THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 1, Issue of January 7, pp. 104–111, 2005
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Printed in U.S.A.

Histone H2A and Spt10 Cooperate to Regulate Induction and Autoregulation of the *CUP1* Metallothionein*

Received for publication, October 7, 2004
Published, JBC Papers in Press, October 21, 2004, DOI 10.1074/jbc.M411437200

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Copper is an essential cellular cofactor that becomes toxic at high levels. Copper homeostasis is tightly regulated by opposing mechanisms that control copper import, export, and copper binding capacity within the cell. High levels of copper induce the expression of metallothioneins, small sulfhydryl-rich proteins with high metal binding capabilities that serve as neutralizers of toxic levels of metals. In yeast, the *CUP1* gene encodes a copper metallothionein that is strongly induced in response to metals and other stress and is subsequently rapidly down-regulated. Activation of *CUP1* is mediated by the copper-responsive transcriptional activator AceI, and also requires the histone acetylase Spt10 for full induction. We have examined the role of histone H2A in the normal regulation of the *CUP1* gene. We have shown that specific H2A mutations in combination with *spt10* deletions result in aberrant regulation of *CUP1* expression. Certain lysine mutations in H2A alleviate the transcriptional defect in *spt10*Δ strains, though *CUP1* activation is still delayed in these mutants; however, *CUP1* shutdown is normal. In contrast, serine mutations in H2A prevent *CUP1* shutdown when combined with *spt10* deletions. In addition, *swi/snf* mutants exhibit both impaired *CUP1* induction and failure to shut down *CUP1* normally. Finally, different Spt10-dependent histone acetylation events correlate with induction and shutdown. Taken together, these data indicate that *CUP1* transcriptional shutdown, like induction, is an active process controlled by the chromatin structure of the gene. These results provide new insights for the role of chromatin structure in metal homeostasis.

All cells must be capable of responding rapidly and specifically to a variety of external stressors, such as sudden changes in temperature, oxygen starvation, DNA-damaging agents, or heavy metals. Metals such as copper, iron, and zinc are essential in enzyme catalysis, protein structure, and respiration. However, these key elements become toxic at high levels. Metal ion homeostasis is tightly controlled, with excess ions exported or sequestered while maintaining sufficient cellular stores to satisfy the cofactor requirements of essential enzymes. Much of this regulation occurs at the level of transcription. The yeast *Saccharomyces cerevisiae* is a powerful model for understanding cellular mechanisms of metal ion homeostasis (1–3).

All transcription occurs in the context of chromatin, a complex structure in which the DNA is wrapped around histone octamers to form nucleosomes. The nucleosome consists of approximately two turns of DNA wrapped around an octamer of histone proteins. Two copies each of histones H2A, H2B, H3, and H4 comprise the core histone octamer. Each core histone contains an N-terminal domain that extends from the surface of the octamer, histone H2A has an additional C-terminal domain. The histone tails are the targets of the majority of the known post-translational modifications of the core histones. These modifications include lysine acetylation and methylation, serine phosphorylation, ubiquitination, sumoylation, and ADP-ribosylation (4–6).

Tail modifications of histones H3 and H4 have been extensively characterized and have been shown to be critical in multiple cellular processes, particularly in transcription (7–9). In contrast, histone H2B modifications have only recently been implicated clearly in transcription (10–12) and DNA double strand break repair (13), and histone H2A/H2AX modifications have primarily been investigated in the process of DNA double strand break repair (14, 15). In one case yeast H2A modification has been implicated in transcription: specific H2A modification sites are required for normal gene silencing at telomeres (16). We have investigated the role of the H2A tails in the regulation of the copper-inducible *CUP1* gene.

The yeast *CUP1* gene encodes a copper metallothionein, which is strongly and rapidly induced in the presence of copper (17). *CUP1* is an excellent model for understanding the role of histone modifications in inducible gene expression. The chromatin organization of the *CUP1* promoter has been extensively characterized (18, 19). In the uninduced state, nucleosomes are organized into defined clusters of positions. The binding of copper to the activator Ace1p leads to its folding and subsequent binding to UASs in the *CUP1* promoter. Ace1p binding results in the repositioning of nucleosomes throughout the *CUP1* promoter and open reading frame, potentially via recruitment of a chromatin remodeling enzyme, leading to increased promoter accessibility. Subsequently, histones H3 and H4 are acetylated (20, 21). This acetylation is dependent on the Spt10p acetyltransferase (21). Spt10p is required for maximal levels of *CUP1* induction, but *CUP1* expression is not eliminated in *spt10* deletion strains. One possibility is that Spt10-dependent acetylation is partially redundant with the putative remodeling activity. Alternatively, other histone modifications in addition to Spt10-dependent acetylation may be important for *CUP1* activation.

We have investigated the roles of the H2A tails in *CUP1* regulation, and have studied the interaction between H2A and Spt10 in regulation of *CUP1*. We show that N- and C-terminal mutations in H2A impair *CUP1* induction in response to copper. Furthermore, H2A C- and N-terminal mutations combined with *spt10* deletions result in striking alterations in the kinetics of *CUP1* regulation. Specific lysine, serine, and threonine mutants impair and/or delay *CUP1* activation to varying degrees in *spt10*Δ strains. Even more strikingly, particular serine

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mutants prevent normal shutdown of *CUP1*, suggesting that an active process of autoregulation is perturbed in these strains that may depend on histone phosphorylation. Consistent with this, phosphorylation of H2A Ser-129 is detected at the *CUP1* promoter coincident with down-regulation. Finally, *CUP1* kinetics are similarly perturbed in a *swi/snf* mutant strain. Taken together, these data indicate that active chromatin modification/remodeling is involved in both the activation and autoregulation of *CUP1*, facilitating the dynamic control of copper homeostasis.

EXPERIMENTAL PROCEDURES

Yeast Strains, Plasmids, and Knockout Constructs—Most strains used in this study are derived from FY406 (*MATa, hta1-htb1::LEU2, hta2-htb2::TRP1, leu2-1, ura3-52, trp1-63, his3-200*) and contain either the wild-type *HTA1-HTB1* locus on a *CEN-ARS* plasmid (*URA3*, JKY28; or *HIS3*, JKY38), or a mutant *hta1* allele + wild type *HTB1* on a *CEN-ARS* plasmid (*HIS3*). The *swi1*Δ strain (JKY20) has the genotype *MATα, swi1::LEU2, lys2-801, ade2-101, leu2-1, his3-200, ura3-99*). The isogenic wild-type strain for the *swi1*Δ strain behaved equivalently to the FY406-derived wild-type strain; the wild-type data reported are from the FY406-derived strain.

