Modulating Acetyl-CoA Binding in the GCN5 Family of Histone Acetyltransferases*

Received for publication, April 5, 2002, and in revised form, May 2, 2002
Published, JBC Papers in Press, May 6, 2002, DOI 10.1074/jbc.M203251200

Michael R. Langer‡, Christopher J. Fry§, Craig L. Peterson¶, and John M. Denu‖

From the ‡Department of Biochemistry and Molecular Biology, Oregon Health & Science University, Portland, Oregon 97201-3098 and the §Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01605

The histone acetyltransferase (HAT) GCN5 is the founding member for a family of chromatin remodeling enzymes. GCN5 is the catalytic subunit of a large multisubunit complex that functions in the regulation of gene activation via acetylation of lysine residues within the N-terminal tails of core histone proteins. Using acetyl-CoA as a co-substrate, the high affinity binding of acetyl-CoA is a critical first step in the reaction. Here, we examine the biochemical and biological importance of a conserved hydroxyl-bearing residue in signature motif A. Interestingly, one major exception is the Saccharomyces cerevisiae GCN5, where an alanine (Ala190) is located in the corresponding position. In related GCN5 family structures, a hydroxyl-containing side chain residue is hydrogen-bonded to the α-phosphate oxygen of CoA. We demonstrate that this key hydrogen bond contributes ~10-fold to the binding affinity of GCN5 HATs for acetyl-CoA. Human p300/CBP-associating factor, human GCN5, and tetrahymena GCN5 displayed dissociation constants (Kd) for acetyl-CoA of 0.64 ± 0.12, 0.56 ± 0.15, and 0.62 ± 0.17 μM, respectively. In contrast, S. cerevisiae GCN5 displayed a Kd of 8.5 μM. When Ala190 was replaced with threonine, the A190T derivative yielded a Kd value of 0.56 ± 0.1 μM for acetyl-CoA, completely restoring the higher affinity binding seen with the GCN5 homologs that naturally harbor a threonine at this position. Detailed kinetic analyses revealed that the A190T derivative was otherwise catalytically indistinguishable from wild type GCN5. We also demonstrate that the A190T allele rescued the slow growth phenotype and the defect in HO transcription caused by a deletion of GCN5. Furthermore, the A190T allele supported wild type levels of transcriptionally targeted and global histone H3 acetylation. In each case, the A190T derivative behaved similarly to wild type GCN5, suggesting that the efficacy of HAT activity by GCN5 is not limited by the availability of nuclear acetyl-CoA pools.

Histone acetyltransferases (HATs) catalyze the transfer of an acetyl group from acetyl-CoA to the acceptor e-amino group of lysine side chains within the N-terminal tails of the core histones, H2A, H2B, H3, and H4. At least four gene families of HATs have been identified in mammals (1, 2). The most characterized HAT family includes the defining member GCN5, whose catalytic domain is conserved from yeast to humans. The GCN5 family of HATs are members of a large superfamily of N-acetyltransferases (referred to as GNATs, for GCN5-like N-acetyltransferases). Exhibiting substrate specificity for non-histone proteins as well as for histones, p300/CBP-associating factor (P/CAF) (3–6) is also a member of the GCN5 family of HATs.

GCN5 has been shown to be essential for full transcriptional activation of a subset of genes in yeast (7–12). The histone acetyltransferase activity of Gcn5p is required for its transcriptional activator function, because mutational inactivation of the histone acetyltransferase domain eliminates this function in vivo (13–15). Although Gcn5p alone can acetylate histone proteins in vitro (16), biochemical analysis has demonstrated that it is a subunit of at least two distinct large multisubunit protein complexes in vivo, ADA and SAGA (17). These complexes, which are targeted to gene promoters by transcriptional activator proteins, have been shown to facilitate the activation of a small number of inducible genes (7), in addition to the cell cycle-regulated HO gene (7, 18). Furthermore, recent studies have shown a more global requirement for GCN5 in gene activation during mitosis (19, 20), presumably because of the barrier posed to the transcriptional machinery by further compaction of chromatin during mitosis. In addition to the promoter-targeted histone acetylation function of Gcn5p during gene activation, it also facilitates the maintenance of a basal level of genome-wide histone acetylation, the function of which is not yet understood (21–23). In mammals, GCN5 also appears to function in at least one multisubunit protein complex and is an essential gene that is critical for development and cellular proliferation (24, 25).

Over the last few years, significant progress has been made toward understanding the structure and catalytic mechanism for the GCN5 family of HATs. With both GCN5 and P/CAF, the catalytic mechanism was shown to involve the initial formation of a ternary complex, where the e-amino attacks the carbonyl carbon of bound acetyl-CoA substrate, with subsequent acetyl transfer to lysine (26–29). In GCN5, Glu173 serves as a general base catalyst in deprotonating the e-amino for facile nucleophilic attack on bound acetyl-CoA (26). The binding of substrates (histone and acetyl-CoA) and the release of products

---

*This work was supported by American Cancer Society Grant RSG-01-029-01-CNE and National Institutes of Health Grants GM59785 (to J. M. D.) and GM49650 (to C. L. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Fellow of the Leukemia and Lymphoma Society of America.

