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Phosphorylation of Serine 10 in Histone H3 Is Functionally Linked In Vitro and In Vivo to Gcn5-Mediated Acetylation at Lysine 14

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Summary

Multiple covalent modifications exist in the amino-terminal tails of core histones, but whether a relationship exists between them is unknown. We examined the relationship between serine 10 phosphorylation and lysine 14 acetylation in histone H3 and have found that, in vitro, several HAT enzymes displayed increased activity on H3 peptides bearing phospho-Ser-10. This augmenting effect of Ser-10 phosphorylation on acetylation by yGcn5 was lost by substitution of alanine for arginine 164 [Gcn5(R164A)], a residue close to Ser-10 in the structure of the ternary tGcn5/CoA/histone H3 complex. Gcn5(R164A) had reduced activity in vivo at a subset of Gcn5-dependent promoters, and, strikingly, transcription of this same subset of genes was also impaired by substitution of serine 10 to alanine in the histone H3 tail. These observations suggest that transcriptional regulation occurs by multiple mechanistically linked covalent modifications of histones.

Introduction

Targeted covalent modification of the amino-terminal tails of the core histones in nucleosomes has emerged as an important mechanism in the regulation of transcriptional activation of RNA polymerase II–transcribed genes. The best understood histone modification is acetylation of lysine residues, whose positions in the amino-terminal tails are evolutionarily conserved (Grunstein, 1997; Mizzen and Allis, 1998). Recent years have seen an explosion of information about both acetyltransferases (HATs) and deacetylases (HDACs) (Struhl, 1998; Suka et al., 1998). Several families of HATs have been identified, including the Gcn5 (Brownell et al., 1996) and MYST (Reifsnyder et al., 1996) families that are conserved throughout eukaryotes and the CBP/p300 (Bannister and Kouzarides, 1996; Ogryzko et al., 1996) family within metazoans. Each of these enzymes displays a characteristic pattern of target lysines within each of the core histones and in nucleosomes (Kuo et al., 1996; Allard et al., 1999; Grant et al., 1999).

The most well-characterized HAT enzymes are those in the Gcn5 family. Gcn5 was the first nuclear HAT protein to be identified (Brownell et al., 1996), and previous studies of Gcn5 as a transcription cofactor in *S. cerevisiae* (Guarente, 1995) set the stage for genetic, biochemical, and structural studies of the enzymatic activity. HAT activity of Gcn5 was shown to be required for Gcn5's function in transcriptional activation (Wang et al., 1998), which correlated with acetylation of histones at Gcn5-dependent promoters (Kuo et al., 1998) and chromatin remodeling itself (Gregory et al., 1998). In vivo, Gcn5 has been shown to exist in at least two high molecular weight protein complexes, a 1.8 MDa SAGA complex and a 0.8 MDa ADA complex (Grant et al., 1997; Saleh et al., 1997). In addition to acetylation activity, these in vivo complexes possess other activities, including interaction with activators for promoter targeting (Utley et al., 1998) and interaction with TBP for regulation of basal factor activity (Dudley et al., 1999; Belotserkovskaya et al., 2000). Taken together, an emerging model is that the SAGA complex is recruited to promoters by interaction with sequence-specific activator proteins, followed by Gcn5-mediated acetylation of histones within the basal promoter of these genes and then general factor recruitment, culminating in heightened transcription from the associated promoters.

There exists a plethora of other covalent histone tail modifications (Bradbury, 1992), stimulating questions concerning the reasons for multiple modifications, and for the close proximity of the sites for acetylation and phosphorylation in the histone H3 tail. For example, a second histone H3 tail modification is phosphorylation at Ser-10, close to one of the favored lysines (Lys-14) for Gcn5-mediated acetylation. Phosphorylation of Ser-10 on histone H3 has been shown to be important during the cell cycle, mitosis, and during transcription. The amino-terminal domain of histone H3 has been implicated in chromatin compaction, and phosphorylation at Ser-10 of histone H3 is tightly correlated with mitotic chromosome condensation and segregation in mammals (Hendzel et al., 1997; Van Hooser et al., 1998; Sauve et al., 1999), *Tetrahymena* (Wei et al., 1998, 1999), and *Xenopus* (de La Barre et al., 2000). In mammalian cells, phosphorylation of histone H3 Ser-10 has also been linked to transcriptional activation of mitogen-stimulated immediate-early response genes, such as *c-fos* and *c-jun* (Mahadevan et al., 1991; Chadee et al., 1999; Sassone-Corsi et al., 1999; Thomson et al., 1999), as well as cAMP-dependent protein kinase A (PKA)-responsive genes (Kogel et al., 1998; DeManno et al., 1999).

The three-dimensional structure of the HAT domain of *Tetrahymena* Gcn5 has been determined in ternary complex with the cofactor CoA and a substrate peptide of the H3 tail (tGCN5/CoA/histone H3) (Rojas et al., 1999). The structure reveals a pronounced groove along the surface of the protein that is occupied by the histone H3 peptide. A particularly striking and unexpected feature of the ternary complex was the mode in which the histone H3 substrate was bound (Rojas et al., 1999). Although the peptide was bound to the protein through an extensive set of hydrogen bond and van der Waals

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interactions, the majority of these interactions were mediated through the backbone region of the peptide. In fact, electron density corresponding to the three N-terminal residues of the peptide (Lys-9, Ser-10, and Thr-11) were not visible and, therefore, these side chain densities were not modeled in the final structure. This observation led to the hypothesis that posttranslational modification of this N-terminal region, such as phosphorylation at Ser-10, may facilitate histone H3 binding affinity and/or specificity.

In this report, we have examined the role of phosphorylation at serine 10 in histone H3. Our results reveal that histone H3 Ser-10 is required for transcriptional activation in vivo. In addition, in vitro, phosphorylation promotes acetylation, and in vivo these modifications are also linked. Moreover, a correlation of our results with the structure of the tGCN5/CoA/histone H3 complex suggest a structural model for how Ser-10 phosphorylation promotes Lys-14 acetylation. Importantly, only a subset of Gcn5-dependent promoters requires phosphorylation of Ser-10, suggesting that the interconnection may be regulatory in nature.

Results

Acetylation of Lys-14 in Histone H3 by Several HATs Is Increased on Peptides that Are Phosphorylated at Ser-10

Histone acetyltransferases in several families are capable of acetylating Lys-14 in the histone H3 amino-terminal tail. These HATs include members of the Gcn5 family (Kuo et al., 1996), the CBP/p300 family (Bannister and Kouzarides, 1996; Ogryzko et al., 1996), and some members of the MYST family (Reifsnyder et al., 1996), such as Esa1 (Allard et al., 1999; Ohba et al., 1999). Because Lys-14 lies very near to Ser-10 in the histone H3 tail, it is possible that the modifications exert a mutual effect upon subsequent modification.