Mutant *hta1* alleles were generated using the QuikChange™ site-directed mutagenesis kit (Stratagene). Mutant *hta1* alleles are exchanged for the wild-type allele by a plasmid shuffle method, in which the strain carrying the *URA3*-marked *HTA1-HTB1* plasmid (JKY29) is transformed with the *HIS3*-marked plasmid containing the mutant *hta1* allele. HIS+ transformants that have lost the wild-type *HTA1* plasmid are selected for using medium supplemented with 5-FOA, which selects against cells expressing *URA3*. *SPT10* was deleted directly in strains containing *hta1* alleles (*HIS3* marked) using a *URA3* gene flanked by 50 bp of *SPT10* homology. This construct was generated via PCR using the following primers to amplify *URA3* from pRS406: 5′-GGATGAGTAAAAAGTGTACC GATCAAGAACAACTCTAGACCT-GTGCGGTATTTCACACCG-3′ and 5′-TATTAGAATTGTGCTCTG-TAAATGTATTAGATTCGTTGTTAAGATTGTACTGAGAGTGCAC-3′.

CUP1 Induction and Detection—To induce *CUP1*, yeast strains are grown to mid-log phase (OD_{600} of ~0.5–0.7) in YEPD, then $CuSO_4$ is added to a final concentration of 1 mM. Cells are incubated at 30 °C for the indicated times, then harvested, and total RNA is isolated using glass bead lysis. Northern blots are performed by standard methods. Signal is detected using a Cyclone Phosphorimager. All blots are simultaneously probed for *ACT1* as a loading control, and data are normalized by dividing the *CUP1* signal by the *ACT1* signal.

Chromatin Immunoprecipitation (ChIP)[1]—ChIPs were performed as described previously (22), except that the quenching step was not included. Cross-linked, sonicated chromatin was immunoprecipitated using antibodies against either acetylated histone H3 (K9 or K14) or hyperacetylated histone H4 (Upstate Biotechnologies). Input and immunoprecipitated material was detected by slot blot.

RESULTS

H2A Tails Are Involved in CUP1 Induction—CUP1 transcription is induced 10–20-fold within 5 min of addition of 1 mM copper. *CUP1* subsequently autoregulates its own expression, and transcription levels drop to a much lower steady-state level by 30–40 min after copper exposure (23, 24). We initially measured *CUP1* expression at three points: in the absence of copper, at 10 min after addition of 1 mM $CuSO_4$, when expression is maximal for wild type, and at 30 min after addition of $CuSO_4$, when *CUP1* is down-regulated. The amount of *CUP1* mRNA is detected by Northern blot and quantitated by phosphorimager. All *CUP1* expression levels are normalized to expression of *ACT1* as a loading control. The average normalized data for at least three independent experiments are presented in each graph, and standard errors are shown.

We have analyzed *CUP1* expression in strains containing a variety of alanine substitutions in the H2A C and N termini (Fig. 1). Most single mutations in the H2A C terminus have minimal effects on *CUP1* induction at 10 min, though alanine substitutions at lysines 120 or 121 show a 2-fold reduction in *CUP1* levels at 10 min (Fig. 1*A*). However, most C-terminal mutations show a reduction in levels of *CUP1* at 30 min after copper addition (Fig. 1*A*).

Because *CUP1* expression changes so rapidly, we confirmed these effects by measuring *CUP1* levels every 5 min in several of these strains, in case we had missed a peak of expression. Fig. 1*B* shows the results of the short time course for two H2A lysine mutants, *htaK120A* and *htaK127A*, compared with wild type. *CUP1* induction is rapid and strong in the wild-type strain, with strong signal within 5 min of copper addition, reaching maximum induction levels by 10 min, and beginning to decrease by 25–30 min (Fig. 1*B*, *top panel*). Both *htaK120A* (*middle panel*) and *htaK127A* (*bottom panel*) exhibit similar induction kinetics compared with wild type, while showing a *CUP1* induction level consistent with that shown in Fig. 1*A*; *i.e.* a 2-fold reduction in *CUP1* mRNA in the *htaK120A* strain *versus* nearly wild-type levels in the *htaK127A* strain.

We have also examined the effects of mutations in the H2A N terminus on *CUP1* expression. This tail appears to play a role in *CUP1* induction comparable to the C terminus, in that alanine substitutions at serine and lysine residues in the N terminus result in reduced levels of *CUP1* mRNA. Induction data for two of these mutants, *htaS2A* and *htaK5A*, are shown in Fig. 1*C*. Both of these strains exhibit ~40% reduction in *CUP1* mRNA levels compared with wild type. Similar results are obtained for other residues in the N terminus (data not shown).

These results indicate that both the N- and C-terminal tails of histone H2A are required for normal induction of *CUP1*. Because all of the residues chosen are sites of potential modification (lysines, serines, and threonine), an obvious question is whether specific modifications of these residues are involved in *CUP1* induction. Indeed, substitution of a glutamine for the lysine at position 127 (a substitution commonly used to mimic lysine acetylation) appears to alleviate the minor *CUP1* induction defect of the alanine mutation at this site (data not shown). Since it is already known that modifications of histones H3 and H4 also occur during *CUP1* induction (20, 21), it will be interesting to determine whether H2A modifications do occur, and cooperate with H3/H4 modifications at *CUP1*.

Different Histone Modifications Coincide with CUP1 Induction and Autoregulation—Previous work has shown that the Spt10 histone acetyltransferase is required for normal induction of *CUP1* (21). *spt10*Δ mutants exhibit delayed induction, reduced levels of *CUP1* mRNA, and sensitivity to high levels of copper. These mutants also result in loss of acetylation of histones H3 and H4 at *CUP1*. We wished to understand the different contributions of Spt10 and H2A to *CUP1* regulation. To address this, we knocked out the *SPT10* gene in strains containing different mutations in H2A. In looking at genetic interactions between H2A and *SPT10*, it is important to note that Spt10 itself is involved in transcription of certain histone genes (25, 26). Spt10 is required for expression of the *HTA2-HTB2* locus; fortunately, our mutant histones are expressed solely from *HTA1* promoters, and their expression is not impacted by the deletion of *SPT10*.