§ To whom correspondence should be addressed: Oregon Health & Science University, Dept. of Biochemistry and Molecular Biology, 3181 SW Sam Jackson Park Rd., Portland, OR 97201-3098. Tel.: 503-494-0644; Fax: 503-494-8393; E-mail: denuj@ohsu.edu.

The abbreviations used are: HAT, histone acetyltransferase; AANAT, arylalkylamine N-acetyltransferase or serotonin N-acetyltransferase; AcCoA, acetyl coenzyme A; DTT, dithiothreitol; hGCN5, human GCN5; P/CAF, p300/CBP-associating factor; hP/CAF, human P/CAF; SmAAT, S. marcescens aminoglycoside 3-N-acetyltransferase; tGCN5, Tetrahymena thermophila GCN5; yGCN5, yeast GCN5 from S. cerevisiae.
AcCoA Binding in the GCN5 Family of HATs

(acyethylated histone and CoA) are strictly ordered (27–29).

Recent structural findings for GCN5 and other related HATs (30–33) have provided additional insights into other key sub-state binding interactions. The structures revealed a conserved α/β globular fold where two roughly orthogonal hydrophobic troughs exist along the surface of the protein. Acetyl-CoA binds within one cleft, whereas the lysine-containing peptide binds within the other. The active site is found at the junction of the two troughs and is marked by the presence of the general base Glu173 (yeast numbering), although the equivalent of this site has not been identified in more distantly related family members. Several interactions between the protein and acetyl-CoA were observed. The majority of the interactions with protein involve the pantothenate and pyrophosphate moieties of CoA and are mediated by the highly conserved (R/Q)XXGX(G/A) sequence (motif A, where X denotes any amino acid). Curiously, immediately C-terminal of the (R/Q)XXGX(G/A) sequence, a threonine or serine is usually found and can be represented as (R/Q)XXGX(G/A)Z where Z is either threonine or serine. Interestingly, the one major exception to this is yeast GCN5, where an alanine is located in the position of Z. In all available GCN5 HAT structures, when Z is a hydroxyl-containing side chain residue, this hydroxyl is hydrogen-bonded to an α-phosphate oxygen of CoA. In the yeast GCN5 structure, where alanine is substituted, no compensating residues appear to function in an analogous fashion, suggesting that yeast GCN5 may have significantly less affinity for acetyl-CoA than the corresponding homologs from other species, because of the loss of this hydrogen bond. If true, does this have important biological implications in terms of the in vivo concentrations of acetyl-CoA substrate and the ability of yeast GCN5 to perform its biological function? Here, we examine biochemically the function of this highly conserved hydroxyl group within the GCN5 family of HATs and probe in vivo the functional relevance and implications of substituting this important residue.

EXPERIMENTAL PROCEDURES

Materials—P81 phosphocellulose disks were purchased from Invitrogen. Dispo-Equilibrium dialyzers were obtained from Amika Corporation. Histone H3 peptide corresponding to the 20 N-terminal residues of human histone H3 and an additional C-terminal cysteine (ART-210), hP/CAF (residues 493–658), hGCN5 (residues 505–670), and yGCN5 and its mutant forms (residues 99–210) were purchased from Fisher, Pierce, or Sigma and were used without further purification.

Expression and Purification of yGCN5 and Homologs—Mutations in the catalytic domain of yGCN5 were generated using the Bio-Rad Muta-gene method and oligonucleotides containing the desired mutations: 5′-CATGATCATTCAACCATGAC-3′ for A190S and 5′-CATGATCATTCAACCATACCC-3′ for A190T. The generated mutations were verified by DNA sequencing. The catalytic domains of gGCN5 (residues 47–210), hP/CAP (residues 493–658), hGCN5 (residues 505–670), and yGCN5 and its mutant forms (residues 99–262) were recombinantly expressed by isoosmotrophic B. thailandicus fermentation and were purified from BL21-DL3 bacteria as described previously (34). Crude bacterial lysates were subjected to ion exchange chromatography on S-Sepharose. Purified fractions (greater than 90%) containing HAT activity, as determined by SDS-PAGE and HAT activity, were pooled, concentrated, and stored at −20 °C until use. The concentration of total protein was determined by the method of Bradford (35). This value was corrected by densitometric analysis to reflect the total concentration of the HAT activity.

Yeast Strains and Media—The yeast strain CY569 (gen5D, H0-lacZ) was used for the analysis of gen5A phenotypes in vivo and is described in Ref. 7. This strain was transformed with expression plasmids encoding wild type Gen5p (CP649), a mutant gen5p containing an A190T mutation (CP914), or no protein (empty vector; BA110). The transformants were selected and maintained on synthetic minimal medium (6.7 g/liter yeast nitrogen base without amino acids (Difco Laboratories), 2% glucose) supplemented with amino acids (minus histidine) as described previously (36). All of the phenotypic analyses were performed in triplicate (three independent transformants) using synthetic minimal medium supplemented with histidine (only for CY569 lacking an expression plasmid) and uracil. Cell doubling times were calculated from cell growth rates, which were determined by measuring the absorbance of growing cultures at 600 nm, using a spectrophotometer. Liquid β-galactosidase assays were performed using cells grown to mid-log phase, as described previously (36).

