mutant and the *hat1,gcn5* double mutant. As we have recently reported, the *gcn5* mutation leads to the absence of HAT-A2 activity (11). In contrast, only the HAT-A1 and HAT-A4 enzymes seem to be present in the double mutant. Thus, in Fig. 5*B* one can observe that incorporation of label into histones H4 and H2A in the first

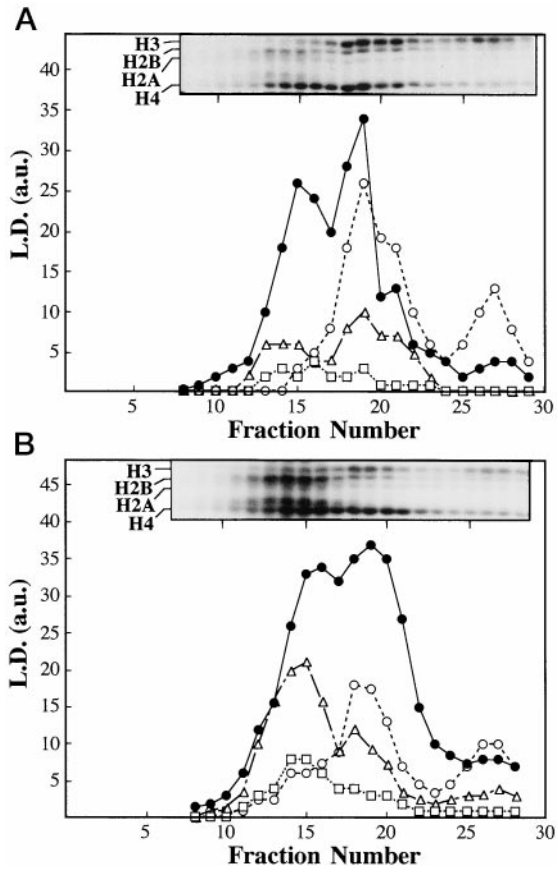

FIG. 4. **Nucleosome acetylating activities in nuclei from wild type and *hat1* mutant yeast strains.** Nuclear extracts from wild type (*A*) and *hat1* mutant (*B*) strain were prepared as described under "Experimental Procedures" and chromatographed on Q-Sepharose. Eluted fractions were dialyzed against buffer B and assayed using chicken erythrocyte nucleosomes as substrate. After incubation, histones were extracted and subjected to SDS-polyacrylamide gel electrophoresis with subsequent analysis by fluorography (*insets*). Relative amounts of radiolabel incorporated into each histone class were determined by densitometry of corresponding bands in the fluorograms. Labeling density (*L.D.*) is plotted against fraction number for each histone. ●, H4; ○, H3; □, H2A; and △, H2B. The *band* running immediately above of H4 is a proteolytic degradation product, probably from histone H2B.

part of the gradient is very similar, whereas in the *gcn5* single mutant there is a striking increase in H4-associated label in the middle of the gradient following the peak of incorporation into H2A. Neither the HAT-A3 or HAT-B enzymes seem to be present in this double mutant. This result provides further evidence that HAT1 is the catalytic subunit of both the HAT-B and HAT-A3 enzymes. In conclusion, the *hat1,gcn5* double mutant behaved as a simple addition of the two single mutants but did not show any new phenotype.

DISCUSSION

The discovery that HAT1 is a catalytic subunit of B type HAT in yeast (25) represents the first major advance in both identifying and cloning the genes that encode the different HATs previously isolated from this organism following biochemical procedures (27, 28, 32, 34). In the present study we have confirmed, by analyzing *hat1* mutants, that HAT1 is the catalytic subunit of the HAT-B type enzyme present in yeast and first described in our laboratory (32).

The main cytoplasmic HAT activity in yeast has been recently identified to be a heteromeric complex of molecular mass greater than 200 kDa, comprised of HAT1, as a catalytic subunit, and HAT2, which seems to increase the affinity of HAT1

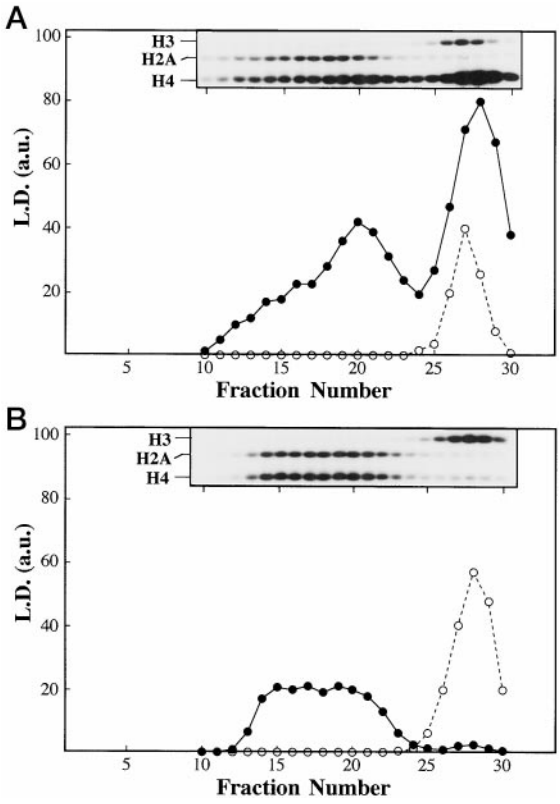

FIG. 5. **Analysis of histone acetyltransferase activities in *gcn5* and *hat1,gcn5* double mutant yeast strains.** Whole cell extracts from *gcn5* (*A*) and *hat1,gcn5* (*B*) mutant strains were prepared and chromatographed on Q-Sepharose as described in Fig. 1. Fluorograms (*insets*) from the histone acetyltransferase assays using protein fractions from the chromatographic elutions were used to determined histone specificity. Labeling density (*L.D.*) values are plotted against fraction number. ○, labeling density for H3; ●, 0.5 × labeling density for H4.