We first compared the expression of *CUP1* in *spt10*Δ cells in the presence of wild-type H2A. Consistent with previous reports (21), we observe both delayed induction and significantly reduced levels of *CUP1* mRNA in the absence of Spt10 (Fig. 2*A*).

It has been shown that histones are acetylated at *CUP1* upon exposure to copper (20, 21), and that this acetylation requires Spt10 (21). However, in another study, AceI-dependent *CUP1*

[1] The abbreviation used is: ChIP, chromatin immunoprecipitation assay.

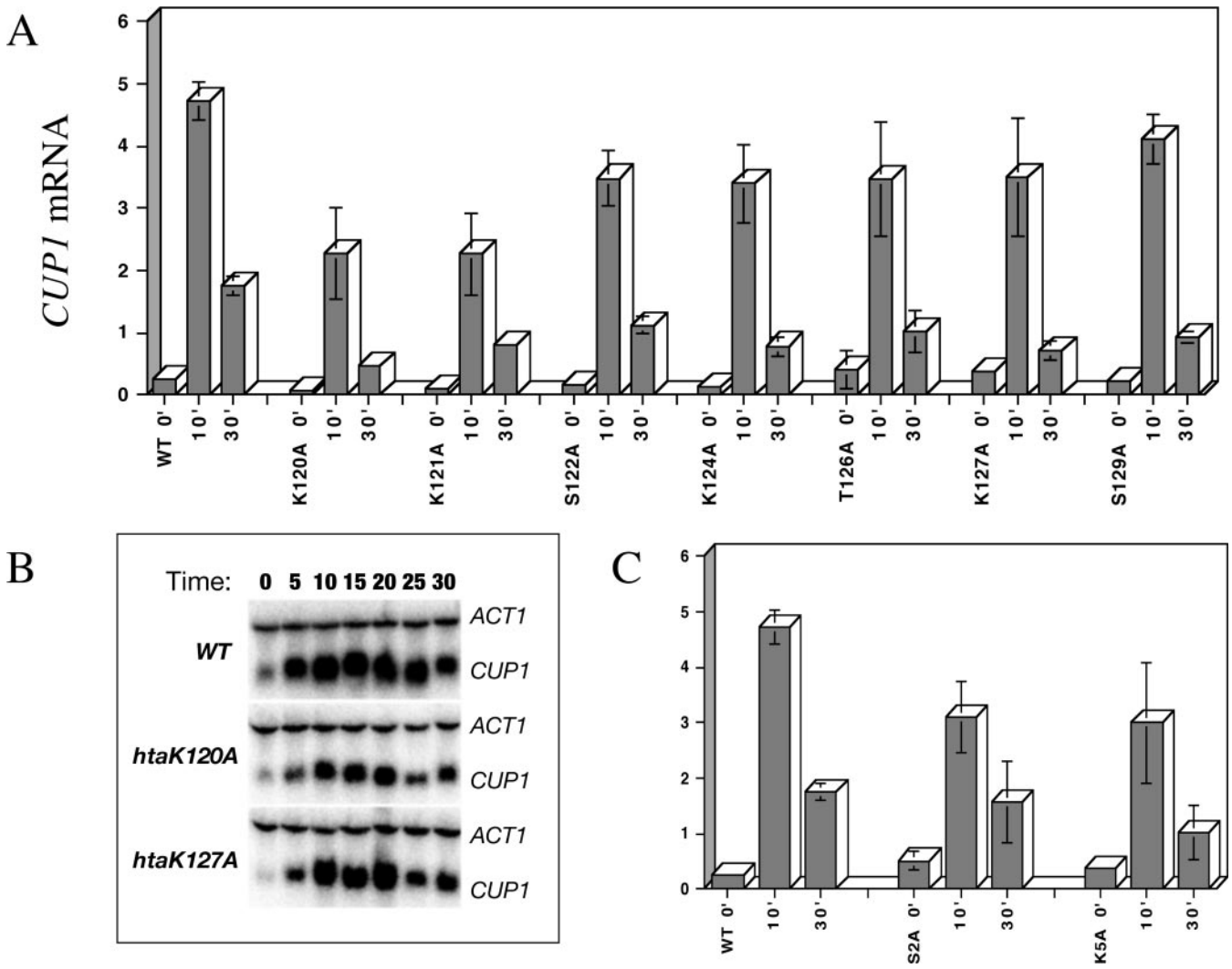

FIG. 1. **Histone H2A point mutations impair *CUP1* induction.** *A*, average *CUP1* mRNA levels in the absence of copper (0′), and 10′ and 30′ after addition of 1 mM $CuSO_4$, for wild-type cells and H2A C-terminal point mutants. Northern blots were quantitated by phosphorimager. Each *bar* represents the average of at least three independent experiments, and standard errors are shown. Amounts of *CUP1* mRNA are normalized to the amount of *ACT1* mRNA as a control for loading. *B*, Northern blots of *CUP1* inductions, showing *CUP1* mRNA levels every 5 min after copper addition for wild type (*top*), *htaK120A* (*middle*), and *htaK127A* (*bottom*). *C*, average *CUP1* mRNA levels as in *A*, for two H2A N-terminal point mutants.

induction resulted in no change in H3 acetylation and a decrease in H4 acetylation (27). These differences could be attributable to different strain backgrounds used in these studies. However, another possibility is that histone modifications at *CUP1* are as dynamic as *CUP1* expression itself, and these different studies were measuring histone acetylation at different relative time points in the *CUP1* expression cycle.

We have performed ChIPs to measure the dynamics of acetylation of histones H3 and H4 in the strains shown in Fig. 2*A*, both to confirm Spt10-dependent acetylation in these strains, and to determine the dynamics of histone acetylation during *CUP1* induction. The results of three independent ChIP experiments are shown in Fig. 2*B*, expressed as the level of immunoprecipitated material normalized to the input levels. These results show that Spt10-dependent H3 and H4 acetylation does occur at *CUP1* in these strains. However, the kinetics of H3 and H4 acetylation are quite different in the wild-type strain. Acetylation of H3 (H3ac9/14) occurs within 10 min of copper addition, coinciding with maximum *CUP1* expression, and decreases coincident with *CUP1* down-regulation (Fig. 2*B*, *top panel*). The extent of H3 acetylation at 10 min is consistent with that observed at a similar time point (15 min) by Shen *et al.* (21). In contrast, H4 acetylation (measured at all sites, H4hyper) shows a decrease 10 min after copper addition, and a striking increase at 30 min after copper addition, when *CUP1* transcription is down-regulated (Fig. 2*B*, *bottom panel*). The decrease in H4 acetylation after 10 min resembles that observed at 15 min by Deckert and Struhl (27). These results suggest that some of the discrepancies seen in previous studies could be explained by the dynamic changes in acetylation during *CUP1* expression.