Plasmids—The wild type Gen5p expression plasmid (CP649) contained the full-length GCN5 gene in pBBS13 (also used as empty vector control BA110; Stratagene). The gen5A190T mutant was constructed using the QuikChange™ XL site-directed mutagenesis kit (Stratagene) with CP649 as template plasmid and the following two oligonucleotides (S′ to 3′): GGTACCGCCTATGACGATCCATATGACATACATCTTAA-AAG and CTITTTAGTCTACCTAGTTGCTCACAAGCGGTAT-CC. The generated mutation and integrity of the full-length GCN5 gene was verified by DNA sequencing using the Primm™ Ready Reaction DyeDeoxy™ terminator cycle sequencing kit (PerkinElmer Life Sciences).

Enzymatic Assays for HATs—HAT activity was measured with [3H]acetetyl-CoA and histone H3 peptide as substrates, using a radioactive P81 filter binding assay as described previously (26). Initial velocities were determined for H3 peptide concentrations between 5 × 10−4 and 5 × 10−6 M Tris, pH 7.5. AcCoA saturation kinetics were evaluated for AcCoA concentrations spanning 0.1 to 100 μM at a fixed 1.5 mM concentration (near saturating) of H3 peptide. H3 peptide saturation kinetics were performed at H3 peptide concentrations spanning 25 μM to 1.0 mM at a fixed 100 μM concentration (saturating) of AcCoA. The data were fitted to Equation 1 and are presented in hyperbolic form.

Isotope Trapping Experiments—Pulse-chase experiments were performed to obtain information regarding both kinetic and equilibrium aspects of the yGCN5-AcCoA complex (37–40). The experiments were performed at 24 ± 1 °C in 5 mM DTT, 50 mM Tris, pH 7.5, as described previously (40). The percentage of the total labeled substrate converted to product in the initial partitioning event was plotted versus the initial H3 peptide concentration, and the data were fitted to Equation 2

where [B] is the initial concentration of the H3 peptide, [P] is the percentage of the complex ([3H]acetetyl-CoA-Y5CN5) partitioned as product (1H]acetetyl-H3 peptide), [Pmax] is the maximum percentage of the complex that can be trapped as product when [B] is increased infinitely, and Kp is the concentration of B that results in half-maximal partitioning.

Determination of the Dissociation Constant for AcCoA Binding to GCN5—The dissociation constant (Kp) for AcCoA in the presence of HAT enzyme was determined by equilibrium dialysis using Dispo-Equilibrium dialyzers (Amika Corp.) as described previously (27). Equilibrium conditions were 5 mM DTT, 50 mM Tris, pH 7.5, and 3–5 μM GCN5 at 4 °C for 40 h. The concentrations of enzyme-bound AcCoA were determined over a range of total AcCoA concentrations spanning 0–40 μM. The data were fitted to Equation 3 and are presented in hyperbolic form.

[AcCoA-Enzyme] = ([Enzyme]tot [AcCoA]tot) / (Kp + [AcCoA]tot) (Eq. 3)

Chromatin Immunoprecipitations—Chromatin immunoprecipitations were performed in triplicate (three independent transformants) using cells grown to mid-log phase and an antibody against diacetylated histone H3 (Upstate Biotechnology, Inc.) as described previously (37). The following modifications were made. After the final ethanol precipitation, the amount of input and immunoprecipitated DNAs was quantified by PCR. For PCR analysis, either 1/500 (input) or 1/50 (immunoprecipitation) of the DNA was amplified using 50 pmol of both HO SCB2 primers (21) and ACT1 primers in 20-μl reactions containing 200 μM dNTPs, 3 mM MgCl2, 2.5 μCi of [32P]dCTP, and 0.25 units of Taq polymerase (Promega). The PCR conditions are as follows: (5′ to 3′) ACAAACGATAGGAGTGGCCCAAG and AATGGCGTGAGGTCAGAAGAGAACC. To determine whether PCR amplification was within the linear range, three consecutive 5-fold dilutions of genomic DNA were included for each PCR primer pair. After 5 min at 95 °C, 26 cycles of 30 s at 95 °C, 30 s at 60 °C, 1 min 72 °C were performed. PCR products were electrophoresed on 10% polyacrylamide gels and dried. The sig-
AcCoA Binding in the GCN5 Family of HATs

AcCoA Saturation Kinetics for HAT Enzymes—To probe the functional significance of the conserved threonine residue and to determine whether the ~10-fold lower AcCoA binding affinity in yGCN5 was due to the lack of this interaction, we generated and kinetically characterized the A190T and A190S mutants of yGCN5. To demonstrate the general importance of this interaction, we kinetically characterized two additional GCN5 family members (hGCN5 from humans and tGCN5 from tetrahymena) that both contain a threonine residue at this position. The steady-state parameters obtained from this analysis are summarized in Table I.