To examine this possibility, we first tested the effect of phosphorylation of Ser-10 on subsequent acetylation by several recombinant HATs synthesized in bacteria, including domains derived from yGcn5, PCAF, p300, and Esa1. Synthetic peptides of 26 residues were used as acetylation substrates and were either unmodified (H3-WT peptide) or bore a phosphoserine residue at position 10 (H3-S10Pi peptide) (Figure 1A). Enzymes were incubated with peptide and [^{3}H]acetyl CoA. Incorporation of [^{3}H]acetate was quantitated by filter binding and scintillation counting.

Yeast Gcn5 exhibited a 4- to 6-fold higher level of activity on the phosphorylated peptide compared to the unmodified peptide (Figure 1B). For comparison, equivalent amounts (Figure 3B) of Gcn5 bearing a substitution mutation that inactivated catalytic function (E173Q) showed low activity (note the different y axis scale) that was not significantly altered on the phosphorylated peptide (Figure 1B). PCAF and p300 HAT enzymes also showed a higher level of acetylation activity on the H3-S10Pi peptide compared to the unmodified peptide (Figure 1B), although the difference in their relative activities on the two peptides were less dramatic than that of yGcn5. In contrast, the Esa1 HAT enzyme, which had an overall lower activity, exhibited slightly lower activity

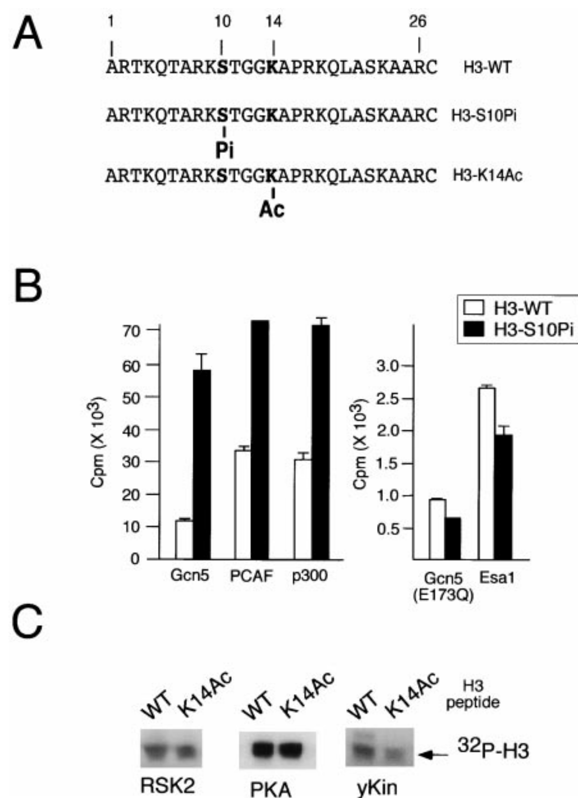

Figure 1. Acetylation of Lys-14 in Histone H3 by Several HATs Is Increased on Peptides that Are Phosphorylated at Ser-10

(A) Sequence and covalent modifications of H3 tail peptides used in the enzymatic assays. The yeast H3 amino-terminal tail (residues 1–26) was synthesized with a carboxyl-terminal cysteine. Pi indicates phosphorylation and Ac indicates acetylation.

(B) The effect of Ser-10 phosphorylation on subsequent acetylation. Purified recombinant HAT domains (20 ng) of yGCN5 (residues 99–262), PCAF (residues 492–695), p300 (residues 398–603), yEsa1 (residues 161–435), and yGCN5(E173Q) were used for liquid HAT assays, with H3-WT peptide or H3-S10Pi peptide. HAT assays were incubated for 10 min at 30°C. Experiments were performed in triplicate, with standard error shown. The background HAT activity (no added enzyme) was 400–500 cpm.

(C) The effect of K14 acetylation on subsequent phoshorylation. In vitro kinase assays were performed with H3-WT and H3-K14Ac peptide, using RSK2 (2 units), PKA (1 unit), or a partially purified yeast H3 Ser-10 kinase complex (yKin). After 30 min incubation with [γ-^{32}P]ATP, the samples were analyzed on SDS-PAGE and autoradiography.

on H3-S10Pi peptide compared to the unmodified peptide (Figure 1B).

We then tested the reverse possibility, that is, whether preacetylation at Lys-14 promotes phosphorylation at Ser-10. We used three sources of histone H3 Ser-10 kinase. These were human Rsk2, human PKA, and a partially purified activity from yeast extracts (W.-S. L., unpublished data). Each kinase phosphorylated the unmodified histone H3 tail peptide but was unaffected (Rsk2 and PKA) or slightly inhibited (yKin) in its ability to phosphorylate the H3-K14Ac peptide (Figure 1C). In summary, acetylation by several histone H3 HATs was improved on prephosphorylated H3, while phosphorylation by several histone H3 kinases was not affected by preacetylated H3.

Serine 10 in the Histone H3 Tail Is in Close Proximity to Arginine 164 in the yGcn5 HAT Domain

These data indicate that histone H3 acetylation is favored on a tail peptide that is prephosphorylated. To begin to understand the mechanism of the promotion of acetylation at Lys-14 on the Pi-S10 peptide, we closely analyzed the three-dimensional structure of the Gcn5 HAT domain in complex with CoA and a histone H3 tail peptide.

One of the surprises from the tGcn5/CoA/histone H3 peptide complex was that the vast majority of protein–peptide interactions were mediated by the backbone atoms of the histone H3 peptide and only Gly13, Lys-14, and Pro-16 were involved in side chain–specific interactions (Rojas et al., 1999). In addition, several of the other side chain residues of the peptide (Lys-9, Ser-10, and Thr-11) were modeled as either alanines or glycines in the final structure due to their respective disorder. Interestingly, although the cβ and oγ atoms of Ser-10 were disordered, the cα atom was clearly pointed into a fairly deep peptide binding cleft of the protein. This region of the peptide binding cleft was largely hydrophobic along its edges but, interestingly, contained a highly basic patch at its base dominated by Arg-113 of tGcn5 (Arg-164 in yGcn5) (Figure 2). Although the aliphatic region of Arg-164 appears to play a role in stabilizing the hydrophobic core of protein, the accessibility of its basic moiety at the base of the peptide binding cleft suggested that it may play a role in accommodating a phosphoserine at position 10 of the histone H3 peptide. In support of this hypothesis was the observation that Arg-164 is strictly conserved within the Gcn5 subfamily of HAT proteins suggesting a functional role.