for histone tails (26). We have demonstrated in our current work that this heteromeric complex is the same as that previously described in our laboratory (32), given that the HAT-B enzyme is not present in either *hat1* or *hat2* mutants. By means of sucrose gradient ultracentrifugation, we have confirmed that the HAT-B enzyme is a complex of high molecular mass, although our estimate of 150 kDa is less than the 200 kDa obtained by gel filtration chromatography (26).

The most important conclusion reached in our study is that HAT1 and HAT2 form part of a nuclear enzyme, HAT-A3, which acetylates histone H4 in its free form. This conclusion is supported by two observations. First, the peak of HAT-A3 enzymatic activity in both total yeast extracts (Fig. 1) and extracts obtained from nuclei (Fig. 2) is lost in *hat1* and *hat2*, and *hat1,hat2* mutants. Moreover, the HAT-A3 enzyme is present in *gcn5* mutant extracts but absent in the fractionation of the *hat1,gcn5* double mutant (Fig. 5). Second, the molecular masses of the catalytic subunits of HAT-B and HAT-A3, obtained by means of the HAT activity gel assays, are identical (Fig. 3). It has been speculated previously that as well as forming part of the HAT-B cytoplasmic enzyme together with HAT2, HAT1 is also present in the nucleus where, upon binding with other proteins, it may be able to acetylate H4 in assembled chromatin. In this regard it has been pointed out that HAT1 is detectable by Western blotting in nuclear extracts (26). The suggestion has also been made that the different HATs could act in the form of heteromeric complexes in such a way that the activity and specificity of each HAT could depend on the association of the catalytic subunit with other proteins (26).

Other authors (14) have proposed that HAT-B type enzymes might be targeted toward nuclear replication forks as well as toward cytoplasmic complexes, and that they use the WD40 protein-protein interaction domain present in HAT2 to this end. In this sense, it has been reported recently that a human HAT activity homologue to *S. cerevisiae* HAT-B enzyme is found in the nucleus of S-phase cells (35). The data given here support this result and definitively pose an important question, *i.e.* what functions do HAT1 and HAT2 (HAT-A3 enzyme) carry out in the nucleus? Under our experimental conditions, the HAT-A3 enzyme is unable to acetylate nucleosomes *in vitro*. This inability could result from the loss of some of the native characteristics of the enzyme, but it is also possible that HAT-A3 may acetylate free histone H4 in the nucleus. One has to bear in mind that *in vivo* chromatin is subject to the disorganization-reorganization processes of the nucleosomal structures that are independent of the chromatin replication phase, *e.g.* the loss of nucleosomes in regulatory regions or repair processes. The histones involved in these processes could be subject to changes in their acetylation patterns in such a way that they can re-assemble in nucleosomes. HAT-A3 enzyme might be responsible for this change of acetylation pattern. It is important to point out that complexes able to assemble histones in chromatin structures, such as CAF-1, have been obtained from the nuclei (20). Histones H3 and H4 only associate with CAF-1 when presenting a cytoplasmic acetylation pattern, despite the presence in the nucleus of HAT type A and histone deacetylase activities that are able to alter this acetylation pattern. The presence of HAT-A3 in the nucleus could guarantee the stability of the complexes formed by CAF-1 and these histones. In yeast, mutations affecting CAF-1 produce an increase in sensitivity to ultraviolet light but do not affect cell viability (36). We have been unable to detect greater sensitivity to ultraviolet radiation in exponentially growing *hat1* and *hat2* mutants.

HAT-A3 function could also be related to the heterochromatin-like complex that silences transcription in telomeric chromatin. To reconstruct nucleosomes in the telomere in each replicative cycle, CAF-1 uses histones H4 and H3 that have a cytoplasmic acetylation pattern. In yeast, the function and organization of telomeric chromatin *in vivo* is affected by mutations in the RLF2 subunit of CAF-1 (37). That the pattern of nascent histone acetylation is retained in silent chromatin (38) suggests that HAT-A3 nuclear activity could be responsible for the maintenance of the cytoplasmic acetylation pattern of histone H4 in telomeres. The function of HAT2 in the nucleus is likely to be the same as in the cytoplasm, *i.e.* to target the catalytic subunit to histones. The existence of a protein family comprising p48 (a component of CAF1 and the human histone deacetylase, HD1) and HAT2 suggests that the processes of acetylation, deacetylation, and chromatin assembly are all closely integrated (14).

The fact that neither the *hat1, hat2*, or *gcn5* mutants nor even the *hat1,hat2* or *hat1,gcn5* double mutants show dramatic alterations in phenotype is noteworthy (Refs. 25 and 26 and this work). The presence of HAT1 and HAT2 in the nucleus makes it even more surprising that their absence does not have profound consequences on cellular growth or cell viability. A frequently suggested explanation is that there are other HAT activities with overlapping functions. This supposition is supported by the existence of at least two other HAT activities as follows: HAT-A1 which is able to acetylate H4 and other histones both in their free form and in nucleosomes, and the enzyme HAT-A4 described here, which is able to acetylate free and nucleosome-bound H4 and H3.

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