The results obtained for tGCN5, hGCN5, and the A190T and A190S mutants of yGCN5 were compared with the kinetic parameters previously obtained with hP/CAF and wild type yGCN5. All of the enzymes exhibited similar catalytic rates (kcat) under saturating conditions of substrates (1.6 ± 0.09,

### RESULTS AND DISCUSSION

**Mutational Analysis of yGCN5**—Previous kinetic analyses of the catalytic domains of hP/CAF and wild type yGCN5 (27–29, 40) indicated a completely ordered kinetic mechanism, where upon AcCoA binding to free enzyme, there is an enzyme isomerization that permits the efficient binding of polypeptide (Scheme 1). The limiting rate in catalytic turnover is the chemistry step (k7; Scheme 1). These initial analyses demonstrated that hP/CAF and yGCN5 have nearly identical rates of catalysis when both substrates are present at saturating levels (kcat = 2.3 ± 0.07 and 1.7 ± 0.12 s–1, respectively). However, it was observed that hP/CAF binds AcCoA ~13-fold more tightly than yGCN5 (Kd = 0.64 ± 0.12 and 8.5 ± 2.6 μM, respectively) (27, 28). Given the similar catalytic efficiencies of GCN5 and P/CAF, it was not clear why there was a dramatic difference in their abilities to bind AcCoA.

To uncover the source of these differences, we compared the CoA-bound structures of yGCN5 with hP/CAF (33), tetrahymena GCN5 (31, 32), yeast HAT1 (30), as well as other GNAT family N-acetyltransferases (SmAAT (41) and AANAT (42)) (Fig. 1). Except for yGCN5, in every case examined, a hydrogen bonding interaction between the α-phosphate oxygen of the pyrophosphate moiety from CoA and a conserved threonine/serine residue (Z in the motif (R/Q)XXGXX(G/A)Z) was observed (Fig. 1). Interestingly, this threonine/serine residue is conserved in all the GCN5 family of HATs except for GCN5 from *Saccharomyces cerevisiae*. In yGCN5, there is an alanine at position 190. In GCN5 from *Schizosaccharomyces pombe*, a serine residue (Ser208) is found at this position (Fig. 2). Moreover, this threonine/serine-mediated interaction is also observed in structural complexes of SmAAT (Thr114) (41) and AANAT (Ser137) (42). Thus, the hydrogen bonding interaction between CoA and the side chain of this conserved threonine/serine may provide the necessary binding energy to potentiate the observed differences in AcCoA binding between hP/CAF and yGCN5.

To probe the functional significance of the conserved threonine residue and to determine whether the ~10-fold lower AcCoA binding affinity in yGCN5 was due to the lack of this interaction, we generated and kinetically characterized the A190T and A190S mutants of yGCN5. To demonstrate the general importance of this interaction, we kinetically characterized two additional GCN5 family members (hGCN5 from humans and tGCN5 from tetrahymena) that both contain a threonine residue at this position (Thr592 and Thr139, respectively) (Fig. 2).

**AcCoA Saturation Kinetics for HAT Enzymes**—To probe the function of the conserved threonine residue, initial AcCoA saturation kinetics were performed to obtain the steady-state kinetic parameters kcat and Km at AcCoA for tGCN5, hGCN5, and the A190T and A190S mutants of yGCN5. Initial velocities as a function of AcCoA concentration were determined at saturating concentrations of histone H3 peptide (Fig. 3). The data sets for each enzyme were plotted in hyperbolic form and were fitted to

### Table I. Steady-state parameters for hP/CAF and GCN5 family members

| Enzyme   | kcat (μM–1s–1) | Km (μM) |
|----------|----------------|---------|
| hP/CAF   | 2.3 ± 0.07     | 0.64 ± 0.12 |
| yGCN5    | 1.7 ± 0.12     | 8.5 ± 2.6 |
| tGCN5    | 0.09 ± 0.01    | 0.07 ± 0.01 |
| hGCN5    | 0.09 ± 0.01    | 0.07 ± 0.01 |
| A190T yGCN5 | 0.01 ± 0.01 | 0.01 ± 0.01 |
| A190S yGCN5 | 0.01 ± 0.01 | 0.01 ± 0.01 |

## AcCoA Binding in the GCN5 Family of HATs

*AcCoA Binding in the GCN5 Family of HATs*
AcCoA Binding in the GCN5 Family of HATs

... is equal to \( k_3/k_2 + k_1 \) (Scheme 1) and is a measure of the concentration of AcCoA required to reach \( \frac{1}{2} k_{\text{cat}} \). Although the \( K_{\text{m,AcCoA}} \) value does reflect the binding affinity of GCN5 for AcCoA, it also reflects the rate of isomerization (\( k_3 \)) and chemical catalysis (\( k_7 \)) and is not necessarily equal to the dissociation constant (\( k_4/k_5 \)). To determine whether changes in the \( K_{\text{m,AcCoA}} \) value represent a change in AcCoA binding affinity, the \( K_{\text{d,AcCoA}} \) values were measured as described below.