Mutation of Arg-164 in Recombinant Gcn5 or in Native Yeast ADA or SAGA Complexes Reduces the Acetylation Advantage on the H3-S10Pi Peptide

Having identified a putative residue, Arg-164, within the Gcn5 HAT domain that may play a role in accommodating phosphoserine at position 10 within the histone H3 tail, we engineered a single alanine substitution at this residue to test whether its mutation was selectively sensitive to phosphorylation at Ser-10 of the histone H3 peptide. First, the Gcn5 wild-type HAT domain enzyme (residues 99–262) was used to establish the linear range of HAT activity. Several concentrations of enzyme were used to test HAT activity on the H3-WT peptide, and the activity was seen to increase linearly with enzyme concentration (Figure 3A). Next, the amount of Gcn5(WT) protein was compared to Gcn5(R164A) to ascertain equivalent amounts of each (Figure 3B). Finally, the acetylation activity of Gcn5(R164A) was compared to the wild-type HAT domain on phosphorylated versus unmodified histone H3 peptide (Figure 3B). The R164 mutant enzyme exhibited similar activity compared to Gcn5(WT) on the H3-WT peptide (suggesting that it does not affect catalysis) but nearly 3-fold lower activity on the H3-S10Pi peptide. This was the reverse relationship compared to the wild-type enzyme, and the overall difference in HAT activity on the H3-S10Pi peptide of Gcn5(WT) compared to Gcn5(R164A) was 7-fold. In addition, a 30 min time course was done to examine the dynamic range of HAT activity and to determine whether the R164A substitution was indeed unaffected on the H3-WT peptide. At every point tested during the linear time course, the activity of Gcn5(R164A) was very near to that of Gcn5(WT) enzyme on the H3-WT peptide but was approximately six times reduced in activity on the H3-S10Pi peptide (data not shown).

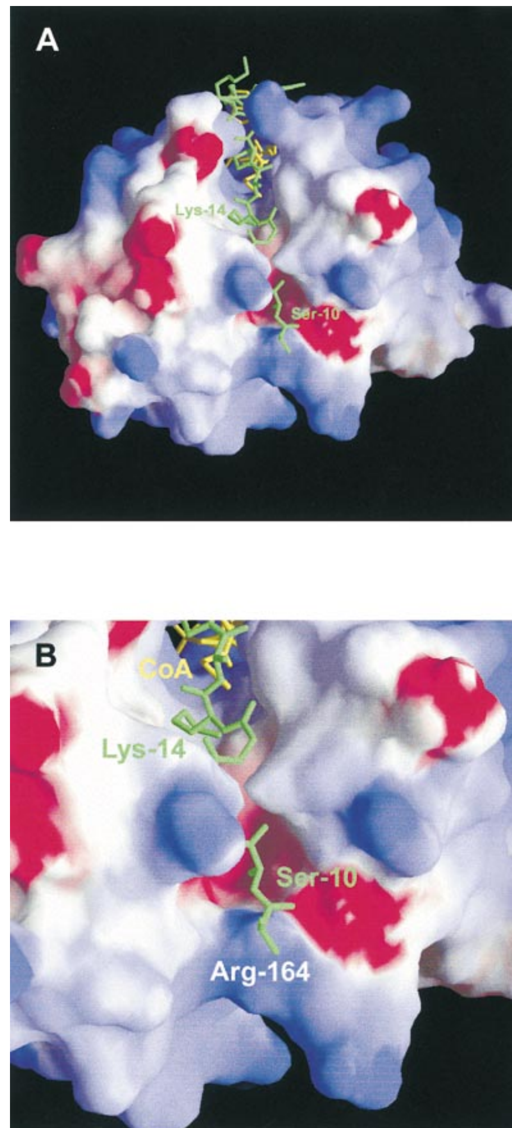

Figure 2. Ser-10 in the H3 Tail Is in Close Proximity to R164 in the yGcn5 HAT Domain

Electrostatic surface of the HAT domain of tGCN5 bound to coenzyme A (yellow) and an eleven amino acid peptide (residues 9–19) from yeast histone H3 (green). The Lys-14 acetylation and the Ser-10 phosphorylation sites are labeled, as well as Arg-164 (yGCN5 numbering). Both overall (A) and close-up views (B) are shown. The model was created with the program GRASP (Nicholis et al., 1991).

To determine the activity of Gcn5(R164A) compared to Gcn5(WT) when incorporated into native yeast HAT complexes, the R164A mutation was engineered into the full-length *GCN5* gene and was integrated into yeast bearing a disruption of the genomic *GCN5* gene. Yeast HAT extracts were prepared from the wild-type strain and from the strain bearing the Gcn5(R164A) mutant, and four

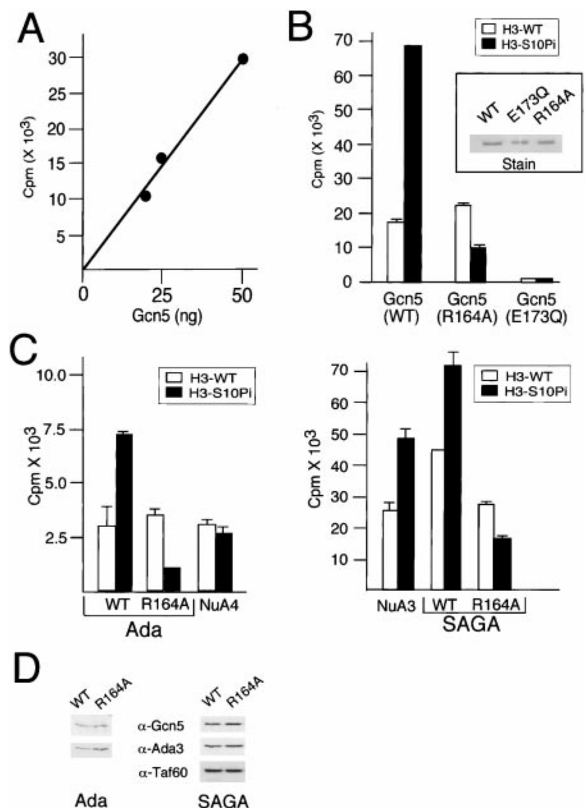

Figure 3. Substitution Mutation of R164 in yGcn5 HAT Protein Reduces the Acetylation Advantage on H3-S10Pi

(A) Linearity of acetylation by recombinant Gcn5 HAT domain as a function of enzyme concentration. Purified wild-type yGCN5 HAT protein (20, 25, or 50 ng) was used for liquid HAT assays with H3-WT tail peptide. HAT assays were performed in triplicate at 30°C for 10 min. Standard errors were $<3\%$.

(B) Acetylation by recombinant Gcn5(WT) and Gcn5(R164A) or Gcn5(E173Q). Purified HAT proteins (20 ng) were used for liquid HAT assays on the indicated peptides, in triplicate at 30°C for 10 min. The standard error is shown. The background HAT activity was 500–600 cpm. Insert shows Coomassie blue staining of 150 ng of each recombinant HAT domain.