These results also indicate that opposing acetylation and deacetylation may be involved in both the activation and subsequent down-regulation of *CUP1*. If Spt10 is responsible for acetylation at both stages of *CUP1* regulation, this protein may be in the unique position of mediating both induction and autoregulation of a single gene. Spt10 has been implicated in both gene activation and repression (25, 28–30), but never for the same gene. We therefore decided to further explore the role of Spt10 in *CUP1* regulation.

*spt10*Δ*-hta1 Double Mutants Alter CUP1 Expression Kinetics*—The results shown in Figs. 1 and 2 indicate that both H2A and Spt10 are involved in the normal induction of *CUP1*. We decided to examine the effect of combining H2A mutations with *spt10* deletion. We selected a number of strains containing H2A mutants in either the C or N terminus, and deleted the *SPT10*

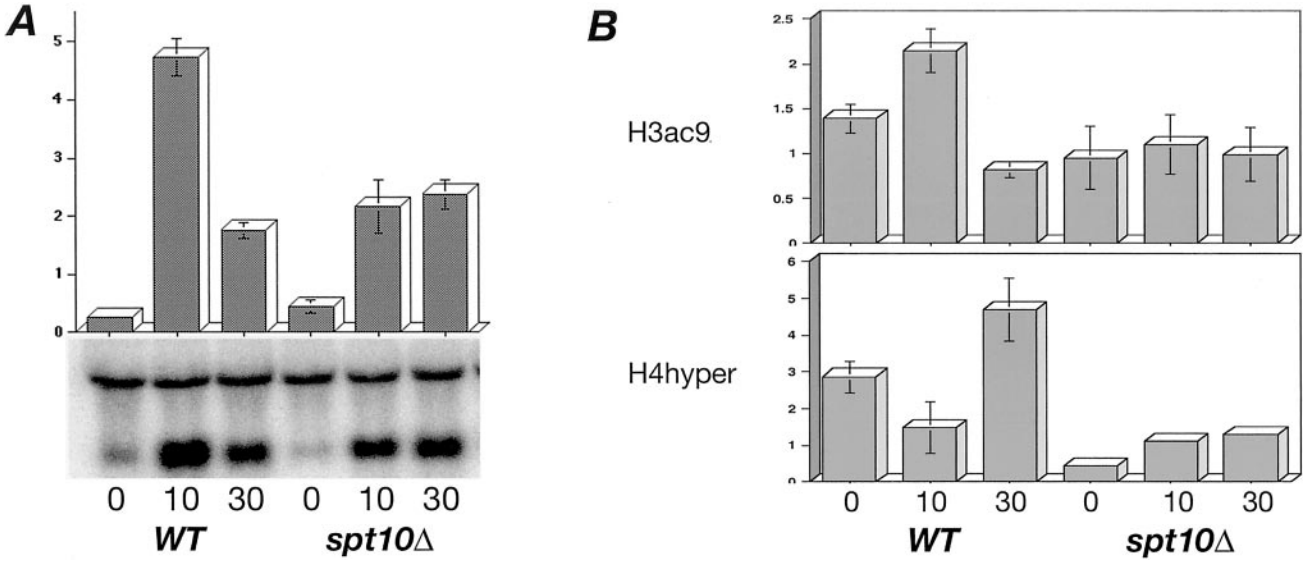

FIG. 2. **Spt10-dependent histone modifications vary with *CUP1* expression kinetics.** *A*, average *CUP1* mRNA levels in the absence of copper (0), and 10′ and 30′ after addition of 1 mM $CuSO_4$, for wild-type cells and *spt10*Δ cells. A representative Northern blot is shown below the graph. Quantitation and normalization of average data are as described in Fig. 1. *B*, ChIPs measuring the levels of histone H3K9 acetylation (*top panel*) and histone H4 acetylation (*bottom panel*) in wild type (*left side*) *versus spt10*Δ (*right side*). Enrichment of the *CUP1* promoter in immunoprecipitated material was detected by slot blot, quantitated by phosphorimager, and normalized to input levels. *Bars* represent ChIP data from three independent copper-treated samples, and standard errors are shown.

gene in these strains. All of the double mutants were viable, though certain combinations (*spt10*Δ combined with *htaS2A*, *htaS122A*, *or htaS129A*) exhibit slow growth phenotypes; in these cases each of the single mutants shows moderately slow growth compared with wild type, which appears to be additive when these mutations are combined.

Combining *spt10*Δ and H2A mutations results in surprising changes in the patterns of *CUP1* expression (Fig. 3). There were changes in the maximum levels of *CUP1* mRNA, and in the autoregulation of *CUP1* transcription. The most striking trend is that in every case, *CUP1* transcription fails to shut down at 30 min after copper addition. In several instances, *CUP1* mRNA levels are still increasing at 30 min.

It has been proposed that the shutdown of *CUP1* is due to the binding of copper by the Cup1p metallothionein, which results in the release of Ace1p from the *CUP1* UAS (31). This mechanism of autoregulation predicts that *CUP1* shutoff simply requires that a sufficient level of Cup1p be synthesized to compete with Ace1p for copper binding. However, several of the mutants shown in Fig. 3 clearly accumulate large amounts of *CUP1* mRNA without shutting down *CUP1* expression. This suggests that *CUP1* autoregulation may instead be a more active process dependent on Spt10 and/or H2A.

To explore this possibility, we performed more extensive analyses of the kinetics of *CUP1* expression in the *spt10-hta1* double mutant strains. We took samples for Northern analysis every 10 min after copper addition, for 1–2 h. The results of 1 h time course are shown in Fig. 4. Representative blots are shown on the left, and the averages of at least three independent experiments are presented in the graphs on the right.

The results from the long time course confirmed that *CUP1* regulation is significantly altered in these strains, and further revealed different patterns of expression in different mutants.