**Equilibrium Dialysis Binding Assays**—To directly assess the AcCoA binding affinity of tGCN5, hGCN5, pCAF, \( yGCN5 \), and the A190T and A190S mutants of \( yGCN5 \), binding assays were performed using equilibrium dialysis. The dissociation constant for AcCoA binding to pCAF and wild type GCN5 were previously determined in the absence of histone H3 peptide via equilibrium dialysis (27, 28). PCAF had a 10–15-fold greater affinity for AcCoA than wild type GCN5. In this current study, the dissociation constants (\( K_{d} \)) were determined as described under “Experimental Procedures” with representative plots shown in Fig. 4. The average \( K_{d} \) values from duplicate experiments are summarized in Table I.

All of the HATs containing a threonine at the relevant position (hP/CAF, hGCN5, tGCN5, and the A190T mutant of \( yGCN5 \)) exhibited equivalent (within error) dissociation constants (0.64 ± 0.12, 0.56 ± 0.15, 0.62 ± 0.17, and 0.56 ± 0.10 \( \mu \text{M} \), respectively), all of which demonstrated a 13–15-fold tighter binding to AcCoA compared with wild type GCN5. Interestingly, the A190S mutant of GCN5 exhibited a 5-fold tighter binding to AcCoA than wild type GCN5. This suggests that a serine hydroxyl at this position may provide a partial rescue of binding affinity by providing a suboptimal hydrogen bonding interaction. The methyl group of threonine may sterically direct the hydroxyl toward the optimal hydrogen bonding orientation with the \( \alpha \)-phosphate oxygen of CoA. Collectively, these results provide strong evidence that this threonine is critical for high affinity AcCoA binding in the GCN5 family of HATs.

**Histone H3 Peptide Saturation Kinetics for HAT Enzymes**—To ensure that mutation of Ala\(^{190} \) to a Ser or Thr did not affect peptide binding and enzyme isomerization steps, H3 peptide saturation kinetics were performed to obtain the steady-state kinetic parameter \( k_{\text{cat}}/K_{\text{m,H3}} \) for tGCN5, hGCN5, and the A190T and A190S mutants of \( yGCN5 \). Initial velocities as a function of histone H3 peptide concentration were determined at saturating concentrations of AcCoA (Fig. 5). The data sets for each enzyme were plotted in hyperbolic form and fitted to Equation 1 using a nonlinear least squares approach. The steady-state parameters obtained from this analysis are summarized in Table I.

The \( k_{\text{cat}}/K_{\text{m,H3}} \) values obtained for tGCN5, hGCN5, and the A190T and A190S mutants of \( yGCN5 \) were compared with those previously obtained with hP/CAF and wild type GCN5. Although kinetic parameters had previously been measured for hP/CAF and GCN5, this is the first time that these parameters had been measured for tGCN5 and GCN5. Quite amazingly, all of the HAT enzymes exhibited similar values for \( K_{\text{m,H3}} \) (357 ± 50, 471 ± 50, 273 ± 80, 352 ± 125, 532 ± 81, and 490 ± 80 \( \mu \text{M} \)) for tGCN5, hGCN5, A190S, A190T, hP/CAF, and wild type GCN5, respectively), and consequently for \( k_{\text{cat}}/K_{\text{m,H3}} \). The catalytic domains of GCN5, tGCN5, and hP/CAF all contain a threonine at the relevant CoA binding interaction and behave similarly with respect to their kinetic parameters. Replacement of alanine at this position in \( yGCN5 \) has no apparent effect on peptide substrate affinity or chemical catalysis. These results suggest that changes in AcCoA binding affinity (as perturbed by substituting \( yGCN5 \) Ala\(^{190} \)) do not affect the ability of HATs to bind H3 peptide and catalyze acetyl...
transfer. Recently, isotope trapping experiments were utilized to demonstrate that yGCN5 undergoes a catalytically relevant isomerization step subsequent to AcCoA binding and prior to H3 peptide binding and catalysis (40). Changes in the rate of isomerization for the A190T mutant would be detected by an alteration in the $k_{\text{cat}}/K_{\text{m,H3}}$ and $k_{\text{cat}}/K_{\text{m, AcCoA}}$ values. Because no change was observed in $k_{\text{cat}}/K_{\text{m, H3}}$ for the A190T or A190S mutants of yGCN5, our results suggested that these amino acid substitutions did not affect the rate of enzyme isomerization. However, isotope trapping experiments would provide a more direct validation of this finding.

Isotope Trapping Experiments—To provide additional evidence that the A190T substitution had no significant effect on the isomerization step, isotope trapping experiments (pulse-
AcCoA Binding in the GCN5 Family of HATs

Isotope partitioning with the A190T mutant of yGCN5.

The percentage of 0.75 μM total [3H]AcCoA partitioned to product during the first turnover of 15 μM A190T derivative (closed diamonds) relative to the amount that dissociates and is diluted into 2.5 mM unlabeled AcCoA was determined for 0–300 μM H3 peptide at 24 °C in 5 mM DTT, 50 mM Tris, pH 7.5. A curve depicting previously published results for the equivalent assay with wild type yGCN5 (dotted line) is included for graphical comparison (40). The data were plotted and fit to Equation 2. The experiment was performed in duplicate with a representative plot displayed.