(C) Acetylation by native yeast ADA or SAGA complexes bearing wild-type or R164A-substituted Gcn5. HAT assays with H3-WT peptide or H3-S10Pi peptide were performed with ADA, NuA4, NuA3, and SAGA complexes and with the same amounts of ADA and SAGA complexes bearing Gcn5(WT) or Gcn5(R164A), as judged from the Western in (D). HAT assays were performed in triplicate at 30°C for 30 min. Standard error is indicated.

(D) Western analysis of native SAGA and ADA complexes. SAGA and ADA prepared from Gcn5(WT) and Gcn5(R164A) mutant were analyzed using the indicated antibodies.

nucleosomal HAT complexes were separated chromatographically, as described previously (Grant et al., 1997). These complexes were two Gcn5-dependent HAT complexes, ADA and SAGA, the Esa1-dependent NuA4 complex, and the NuA3 complex, which acetylates histone H3 but whose HAT enzyme is not yet known. Acetylation by each of the four complexes was tested on the H3-WT and H3-S10Pi peptides. The two Gcn5-dependent complexes, ADA and SAGA, showed a roughly 2-fold higher acetylation activity on modified compared to unmodified peptide (Figure 3C). ADA and SAGA complexes were then isolated from the strain expressing Gcn5(R164A). Similar amounts of complexes bearing wild-type and the Gcn5(R164A) mutant were established by Western analysis, using antibodies to detect multiple subunits of each complex (Figure 3D). The Gcn5(R164A) ADA and SAGA complexes showed similar levels of activity on the H3-WT peptide but lower levels of activity on the H3-S10Pi peptide (Figure 3C), a behavior similar to recombinant Gcn5(R164A) (Figure 3B). The NuA3 complex also showed increased activity on the H3-S10Pi peptide. In contrast, the NuA4 complex showed similar activity on modified and unmodified peptides (Figure 3C), just as recombinant Esa1 was unaffected by phosphorylation (Figure 1B). In summary, recombinant Gcn5 or native Gcn5-containing complexes, as well as the NuA3 complex, exhibit improved acetylation activity on phosphorylated H3 tail peptide compared to unmodified peptide. This advantage of the ADA and SAGA complexes was eliminated in strains bearing Gcn5(R164A), the residue implicated to play a role in mediating the interaction of histone H3 phosphoserine 10 with Gcn5.

The R164A Mutation in Gcn5 Lowers Growth and Transcriptional Activation In Vivo in a Subset of Conditions and Promoters that Require Gcn5 HAT Activity

The R164A mutation lowered acetylation by Gcn5, or complexes bearing Gcn5, selectively on the H3-S10Pi peptide. Next, we determined the effect of the R164A substitution in Gcn5-dependent assays in vivo. First, the effect of the mutation was examined in several assays in which Gcn5 function is required for optimal growth. The effect of Gcn5(R164A) was compared to three previously characterized Gcn5 mutants. These were Gcn5 disruption, Gcn5(E173Q) bearing a substitution of a key catalytic residue, and a double substitution mutant [Gcn5(YI135AA)] that eliminates a tyrosine side chain that makes specific contact with the histone peptide (Rojas et al., 1999). Previous data indicates that Gcn5(E173Q) is very defective in vivo (Trievel et al., 1999), while Gcn5(YI135AA) displays partial defects (Wang et al., 1998).

Two assays were used to examine overall growth in several media that induce different biosynthetic pathways: first, a qualitative colony size assay and, second, a quantitative growth rate assay. Each strain grew well and exhibited comparable growth rates on synthetic complete media (Figure 4, SC, first panel from the left), which is not restrictive for growth of *gcn5*⁻ (Marcus et al., 1994). In minimal media, the Gcn5(R164A) substitution had little effect, which can be seen both in colony size and growth rate (Figure 4, second panel), while the *GCN5* disruption or strains bearing Gcn5(E173Q) or Gcn5(YI135AA) caused poor yeast growth, as observed previously (Wang et al., 1998; Trievel et al., 1999). In ethanol/glycerol media (Figure 4, middle panel), the strains bearing Gcn5(R164A) and Gcn5(YI135AA) grew more poorly in both assays than the Gcn5(WT) strain, although not as poorly as *gcn5*⁻ or Gcn5(E173Q). In nitrogen starvation media or media containing 3-AT (Figure 4, two panels on the right), Gcn5(R164A) grew as poorly as any of the other Gcn5 mutant strains. Thus, the R164A substitution resulted in selective growth effects compared to other mutations that affect activity of the Gcn5 HAT domain.