Fig. 4*A* shows that significant (though not complete) *CUP1* shutoff occurs with only a slight delay in the *spt10*Δ strain, despite the low levels of *CUP1* mRNA actually produced. This is consistent with the severe sensitivity to copper exhibited by *spt10*Δ cells (21), in that these cells do not appear to synthesize sufficient *CUP1* mRNA for protection from the excess copper.

When the *spt10* deletion is combined with mutations in H2A, the delay in induction observed in *spt10*Δ cells is preserved; however, most of these double mutants produce wild-type levels of *CUP1* mRNA. Fig. 4*B* shows the results for *spt10*Δ-*htaK5A* and *spt10*Δ-*htaK127A*, which combine the *spt10* deletion with a lysine-to-alanine mutation in each tail of H2A. Both of these strains show a peak of *CUP1* expression at 20 min, coincident with the peak of expression in the *spt10*Δ mutant alone. However, these strains produce wild type or nearly wild-type levels of *CUP1* mRNA, and exhibit a normal pattern of autoregulation. Spt10 (presumably via Spt10-dependent histone acetylation) is required for rapid induction of *CUP1* within 5–10 min of copper exposure, and this rapid response is still absent in the double mutants. However, elimination of a positive charge on either H2A tail appears to alleviate the defect in the level of *CUP1* induction in *spt10*Δ, possibly by mimicking an acetylation event that can partially substitute for an acetylation event that has been lost.

In contrast, the *spt10*Δ-*htaT126A* double mutant exhibits kinetics indistinguishable from *spt10*Δ alone (Fig. 4*C*). The nearby *htaS129A* mutation combined with *spt10*Δ, however, exhibits delayed (and incomplete) *CUP1* shutoff, despite the accumulation of levels of *CUP1* mRNA comparable to wild type.

The most striking misregulation of *CUP1* occurs in *spt10*Δ-*htaS2A* and *spt10*Δ-*htaS122A* strains, shown in Fig. 4*D*. These strains show delayed induction (peaking at 20–30 min), but achieve wild-type levels of induction. What is surprising about these mutants is that they completely fail to autoregulate *CUP1*, but rather continue to express *CUP1* at close to peak levels. This level of expression continues for at least 2 h (data not shown and Fig. 5, *below*).

These results strongly suggest that an active mechanism for *CUP1* shutoff has been lost in these mutants. This regulatory mechanism is presumably not dependent on Ace1p displacement due to copper binding by Cup1p. The accumulation of *CUP1* mRNA in these mutants far exceeds that of wild-type cells at the time of *CUP1* shutoff in wild type; presumably a concomitant accumulation of Cup1p metallothionein also occurs. It is formally possible that translation of *CUP1* mRNA is impaired in these mutants, though there is no general translational defect apparent in these mutants (with or without

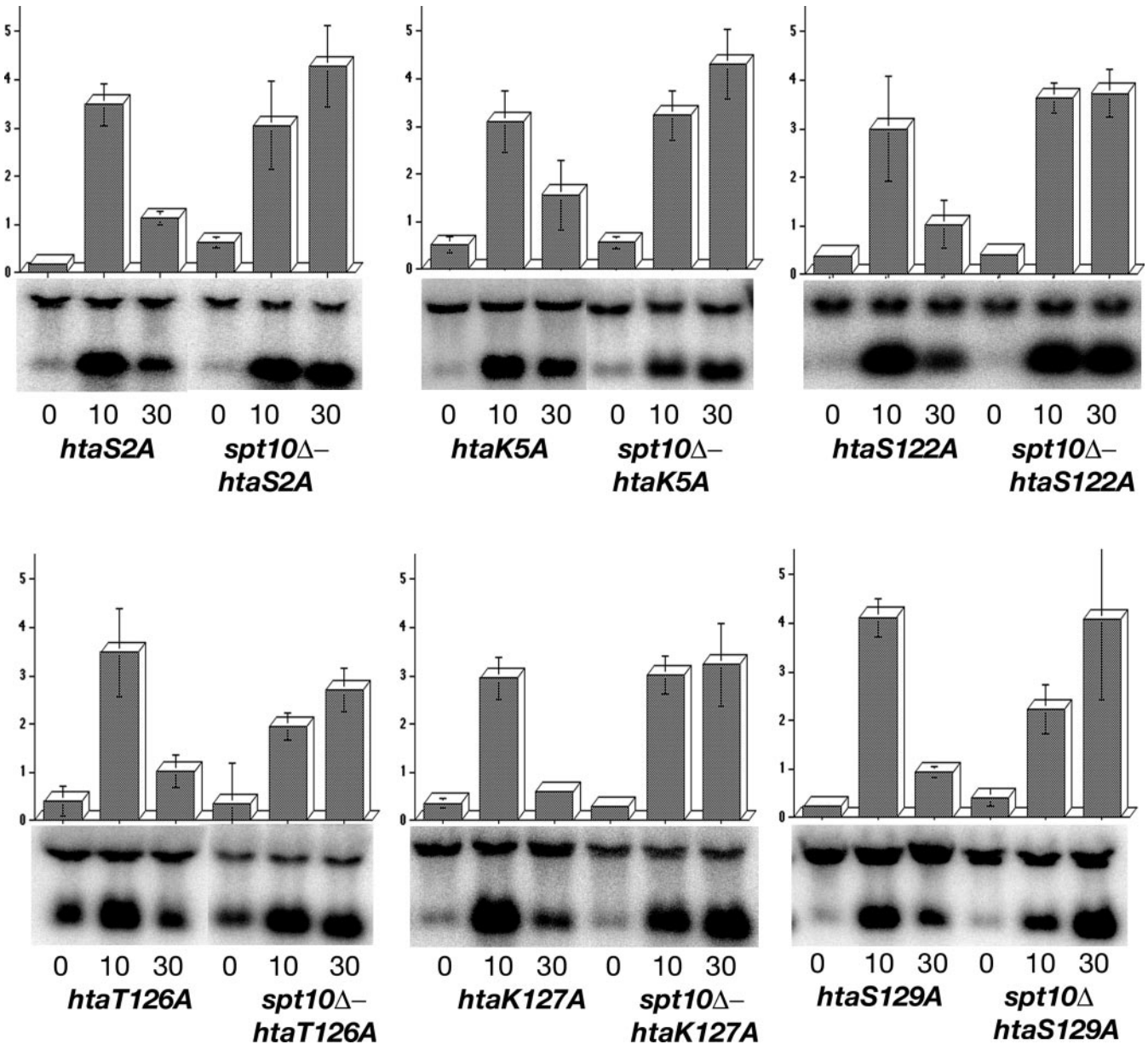

FIG. 3. **Deletion of *SPT10* leads to altered *CUP1* expression kinetics in strains bearing H2A point mutants.** Each panel shows the comparison of *CUP1* expression in H2A point mutants alone to H2A point mutants combined with *spt10* deletions. Representative Northern blots are shown below each graph. Quantitation and normalization of average data are as described in the legend to Fig. 1. All graphs have the same scale as the graph shown in Fig. 2*A* for ease of comparison.

copper) when total proteins are run on SDS-PAGE (data not shown). Cup1p protein itself is known to be quite stable, persisting well after *CUP1* transcription shutdown (24).