For the A190T mutant of yGCN5, the maximum percentage of labeled substrate partitioned to product from the [3H]acetyl-CoA substrate from a [3H]acetyl-CoA-HAT complex that dissociates to a large pool of unlabeled AcCoA substrate (effectively irreversible) versus the amount of labeled substrate from the complex that goes on to generate products. This experiment was previously performed with wild type yGCN5, and the inability to convert all of the labeled substrate to product at high concentrations of histone H3 peptide led to the conclusion that the AcCoA yGCN5 complex exists in two isomeric forms, one of which is competent to perform chemistry (40).

For the A190T mutant of yGCN5, the maximum percentage of labeled substrate partitioned to product by the A190T mutant of yGCN5 proved to be saturable at low concentrations of histone H3 peptide. The data were plotted as a function of the initial H3 peptide concentration and were fitted to Equation 2 (Fig. 6). As with wild type yGCN5, the maximum percentage of labeled substrate partitioned to product by the A190T mutant of yGCN5 proved to be saturable at low concentrations of histone H3 peptide (K_{H3} = 9.42 ± 1.35 and 7.81 ± 2.03 μM for wild type yGCN5 and A190T, respectively), and identical ([[^3]H]P_{max} = 8.76 ± 0.23 and 9.12 ± 0.75%) for wild type yGCN5 and A190T, respectively). These results indicate that at saturating concentrations of AcCoA, enzyme isomerization in the A190T mutant is not compromised relative to that observed for wild type yGCN5.

Functional Significance of A190T GCN5 in S. cerevisiae.—We demonstrated that the conserved threonine/serine residue within the (R/Q)XXGXG/G/AZ (where Z is T or S) is critical for the high affinity AcCoA binding relative to that observed with yGCN5, where Z is an alanine residue. Having shown that the A190T substitution in yGCN5 can rescue this lower affinity (−10-fold), we pondered whether increasing the effectiveness of AcCoA binding in the A190T yGCN5 mutant would have any biological consequences on the ability of this enzyme to acetylate histones in vivo and promote transcriptional activation. We reasoned that if AcCoA availability were rate-limiting in the GCN5-catalyzed acetylation of histones, then one might predict the A190T derivative to behave as a more effective activator of transcription. On the other hand, if AcCoA availability is not a determinant in the function of GCN5, then the A190T derivative may behave identically to that of wild type enzyme. To examine these questions, we constructed isogenic yeast strains that contained a deletion of the endogenous GCN5 gene and harbored a plasmid that expressed either wild type Gcn5p or a Gcn5p derivative containing the A190T mutation (GCN5-A190T). A strain containing an empty expression plasmid was used as an additional negative control. To determine whether Gcn5p-A190T was more effective than wild type Gcn5p in vivo, we first compared the ability of the two alleles to rescue the slow growth phenotype of a gcn5Δ strain in minimal medium (43). The yeast strains were grown in minimal medium, and the cell doubling times were calculated. As shown in Table II, the strains containing gcn5-A190T and wild type GCN5 grew equally well, because ectopic expression of either allele reduces the doubling time of a gcn5Δ strain by approximately 1 h. We next determined whether Gcn5-A190T was a more effective activator of transcription than wild type Gcn5p by comparing the ability of the two alleles to rescue a defect in HO gene expression, because deletion of GCN5 results in a dramatic decrease in expression of an HO-lacZ fusion gene (7, 18). As shown in Table II, expression of either wild type Gcn5p or Gcn5-A190T restored HO-lacZ expression to equal levels, suggesting that the A190T derivative is not a better transcriptional activator than wild type Gcn5p.

The transcription of Gcn5p target genes is critically dependent on the histone acetyltransferase activity of Gcn5p, because mutations that abolish this catalytic function significantly reduce the ability of Gcn5p to activate transcription (13–15). Although the wild type and A190T alleles of GCN5 do not differ in their ability to activate transcription of the HO gene, it is still possible that the histone acetyltransferase activity of the A190T derivative exceeds that of wild type Gcn5p, supporting higher levels of histone H3 acetylation in vivo. However, this difference in catalytic activity would not be detected through changes in gene expression if both enzymes maintained histone acetylation levels that supported the threshold required for transcriptional activation. Therefore, to determine whether Gcn5-5A190T supported higher levels of histone acetylation than wild type Gcn5p, we used a formaldehyde cross-linking chromatin immunoprecipitation protocol to analyze transcriptionally targeted histone H3 acetylation at the HO promoter (21) in yeast strains grown to mid-log phase in SD-minimal medium. Deletion of Gcn5p results in a 2–3-fold decrease in H3 acetylation at the HO promoter in log phase cells (data not shown and Ref. 19). As shown in Fig. 7 (A and B), Gcn5-A190T and wild type Gcn5p support similar levels of H3 acetylation when targeted to the HO promoter, ~2-fold above levels observed in the Gcn5Δ strain. We also determined whether Gcn5-A190T supported higher levels of global histone acetylation using chromatin immunoprecipitation analysis to measure histone H3 acetylation levels in the ACT1 coding region. As shown in Fig. 7C, Gcn5-A190T and wild type Gcn5p support similar levels of H3 acetylation in the ACT1 coding region,