The R164A substitution was then tested for effects on

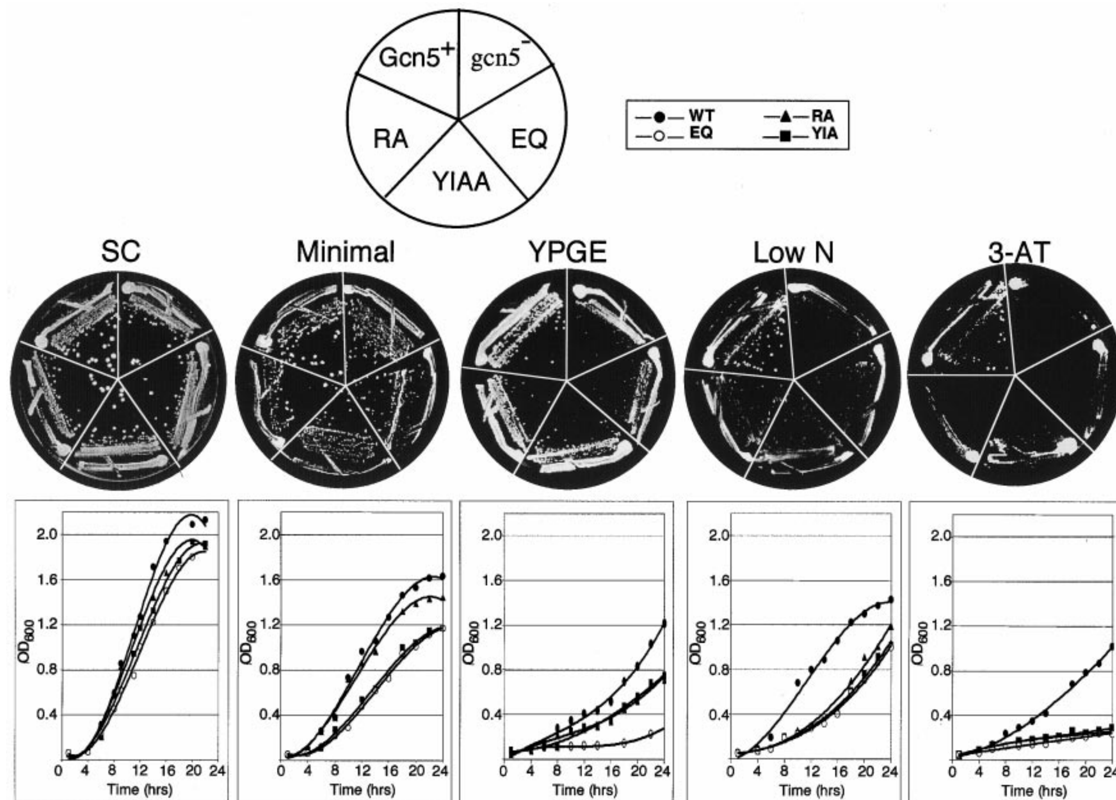

Figure 4. Growth Assays of Yeast Bearing Wild-Type or Mutant Gcn5

GCN5-disrupted yeast containing wild-type Gcn5 (Gcn5$^+$), Gcn5(R164A) (RA), Gcn5(E173Q) (EQ), Gcn5(YI135AA) (YIAA), or vector alone (*gcn5*$^-$) were streaked for single colonies onto media and were grown at 30°C for 4–7 days (top panel) or grown in liquid culture for 24 hr, and sampled at the indicated times (bottom panel). The media are synthetic complete (SC), minimal, synthetic with glycerol and ethanol substituted for glucose (YPGE), synthetic complete with 100 mM 3-AT (AT), or nitrogen starvation (Low N).

transcription. Gcn5 is required for full activation by the test activator LexA-GCN4 in a β-gal reporter assay (Wang et al., 1998). The Gcn5(E173Q) substitution lowered transcription to *gcn5*$^-$ levels, while Gcn5(R164A) had an intermediate effect on LexA-GCN4's ability to activate, similar to Gcn5(YI135AA) (Figure 5A). In contrast, none of the substitution mutations in Gcn5 had an effect on the ability of LexA-HAP4, a previously characterized Gcn5-independent activator (Marcus et al., 1994), to activate the same reporter (Figure 5A). Thus, the R164A substitution affected transcription driven by a known Gcn5-dependent activator, but not by a Gcn5-independent activator.

Transcription levels of several endogenous Gcn5-dependent genes were then tested. RNA was prepared from *GCN5*$^+$ and *gcn5*$^-$ strains and from strains bearing the Gcn5 substitutions R164A, E173Q, and YI135AA. *HO*, *PCL2*, and *CDC6* RNA levels were analyzed by quantitative S1 nuclease assay from RNA prepared from cells grown in rich media, and *HIS3* transcription was analyzed from cells grown in media supplemented with 3-AT, an inducer of the *HIS3* gene. Transcription of each gene was affected by *gcn5*$^-$ and the Gcn5(E173Q) catalytic and the Gcn5(YI135AA) peptide interaction substitutions (Figure 5B and quantitated in Figure 5C), indicating that Gcn5's acetylation function is required for transcription of each gene. With respect to the effect of the Gcn5(R164A) substitution, the four genes tested fell into two groups. Transcription from two of the genes, *HIS3* and *HO*, was dramatically lowered in the strain bearing Gcn5(R164A). In contrast, transcription of *PCL2* and *CDC6* was not affected by the Gcn5(R164A) mutation (Figures 5B and 5C). These results indicate that, similar to the selective effect of Gcn5(R164A) in the Gcn5-dependent growth assays (Figure 4), transcription of only a subset of Gcn5-dependent genes is lowered by Gcn5(R164A).

The Same Subset of Gcn5-Dependent Promoters that Are Affected by the R164A Mutation Are Also Affected by Histone H3 S10A Mutation

These data demonstrate that R164 is required for Gcn5's function at some, but not all, promoters, suggesting that phosphorylation of serine 10 is important at some, but not all Gcn5-regulated promoters. To test this directly, we examined, at the same constellation of Gcn5-regulated promoters, the effect of substituting Ser-10 in histone H3 to alanine [H3(S10A)]. RNA was prepared from a yeast strain bearing H3(S10A) and its wild-type parent. Strikingly, transcription from the two promoters reduced by the R164A substitution in Gcn5 (*HIS3* and *HO*) was also reduced by S10A in histone H3, while the two promoters that were not affected by Gcn5(R164A) (*PCL2 and CDC6*) were not affected by H3(S10A) (Figures 6A and 6B). Moreover, the magnitude of the effect of the H3(S10A) substitution was comparable to that of Gcn5(R164A). Thus, Ser-10 in histone H3 is required for transcription of some, but

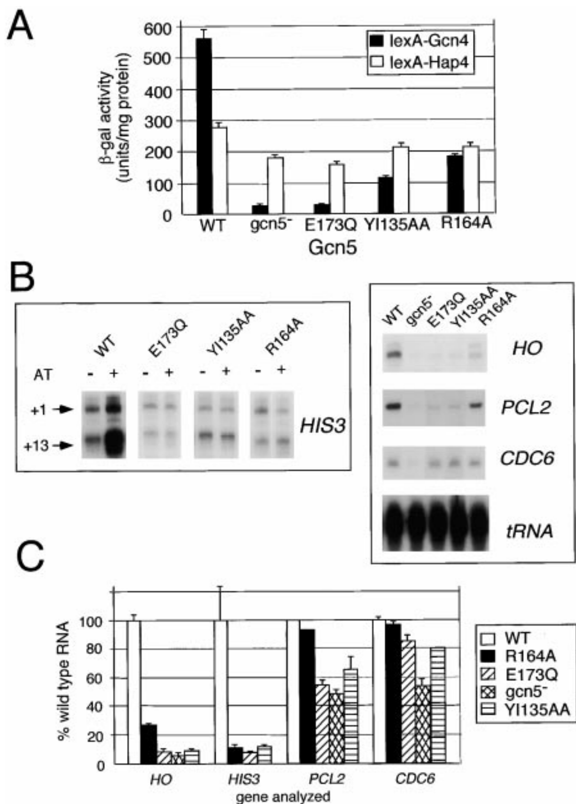

Figure 5. Transcription Analyses of Various Genes in Yeast Bearing Wild-Type or Mutant Gcn5

(A) β-galactosidase reporter assays of LexA-GCN4 activity. Gcn5-deleted cells containing Gcn5(WT) or substitution mutants Gcn5(R164A), Gcn5(E173Q), or Gcn5(YI135AA) were cotransformed with a plasmid expressing LexA-GCN4 or LexA-HAP4 and a reporter plasmid bearing LexA binding sites. The yGCN5-deleted strain transformed with vector alone was used as a negative control (*gcn5⁻*). Cells were grown in minimal medium to optical density of 0.8–1.0 (A_{600} nm) for the β-gal assays. β-galactosidase (β-gal) activity was determined as units per milligram of protein. Error bars represent the standard deviation from the mean in four independent experiments.