What might be the mechanism of this active shutoff? Several possibilities present themselves. A specific repressor could bind to *CUP1* and displace RNA polymerase or other positive factors (besides Ace1p) required for sustained transcription. Deacetylation of Spt10-acetylated histones H3 and H4 could be required to reset the "off" state for *CUP1*. A chromatin remodeler could act to return the nucleosomes at *CUP1* to the positions normally occupied in the uninduced state. Whatever the mechanism, it is clear that this shutoff can be mediated by Spt10 or by serines in the H2A tails; loss of either alone does not impair shutoff, but the double mutants can. Any of the activities proposed could, for example, be recruited by Spt10p and stabilized at *CUP1* by binding to (phosphorylated?) serines in H2A.

swi/snf Mutants Also Alter CUP1 Expression Kinetics—One explanation for the loss of autoregulation in certain *spt10*Δ-*hta1* double mutants is that Spt10 and H2A (possibly phosphorylated H2A) are required to recruit chromatin remodeling activity to "reset" the *CUP1* chromatin to the off state. To test this possibility, we measured *CUP1* expression in a *swi/snf* mutant strain.

It has been shown previously that the SWI/SNF complex is not required for histone H3 acetylation at the *CUP1* promoter, and that significant *CUP1* transcription is detected at 30 min after copper addition in *swi2*Δ cells (20). However, this earlier study did not examine the kinetics of *CUP1* regulation. We have therefore extended these earlier studies to measure both the induction and autoregulation of *CUP1* in a *swi1*Δ strain, shown in Fig. 5.

Surprisingly, *CUP1* expression in the *swi1*Δ strain reveals kinetics reminiscent of a combination of both the *spt10*Δ strain and the *spt10*Δ-*htaS2A*, *spt10*Δ-*htaS122A* strains (Fig. 5*A*; compare with Fig. 4). *swi1*Δ cells exhibit delayed *CUP1* induction and reduced overall levels of *CUP1* mRNA, similar to that