### Table II

| Yeast strain | Doubling time (h) | HO-lacZ β-gal activity (Miller units) |
|-------------|------------------|--------------------------------------|
| gcn5 Δ      | 3.91 ± 0.08      | 0.14 ± 0.04                           |
| + Wild type | 3.07 ± 0.17      | 1.58 ± 0.33                           |
| + A190T     | 3.06 ± 0.07      | 1.35 ± 0.30                           |
| + Vector    | 4.07 ± 0.22      | 0.06 ± 0.02                           |

Expression of wild type GCN5 and gcn5-A190T equally rescue slow growth kinetics and reduced HO-lacZ activity because of a gcn5 deletion.
AcCoA Binding in the GCN5 Family of HATs

About 2-fold greater than the level of H3 acetylation observed in the gcn5Δ strain. Taken together, our data suggest that the A190T mutation does not increase the histone acetyltransferase activity of Gcn5p in vivo.

In summary, we have demonstrated that threonine/serine in the highly conserved motif (R/Q)XGGX(G/A)(T/S) greatly influences the binding affinity of GCN5 HATs for AcCoA. One notable exception is the S. cerevisiae yGCN5, which contains an alanine residue at this position. Site-directed replacement of alanine with a threonine (A190T) in yGCN5 completely restores high affinity AcCoA binding. To explore the consequences of increasing AcCoA substrate affinity in yGCN5, we explored the efficiency of the A190T derivative to rescue the Gcn5pΔ yeast strain and, interestingly, detected no biological consequences as assessed by the standard methodologies used in this study. Thus, the regulated function of yGCN5 does not appear to depend on the availability of AcCoA in the nucleus. At least in S. cerevisiae, GCN5 may be regulated solely by its ability to be recruited to specific loci and by its ability to efficiently access the histone tails of chromatin. We have recently demonstrated that ADA2, a protein found in complex with yGCN5, can function as a cofactor of GCN5-catalyzed acetylation of histones by increasing the kcat (catalytic efficiency) and decreasing the Km value (for histones) of the enzyme.

Interestingly, the ADA2-GCN5 complex did not exhibit changes in the efficiency of AcCoA binding, again suggesting that regulation via modulation of AcCoA binding affinity is unnecessary in the yeast. Why then is this threonine/serine residue so highly conserved in nearly all other distinct or eukaryotic members of the GCN5 family? Is the availability of AcCoA limiting in other organisms, and therefore these GCN5 homologs require higher affinity for obtaining the required level of acetylation? To the best of our knowledge, the difference in the levels of cellular AcCoA between diverse eukaryotes is not known. For the A190T derivative to function similarly to GCN5 in vivo, our data suggest that the nuclear AcCoA concentration in S. cerevisiae would be at least 8 μM, which is ~3-fold higher than the observed Km values seen with GCN5. At levels ≥8 μM, GCN5 would be at ≥75% maximal efficiency, which is within the error of our functional assays. In higher eukaryotes, it may be that the need to saturate the AcCoA-binding site of HATs has provided the necessary evolutionary pressure to add and maintain this important hydrogen bond. Alternatively, this interaction may be an evolutionary remnant, stemming from a common ancestral transferase gene that required tighter AcCoA binding for its biological function. Supporting the latter assertion, many distantly related acetyltransferases (Fig. 2 and Ref. 3), which acetylate small molecules, contain a threonine or serine in the signature motif (R/Q)XGGX(G/A)(T/S). Moreover, other potential hydrogen bond-forming residues can be found at this site in more diverse acetyltransferases (3). Therefore, our biochemical findings can be extrapolated to other acetyltransferases in the GNAT family. As we have shown, alteration of this site only affects the affinity for AcCoA; no other catalytic step in the reaction was compromised. Use of mutations at this site should prove extremely useful in probing the role of modulating AcCoA binding affinity in the cellular function of diverse acetyltransferases.

These future studies will be particularly insightful and will address the question of the role of co-enzyme and co-substrate levels in acetyltransferase cellular function.

Acknowledgment—We thank Michael Jackson for helpful comments on the manuscript.