(B) Representative S1 nuclease assays of several Gcn5-dependent yeast genes. Probes for S1 analysis were specific for *HIS3*, *HO*, *PCL2*, *CDC6* RNA, and tRNA probe as the internal control. The strains contained wild-type *GCN5* or substituted *GCN5* mutants.

(C) PhosphorImager quantitation of the S1 assays. The averages and standard deviations are shown from triplicate experiments, at minimum.

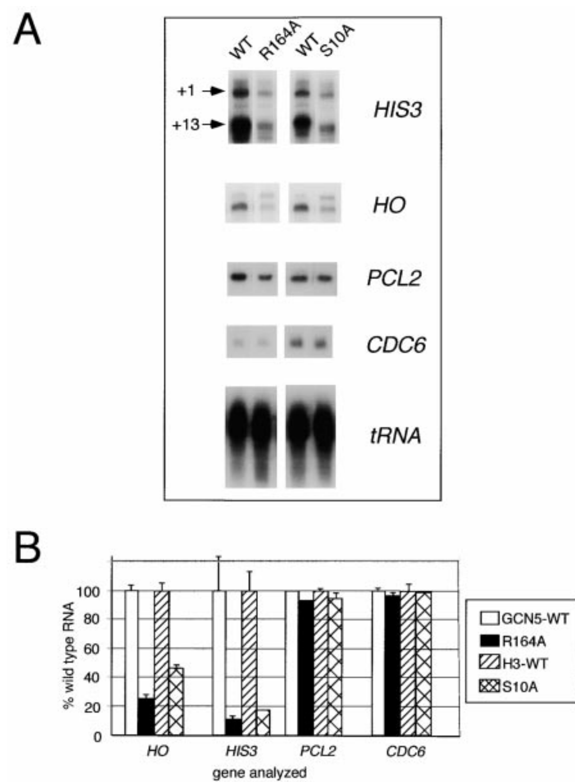

Figure 6. Transcription Analysis of Various Genes in Yeast Bearing Wild-Type or Mutant H3

(A) Comparison of RNA levels in strains bearing Gcn5(R164A) and histone H3(S10A). RNAs were purified from strains as indicated. (Left panel) Gcn5(WT) and Gcn5(R164A) substitution. (Right panel) histone H3 wild-type and S10A-substituted H3. S1 assays were performed as described as above.

(B) PhosphorImager quantitation of the S1 assays. Same as in Figure 5C.

not all, Gcn5-regulated genes, and the same subset of Gcn5-regulated genes require R164 in the Gcn5 HAT domain.

Discussion

It has long been known that multiple sites of covalent modification occur in the amino-terminal tails of the core histones (Bradbury, 1992); however, it has not been known whether these modifications are functionally linked. The data presented describe the mechanistic linkage of two modifications in the histone H3-terminal tail. We have found that phosphorylation of Ser-10 and accommodation of the phosphate by the HAT domain of Gcn5 promotes subsequent acetylation at Lys-14 by Gcn5. This sequence of events is linked to transcriptional upregulation of affected genes. The evidence that this pathway exists and is important for gene regulation derives from enzymatic, structural, and functional evidence.

First, our data indicate that phosphorylation of Ser-10 of the H3 tail increased acetylation by Gcn5 and that substitution mutation at R164 in Gcn5 selectively reversed the acetylation advantage, such that the nonphosphorylated tail was acetylated better than the phosphorylated tail. Arginine 164 was targeted for mutagenesis based on examination of structural data of the detailed association of residues within the Gcn5 HAT domain with histone H3 peptide. The effect of the R164A substitution in Gcn5 was true both for the recombinant HAT domain and for native yeast protein complexes (ADA and SAGA) containing Gcn5. Importantly, the R164A substitution did not lower acetylation on the unmodified peptide, strongly suggesting that the alteration does not affect enzymatic HAT activity itself, but rather, recognition of the phosphorylated peptide.

Second, the R164A substitution in the HAT domain lowered activation of a subset of Gcn5-regulated promoters in vivo (i.e., certain promoters that required the acetylation

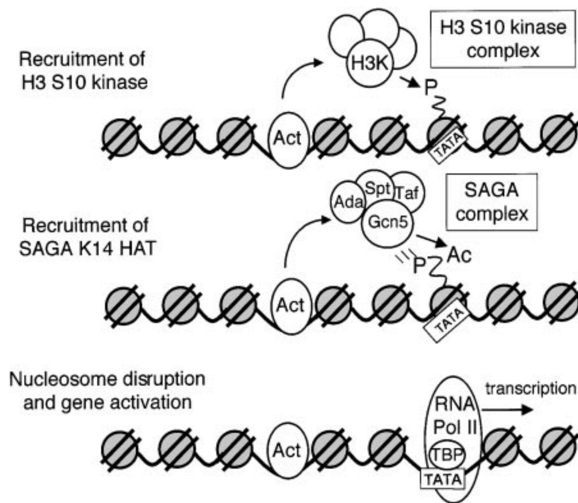

Figure 7. Schematic Model for the Role of Histone H3 Serine 10 Phosphorylation in SAGA Function

(Top) A putative histone H3 Ser-10 kinase protein complex is recruited to relevant promoters by interaction with a DNA-bound activator. (Middle) The SAGA HAT complex is recruited by interaction with a DNA-bound activator and is aided by association of Gcn5 HAT domain with phosphorylated Ser-10 on histone H3. (Lower) The combination of Ser-10 phosphorylation and Lys-14 acetylation results in activated transcription.

function of Gcn5 also required intact Arg-164 [*HO* and *HIS3*], while other promoters that required Gcn5 acetylation did not require intact Arg-164 for function [*CDC6* and *PCL2*]). Finally, analysis of the S10A mutation in the histone H3 tail revealed a very similar relationship: the identical Gcn5-dependent promoters that required Arg-164 in Gcn5 also required intact Ser-10 in H3, while the Gcn5-dependent promoters that did not require Arg-164 did not require Ser-10. These data support the model that Gcn5-bearing HAT complexes function more efficiently when histone H3 is modified. The promoter selectivity in vivo suggests that histone H3 Ser-10 phosphorylation is regulatory for transcription and occurs at certain promoters to specifically improve the activity of HAT complexes.