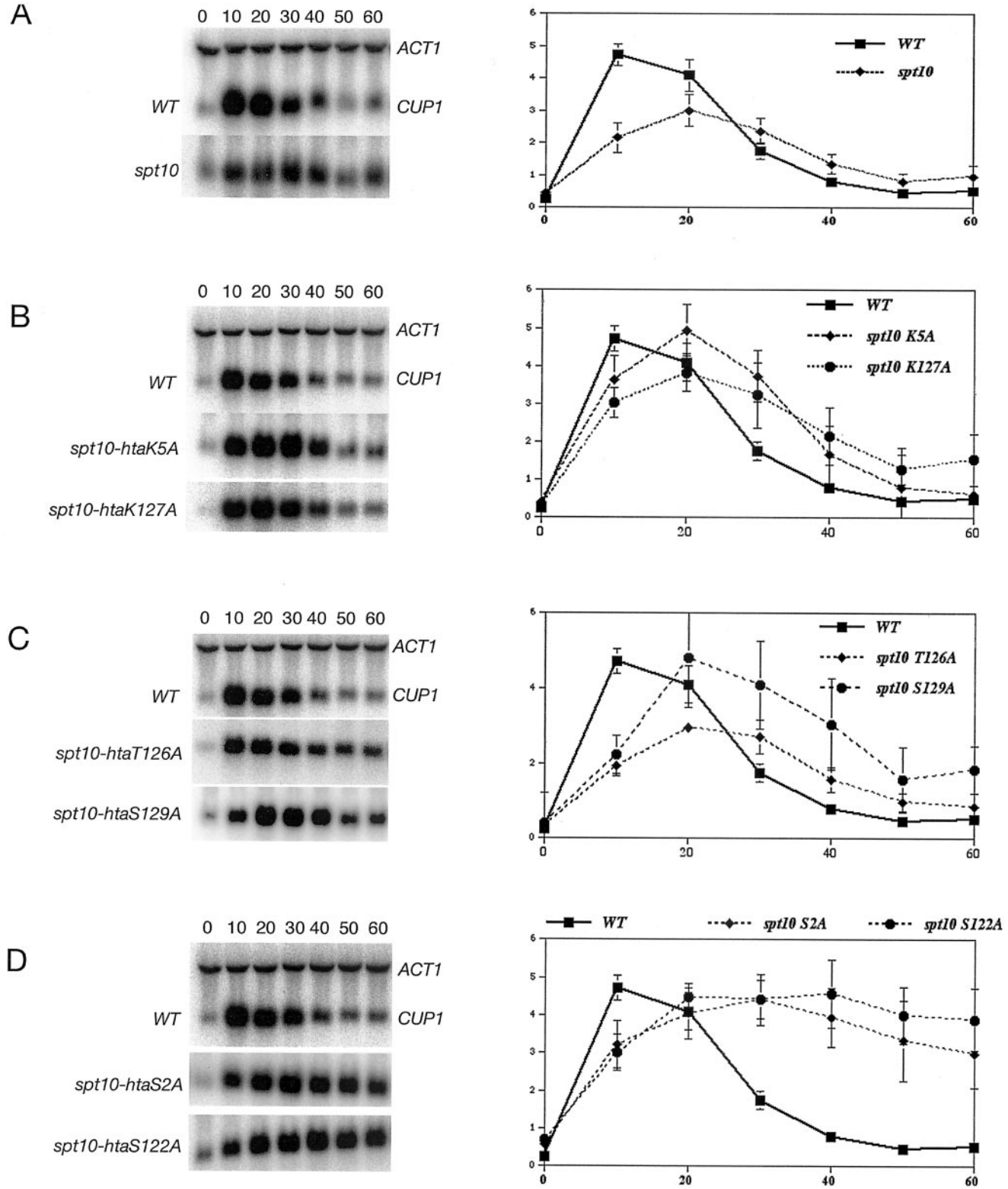

FIG. 4. ***spt10* deletion in H2A mutant strains affects both induction and shutdown of *CUP1*.** 60-min time courses of *CUP1* inductions are shown; representative Northern blots are shown on the *left*, average mRNA levels for 3 or more independent experiments are graphed on the *right*. Quantitation and normalization of average data are as described in the legend to Fig. 1. *A*, wild type (*squares*) *versus spt10*Δ (*diamonds*). Note that while *spt10*Δ cells show delayed induction and lower overall levels of *CUP1* mRNA, down-regulation of *CUP1* similar to wild type occurs in this strain. *B*, wild type (*squares*) *versus spt10*Δ-*htaK5A* (*diamonds*) and *spt10*Δ-*htaK127A* (*circles*). *C*, wild type (*squares*) *versus spt10*Δ-*htaT126A* (*diamonds*) and *spt10*Δ-*htaS129A* (*circles*). *D*, wild type (*squares*) *versus spt10*Δ-*htaS2A* (*diamonds*) and *spt10*Δ-*htaS122A* (*circles*). Note the failure of these strains to down-regulate *CUP1* expression.

seen for *spt10*Δ cells. However, as is the case for *spt10*Δ-*htaS2A* and *spt10*Δ-*htaS122A* strains, the *swi1*Δ cells also fail to shut off *CUP1* transcription. This suggests two roles for SWI/SNF in *CUP1* regulation. First, SWI/SNF may be involved in the initial Ace1p-dependent repositioning of nucleosomes that occurs during *CUP1* induction (19). This repositioning is independent of Spt10-dependent histone acetylation (21); therefore, Spt10-dependent acetylation and nucleosome repositioning may be par-

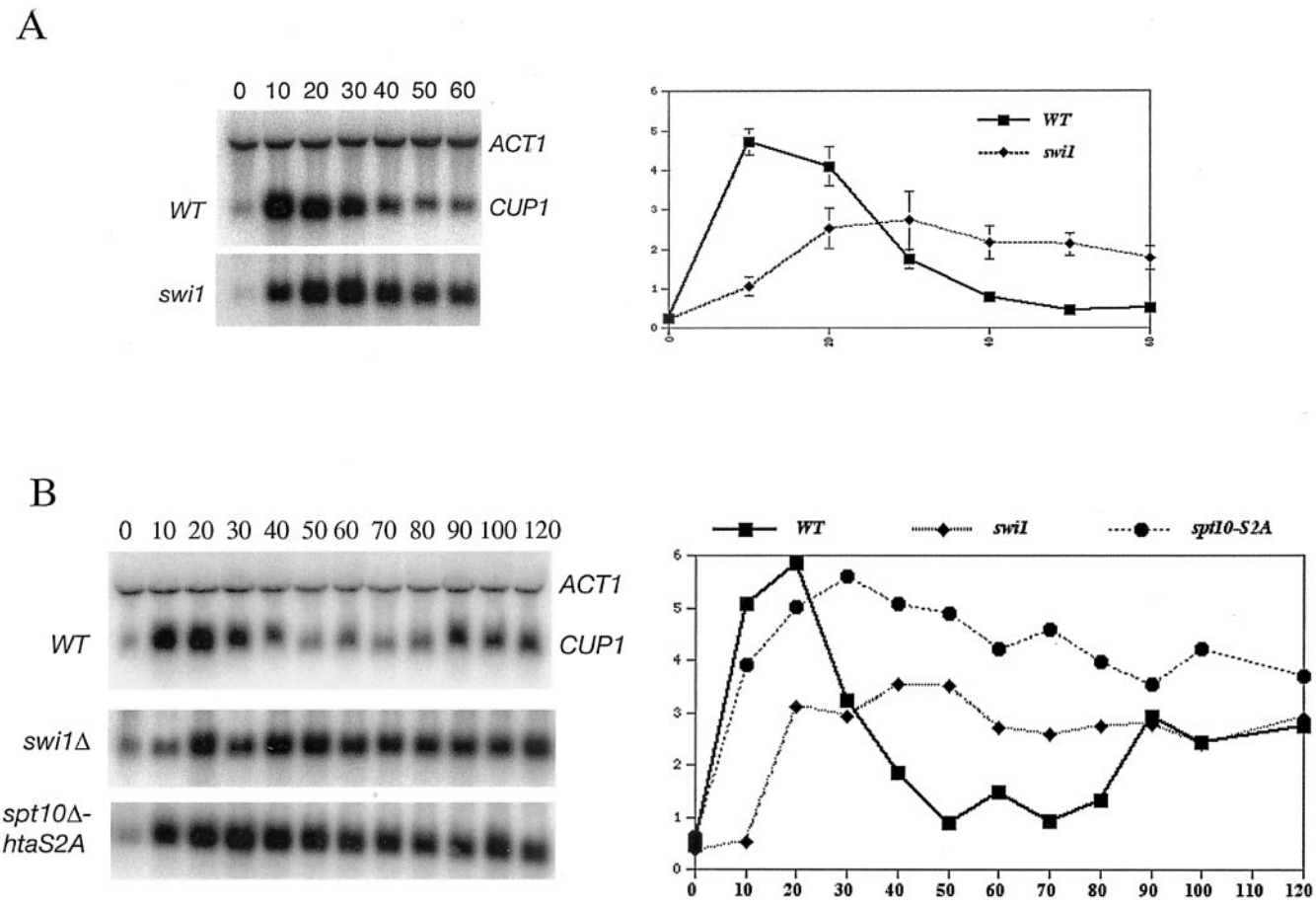

FIG. 5. ***swi1*Δ deletion also alters *CUP1* expression kinetics.** *A*, 60-min time course comparing wild type (*squares*) and *swi1*Δ *CUP1* mRNA levels. Representative Northern blots are shown on the *left*, average mRNA levels for 3 or more independent experiments are graphed on the *right*. Quantitation and normalization of average data are as described in the legend to Fig. 1. *B*, 120-min time courses for wild type (*squares*), *swi1*Δ (*diamonds*), and *spt10*Δ-*htaS2A* (*circles*). The graph is a quantitation of the Northern blots on the *left*.

tially redundant mechanisms facilitating access to the *CUP1* promoter. Loss of either mechanism delays induction and reduces transcription efficiency; we predict that loss of both mechanisms might result in failure to induce *CUP1*. Experiments are currently underway to test this prediction.