L. A. Boyer, M. R. Langer, K. A. Crowley, S. Tan, J. M. Denu, and C. L. Peterson, submitted for publication.
REFERENCES
1. Mizzen, C. A., and Allis, C. D. (1998) Cell. Mol. Life Sci. 54, 6–20
2. Kouzarides, T. (1999) Curr. Opin. Genet. Dev. 9, 40–48
3. Neuwald, A. F., and Landsman, D. (1997) Trends Biochem. Sci. 22, 154–155
4. Yang, X. J., Ogryzko, V. V., Nishikawa, J., Howard, B. H., and Nakatani, Y. (1996) Nature 382, 319–324
5. Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (1996) Cell 87, 953–959
6. Bannister, A. J., and Kouzarides, T. (1996) Nature 384, 641–643
7. Pollard, K. J., and Peterson, C. L. (1997) Mol. Cell. Biol. 17, 6212–6222
8. Georgakopoulos, T., and Theodor, G. (1992) EMBO J. 11, 4146–4152
9. Brownell, J. E., and Allis, C. D. (1996) Curr. Opin. Genet. Dev. 6, 176–184
10. Wade, P. A., Pruss, D., and Wolfe, A. P. (1997) Trends Biochem. Sci. 22, 128–132
11. Grunstein, M. (1997) Nature 390, 349–352
12. Roth, S. Y., Denu, J. M., and Allis, C. D. (2001) Annu. Rev. Biochem. 70, 81–120
13. Wang, L., Liu, L., and Berger, S. L. (1998) Genes Dev. 12, 640–653
14. Zhang, W., Bone, J. R., Edmondson, D. G., Turner, B. M., and Roth, S. Y. (1998) EMBO J. 17, 3155–3167
15. Kuo, M. H., Zhou, J., Jambeck, P., Churchill, M. E., and Allis, C. D. (1998) Genes Dev. 12, 627–639
16. Tae, C., Georgieva, E. I., Ruiz-Garcia, A. B., Sendra, R., and Hansen, J. C. (1998) J. Biol. Chem. 273, 32388–32392
17. Grant, P. A., Duggan, L., Cote, J., Roberts, S. M., Brownell, J. E., Candau, R., Ohba, R., Owen-Hughes, T., Allis, C. D., Winston, F., Berger, S. L., and Workman, J. L. (1997) Genes Dev. 11, 1640–1650
18. Breeden, L., and Nasmyth, K. (1987) Cell 48, 389–397
19. Krebs, J. E., Fry, C. J., Samuels, M. L., and Peterson, C. L. (2000) Cell 102, 587–598
20. Fry, C. J., and Peterson, C. L. (2001) Curr. Biol. 11, R155–R197
21. Krebs, J. E., Kuo, M. H., Allis, C. D., and Peterson, C. L. (1999) Genes Dev. 13, 1412–1431
22. Kuo, M. H., vom Baur, E., Struhl, K., and Allis, C. D. (1999) Mol. Cell 4, 1309–1320
23. Voglewede, M., Wu, J., Suka, N., and Grunstein, M. (2000) Nature 406, 495–498
24. Xu, W., Edmondson, D. G., Evrard, Y. A., Wakamiya, M., Behringer, R. R., and Roth, S. Y. (2000) Nat. Genet. 26, 229–232
25. Yamazuchi, T., Yamazuchi, J., Kawai, T., Tamura, T., Yamashita, T., Bae, N., Westphal, H., Ozato, K., and Nakatani, Y. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 11303–11306
26. Tanner, K. G., Trievel, R. C., Kuo, M. H., Howard, R., Berger, S. L., Allis, C. D., Marmorstein, R., and Denu, J. M. (1999) J. Biol. Chem. 274, 18157–18166
27. Tanner, K. G., Langer, M. R., and Denu, J. M. (2000) Biochemistry 39, 11961–11969
28. Tanner, K. G., Langer, M. R., Kim, Y., and Denu, J. M. (2000) J. Biol. Chem. 275, 20394–20405
29. Luo, O. D., Courtne, A. D., Vassilev, A., Marzilli, L. A., Cotter, J. R., Nakatani, Y., and Cole, P. A. (2000) J. Biol. Chem. 275, 21953–21959
30. Dutnall, R. N., Tatfrov, S. T., Sternslanz, R., and Ramakrishnan, V. (1998) Cell 94, 427–438
31. Lin, Y., Fletcher, C. M., Zhou, J., Allis, C. D., and Wagner, G. (1999) Nature 400, 86–89
32. Rojas, J. R., Trievel, R. C., Zhou, J., Mo, Y., Li, X., Berger, S. L., Allis, C. D., and Marmorstein, R. (1999) Nature 401, 93–98
33. Clements, A., Rojas, J. R., Trievel, R. C., Wang, L., Berger, S. L., and Marmorstein, R. (1999) EMBO J. 18, 3521–3532
34. Kim, Y., Tanner, K. G., and Denu, J. M. (2000) Anal. Biochem. 280, 308–314
35. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
36. Stern, M., Jensen, R., and Herskowitz, I. (1994) J. Mol. Biol. 248, 953–968
37. Rose, I. A., O’Connell, E. L., and Litwin, S. (1974) J. Biol. Chem. 249, 5163–5168
38. Rose, I. A. (1980) Methods Enzymol. 64, 47–59
39. Rose, I. A. (1980) Methods Enzymol. 64, 47–59
40. Langer, M. R., Tanner, K. G., and Denu, J. M. (2000) J. Biol. Chem. 276, 31321–31331
41. Wolf, E., Vassilev, A., Makino, Y., Sali, A., Nakatani, Y., and Burley, S. K. (1998) Cell 94, 439–449
42. Hickman, A. B., Namboodiri, A. M., Klein, D. C., and Dyda, F. (1999) Cell 95, 361–369
43. Marcus, G. A., Silverman, N., Berger, S. L., Horiuchi, J., and Guarente, L. (1994) EMBO J. 13, 4807–4815