One additional point is that these data implicate phosphorylation of histone H3 in transcriptional activation in vivo. Previous evidence from the mammalian system indicated that increased histone H3 Ser-10 phosphorylation correlates with induction of immediate-early response genes *c-jun* and *c-fos*. Rsk2, a member of the pp90 Rsk kinase family (Blenis, 1993), was previously identified in these signaling pathways (De Cesare et al., 1998) and has been recently found to phosphorylate Ser-10 of histone H3 (Sassone-Corsi et al., 1999). Our mutational analysis of histone H3 Ser-10 provides direct genetic evidence that histone phosphorylation is linked to gene activation.

The findings that Ser-10 phosphorylation improved Lys-14 acetylation but that Lys-14 acetylation had no effect or was slightly inhibitory to Ser-10 phosphorylation suggest a potential pathway of modification. At promoters where Ser-10/Lys-14 are doubly modified, Ser-10 phosphorylation must occur prior to Lys-14 acetylation, in order for a mutual effect to be manifested. It is also possible that Ser-10 is phosphorylated at many or all promoters, but that Gcn5, for example, requires the modification only at certain promoters. Several arguments weigh against constitutive histone H3 Ser-10 phosphorylation. Analysis of the mammalian Rsk2 kinase argues for promoter selectivity. The specificity of the transcriptional response to Rsk2 by *c-fos* and *c-jun* (De Cesare et al., 1998) and the finding that phosphorylation of Ser-10 in histone H3 increases dramatically following mitogen induction (Sassone-Corsi et al., 1999) argue against the modification occurring at all promoters and indicate that, like acetylation, phosphorylation is a specific induced modification used to activate promoters. Additional evidence for a pathway of mitogen-induced phosphorylation proceeding and linked to subsequent acetylation is provided by the accompanying paper (Cheung et al., 2000 [this issue of *Molecular Cell*]).

Second, the finding that the Gcn5 family and CBP, but not Esa1, are responsive to Ser-10 phosphorylation also indicates promoter specificity in the effect and that phosphorylation is not an obligatory prelude to acetylation. Given the widespread role of CBP in activation of promoters in higher eukaryotes, it is a clear possibility that Ser-10 phosphorylation is used in a regulatory role for a subset of CBP/p300-dependent promoters. Interestingly, Rsk2 has been shown to phosphorylate the CBP-dependent activator CREB (Xing et al., 1996), raising the specter of further functional interplay and cooperativity between acetylation and phosphorylation. Consistent with this proposal, many transcription factors have been shown to be acetylated (Berger, 1999), and several of these have known phosphorylation sites in the general vicinity of acetylation.

Our finding that phosphorylation of Ser-10 affects acetylation of Lys-14 suggests the possibility of additional cross-talk between modifications. Serine 10 is adjacent to Lys-9 and near Lys-18, which are both acetylated by Gcn5 within SAGA (Grant et al., 1999) and by CBP/p300 (Ogryzko et al., 1996; Sato et al., 1997). Thus, it will be important to investigate the mutual positive or negative influences of acetylation/phosphorylation of these sites. The many additional sites of covalent modifications, including methylation and ubiquitination, also represent possible regulatory interactions.

What might be the physiological function of interconnected modifications? There are two general models for the role of modifications of histones (Turner, 1993; Mizzen et al., 1998). First, modifications may physically perturb chromatin structure, and phosphorylation, like acetylation, increases negative charge in the tails, to potentially alter the ionic environment in a similar fashion. A second hypothesis is that the covalent modifications function as interaction signals for additional proteins, and thus the presence of both modifications may serve as a highly specific chemical marker to promote interactions with additional regulatory proteins. This proposal has been put forth by several researchers and has been codified recently as the "histone code" hypothesis (Strahl and Allis, 2000). In the case of acetylation, there is experimental evidence to support both models, including acetylation causing chromatin disruption (Lee et al., 1993; Vettese-Dadey et al., 1996; Tse et al., 1998) and specific acetylation states promoting interaction of both repressive (Hecht et al., 1995; Edmondson et al., 1996) and activating (Dhalluin et al., 1999; Ornaghi et al., 1999) proteins. Thus, these models may not be mutually exclusive and each may incorporate more finely tuned regulation and synergistic effects

caused by increased number and alternate patterns of modifications.

The detailed structural mechanism by which Ser-10 phosphorylation promotes acetylation of Lys-14 of histone H3 and the role played by Arg-164 in mediating this effect awaits the structure determination of a ternary Gcn5 HAT complex bound to a Ser-10 phosphorylated histone H3 peptide. Nonetheless, the functional data suggests that Ser-10 phosphorylation of histone H3 affects histone tail interactions with the Gcn5 HAT domain and that Arg-164 plays an important role in mediating this effect. We entertain the possibility that Arg-164 makes direct hydrogen bonds with the phosphooxygens of the phosphoserine, although the largely hydrophobic peptide cleft would have to make significant adjustments to accommodate the somewhat bulky phosphoserine. Alternatively, phosphorylation of Ser-10 may cause adjustments of nearby histone residues to make alternative, and potentially more favorable, HAT domain interactions through Arg-164.

The data lead to a model for the functional interplay of covalent modifications of the histone H3 amino-terminal tail at certain Gcn5-dependent promoters. In this model (depicted in Figure 7), phosphorylation of Ser-10 is achieved by recruitment of a kinase complex to certain promoters, perhaps by interaction with a DNA-bound activator analogous to recruitment of HAT complexes (Silverman et al., 1994; Barlev et al., 1995; Utley et al., 1998). This would be followed by activator recruitment of HAT complexes, aided by the prior phosphorylation of Ser-10, which would either increase the affinity of the HAT complexes with nucleosomes, or increase HAT catalytic activity. Because not all Gcn5-dependent promoters are affected by H3 phosphorylation, it is unclear whether certain DNA-bound activators may have evolved the ability to recruit both types of complexes, or whether different activators exist at the same promoters to recruit a putative kinase complex or an acetylation complex. The doubly modified histone H3 tail destabilizes nucleosomes and/or targets additional regulatory proteins to upregulate affected promoters beyond the effect achieved by acetylation alone.

It will be crucial to distinguish between the two distinct models for the role of covalent modifications in gene regulation (and other chromatin processes). Our data are consistent with either model but suggest that the presence of multiple modifications result in increased flexibility and another level of gene regulation. This regulation would include both combinatorial and synergistic effects to set the overall range of control attainable in gene regulation.