The results shown in Fig. 5*A* also suggest a second role for SWI/SNF, in *CUP1* shutoff. While *CUP1* mRNA levels in the *swi1*Δ strain never achieve wild-type levels, the ongoing transcription eventually leads to combined levels of *CUP1* mRNA in excess of wild type. This is even more apparent in 2-h time courses; examples of which are shown in Fig. 5*B*. After ~90 min of copper exposure, wild-type cells begin to reactivate *CUP1,* though at less than half the level of the initial burst of *CUP1* expression (Fig. 5*B*, *top panel*). This is similar to previous observations of the *CUP1* response to 1 mM copper ion (24). In contrast, the *swi1*Δ (Fig. 5*B*, *middle panel*) and *spt10*Δ-*htaS2A* (Fig. 5*B*, *bottom panel*) strains maintain steady-state *CUP1* expression at close to the maximum level achieved by the respective strains. One model to explain these results is that Spt10 is required to recruit SWI/SNF to *CUP1*, while interactions with H2A are required to maintain SWI/SNF at *CUP1*, or perhaps phosphorylation of H2A triggers the "resetting" activity needed to turn off *CUP1* expression.

DISCUSSION

We have shown that the normal kinetics of *CUP1* induction and autoregulation depend on a complex interplay between chromatin modifying enzymes and their histone targets. We have also shown that the shutoff of *CUP1* may be a more active, regulated process than was previously suspected. The fact that different mutations in chromatin modifiers (*spt10*Δ + *hta1 alleles versus swi1*Δ) result in similar alterations in shutdown kinetics argues that chromatin remodeling plays an active role in turning off *CUP1* shortly after its initial activation. Shut off may normally involve the concerted action of several activities: the acetylation of histone H4 by Spt10, the deacetylation of H3, phosphorylation of H2A, and resetting of nucleosome positions by SWI/SNF. Some of these effects could be indirect; for example, SWI/SNF could be required for normal expression or recruitment of a repressor needed for *CUP1* regulation. Detection of SWI/SNF at *CUP1* by ChIP, and measurement of nucleosome positioning during shut off, would support a direct role for SWI/SNF remodeling.

The sensitivity of yeast cells to copper toxicity varies with respect to cell cycle stage, with cells in G_2/M being more sensitive to killing by copper than cells in G_1/S (32). This sensitivity has been linked to variations in basal levels of Cu,Zn-superoxide dismutase (Sod1) activity throughout the cell cycle (33). An alternative explanation for the differences in *CUP1* expression observed in the present study could be that different mutants have different cell cycle rates or different distributions of cell cycle stage occupancies, resulting in differential responses to copper. By monitoring budding, we do observe that a larger percentage of logarithmically growing *spt10*Δ-*htaS2A* cells occupy G_2/M compared with wild type or *spt10*Δ alone (~85% *versus* ~40–60%). Because this is the most copper-sensitive stage of the cell cycle, this suggests that 1 mM copper could be more stressful for this strain, perhaps triggering a specific bypass of the normal *CUP1* autoregulation process.

This "extra" stress could activate non-AceI-dependent pathways, such as the oxidative stress response, which could activate or maintain *CUP1* expression in parallel with the AceI-dependent response. Future experiments will test whether alternate stress pathways are contributing to the response to copper in these strains. We do observe phosphorylation of histone H2A at Ser-129 in copper-treated cells,[2] a modification normally associated with DNA double strand breaks (34). H2A phosphorylation has not been reported for other types of DNA damage; *Drosophila* H2Av, which is phosphorylated in response to double strand breaks, is not phosphorylated in response to oxidative stress (35). It will be important to test whether yeast H2A is phosphorylated at Ser-129 (or Ser-2, Ser-122) in response to oxidative stress *per se*.

Numerous studies have addressed the roles of histone modification and chromatin remodeling in gene silencing or repression, particularly in epigenetic silencing (for some recent reviews, see Refs. 8 and 36–39). However, there have been very few studies of the role of chromatin modifications in the process of shutting down an active gene. For many genes it is clear that shutdown is rapid and regulated, such as for genes transiently expressed during the cell cycle, or genes that are autoregulated by their own products (such as *CUP1*). For genes for which rapid shutdown is important, it is likely that removal of specific activator(s) is only one part of the shutdown process. Besides their roles in repression or silencing, histone deacetylases have been implicated in transcription shutoff/chromatin resetting in some cases (22, 40, 41). One of these studies also implicated SWI/SNF in feedback repression (40). However, a dependence of normal shutdown kinetics on chromatin has not been addressed prior to this study.

One question that arises from this study is what is the role of rapid *CUP1* shutoff in normal copper metabolism? High levels of Cup1 are known to protect cells in high copper conditions; regulated shut off of *CUP1* suggests that overexpression of *CUP1* could have serious repercussions for copper homeostasis. Cells may have evolved a rapid response to high copper levels, followed by a shut down for "reassessment." If high copper levels persist, AceI is reactivated and appropriate levels of *CUP1* transcription can resume. If the initial burst of Cup1 production was sufficient to bind the free copper, then it would be advantageous to not make excess Cup1, which could begin to compete with copper-dependent enzymes for their essential cofactor. This model leads to two possible testable outcomes for the mutants that do not shut down *CUP1* appropriately: 1) that excess copper ion depletion might lead to induction of genes involved in copper uptake (*i.e.* simulation of a copper-starved state), and 2) that these strains might show decreased viability over time, as essential copper reserves are depleted.

This research emphasizes the complexity of copper homeostasis. Defects in copper homeostasis are responsible for serious disease in humans, including diseases of copper overload (Wilson's disease), copper deficiency (Menkes disease), and carcinogenesis (42, 43). Detailed understanding of the regulation of the genes responsible for copper homeostasis provides not only insight into the complex interactions of chromatin remodeling in transcriptional control, but also provides critical understanding of processes essential to human health.

[2] H.-C. Kuo and J. E. Krebs, unpublished data.

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