Experimental Procedures

Yeast Strains and Media

The *S. cerevisiae* strains used in this study are: FY1370, *MATα gcn5Δ::HIS3, his3Δ200 leu2Δ1 ura3–5 trp1::hisG* (Roberts and Winston, 1997); YIA, isogenic to FY1370, but *URA3::pRS306-GCN5-YIA* (Wang et al., 1998); R164A, isogenic to FY1370, but *URA3::pRS306-GCN5-R164A*; E173Q, isogenic to FY1370, but *URA3::pRS306-GCN5-E173Q*; JHY90, *MATa ura3–52 ade2–101 his3Δ1 leu2–3,112 trp1–289 lys2–801 Δ(hht1-hhf1) Δ(lyhht2-hhf2) pJH18[CEN TRP1 HHT2-HHF2]*; JHY91, isogenic to JHY90, but *pJH15[CEN TRP1 hht2–3 (S10A) HHF2*. Yeast strain FY1370 Δ*gcn5* was described previously (Roberts and Winston, 1997) and was used for integration of the *GCN5* substitution mutations.

Standard yeast media, rich (YEPD), synthetic complete (SC), and minimal media and genetic manipulations were used (Rose et al., 1990). Limiting nitrogen media contains 0.17% yeast nitrogen base without amino acids or ammonium sulfate, 2% dextrose, 2% Bacto agar, and supplemented with the required amino acids. Histidine limitation was accomplished by supplementing minimal media with 100 mM 3-amino-1,2,4-triazole (3-AT).

GCN5 Mutagenesis and Plasmid Constructs

Single amino acid substitutions, R164A, changing Arg to Ala (codon CGA to GCA), and E173Q, changing Glu to Gln (codon GAA to CAA), were generated in pRSET-yGCN5 and in pRS306-yGCN5 (QuickChange kit [Stratagene]). For integration of wild-type and mutant *GCN5* into yeast, constructs were prepared in vector pRS306 bearing *GCN5* and its natural promoter. The pRS306-*yGCN5* constructs were linearized with NsiI and transformed into the *gcn5* deleted strain. pRSET-*yGCN5*-HAT was used for overexpression of yGCN5 HAT protein in bacteria. All mutations were confirmed by sequencing.

Protein Preparation and Western Analysis

The purification of yGCN5 HAT protein was performed as described (Trievel et al., 1999). Briefly, the protein was purified with sequential SP-Sepharose (Amersham Pharmacia), CoA-agarose (Sigma), and Superdex 75 (Amersham Pharmacia) chromatographies. Purified protein was concentrated to 10 mg/ml, and purity was assessed on SDS-PAGE. The purification of GST-p300 and GST-pCAF HAT proteins was performed as described (Liu et al., 1999).

Anti-Ada3 and anti-Gcn5 antibody dilutions were 1:4000. Anti-$Taf_{II}60$ and anti-H3 S10Pi antisera were diluted 1:6000 and 1:5000, respectively. Immunodetection was done using chemiluminescence (ECL kit; Amersham).

In Vivo Transcription and RNA Assays

For β-galactosidase assays, strains were cotransformed with pRS414-*lexA-Gcn4* (Hope and Struhl, 1986) and LexA-8x-LacZ reporter (Candau and Berger, 1996). The cells were grown to an optical density of 0.8 at 600 nm, and extracts were prepared and assayed for β-galactosidase activity as described (Rose et al., 1990).

In vivo RNA analysis was done by S1 nuclease assay. Total RNA was isolated from yeast cultures grown in YPD medium to an optical density at 600 nm of 0.8–1.2, using hot phenol as described (Belotserkovskaya et al., 2000). To derepress *HIS3* transcription, 40 mM 3-aminotriazole (3-AT) was added to the SC medium for 2 hr prior to RNA isolation. Each RNA sample (40–80 μg) was hybridized with an excess of ^{32}P end–labeled probes. S1 probes were synthesized (IDT Corp), and approximately 0.5 pM of oligonucleotide probe was resuspended with 40 μg (for HIS3 and tRNA) or 80 μg (for HO, CDC6, and PCL2) of total yeast RNA in 10 μl of H_2O. The annealing reaction and S1 digestion reaction was described (Belotserkovskaya et al., 2000). The samples were analyzed on 10% SDS-PAGE, followed by autoradiography and representative experiments are shown. Quantitation was performed by PhosphorImager (Molecular Dynamics). RNA was prepared a minimum of three times, and each experiment was performed in duplicate. Oligonucleotides complementary to the genes assayed by S1 nuclease analysis are as follows: HIS3, 5′-GGTTTCATTTGTAATACGCTTTACTAGGGCTTTCTGCTCTGTCAT CTTTGCCTTCGTTTATCTTGCCTGCTCATTTT-3′; tRNA, 5′-GGAAT TTCCAAGATTTAATTGGAGTCGAAAGCTCGCCTTA-3′; HO, 5′-GCC CTGTGTGACATTTATGACGCGGGCAGCGGAGCCATCTGCGCACA TAACGTAAGAGTTAGCCCACCGC-3′; CDC6, 5′-CATTCAGATCTT GGAAGGAATCGAAAATCTTCTGGAAAATGGAGGACGGCTCTCCC AACGGCGCGT-3′; PCL2, 5′-GATAAATTCGATCTTCTAGATTCCAA CGTGGAGAGGGTC GAAGTGGAAGCTAAAGATGGGCGCTCA-3′.

HAT Complex and Kinase Complex Purification

Nucleosomal HAT complexes were prepared as previously described (Grant et al., 1997). Peak Mono Q fractions of ADA, NuA4, NuA3, and SAGA were determined by HAT assay and Western analysis. Peak Mono Q fractions of yeast H3 S10 kinase complex were further purified using Superose 6 HR 10/30 column chromotography (Pharmacia), and kinase activity was determined by Western analysis using anti-H3S10-phosphorylated antibody.

HAT and Kinase Assays

Liquid HAT assays were modified from previously described methods (Grant et al., 1997): 1 pmol (20 ng) of enzyme sample and either 1.5 nmol peptide or 10 μg free histone were incubated 2–30 min in HAT buffer (pH 7.6). Reactions were stopped by pipetting 30 μl onto a square of P81 filter paper (Whatman), then washed four times in 50 mM $NaHCO_3/Na_2CO_3$ (pH 8.5), and once in acetone. Incorporated counts were determined by scintillation counting. Free core histones were calf thymus (Sigma). Histone H3 peptides were from the Protein Chemistry Core Facility at the Baylor College of Medicine. Peptides were lyophilized to dryness and stored at −70°C and then resuspended in an equal volume of deionized, distilled water before use.

Kinase assays were performed in 30 μl volume containing 50 mM HEPES (pH 7.5), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM DTT, 2 mM sodium orthovanadate, 10 μCi [(3000 ci/mmol)γ-^{32}P]ATP, and 50 μM peptide substrate (30°C for 30 min). The samples were electrophoresed on 20% SDS-PAGE, dried, and exposed to X-ray film. Kinases were: a partially purified yeast H3-Ser-10 kinase complex (W.-S. L., unpublished data), c-AMP dependent protein kinase (PKA; New England Biolabs), and RSK2 kinase (Upstate).

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