Molecular Microbiology (2001) **40**(5), 1165–1174

The *Candida glabrata* Amt1 copper-sensing transcription factor requires Swi/Snf and Gcn5 at a critical step in copper detoxification

Keith A. Koch,[1†] Stéphane Allard,[2] Nicholas Santoro,[1] Jacques Côté[2] and Dennis J. Thiele[1*]
[1]*Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, MI 48109-0606, USA.*
[2]*Laval University Cancer Research Center, Hotel-Dieu de Quebec (CHUQ), Quebec City, Canada G1R 2J6.*

Summary

The yeast *Candida glabrata* rapidly autoactivates transcription of the *AMT1* gene in response to potentially toxic copper levels through the copper-inducible binding of the Amt1 transcription factor to a metal response element (MRE) within a positioned nucleosome. Our previous studies have characterized the role of a 16 bp homopolymeric dA:dT DNA structural element in facilitating rapid Amt1 access to the *AMT1* promoter nucleosomal MRE. In this study, we have used the genetically more facile yeast *Saccharomyces cerevisiae* to identify additional cellular factors that are important for promoting rapid autoactivation of the *AMT1* gene in response to toxic copper levels. We demonstrate that the Swi/Snf nucleosome remodelling complex and the histone acetyltransferase Gcn5 are both essential for *AMT1* gene autoregulation, and that the requirement for these chromatin remodelling factors is target gene specific. Chromatin accessibility measurements performed *in vitro* and *in vivo* indicate that part of the absolute requirement for these factors is derived from their involvement in facilitating nucleosomal access to the *AMT1* promoter MRE. Additionally, these data implicate the involvement of Swi/Snf and Gcn5 at multiple levels of *AMT1* gene autoregulation.

Introduction

The transition metal copper (Cu) is both essential and, at high levels, toxic to cells. The redox properties of Cu that make it a suitable cofactor for enzymes such as Cu,Zn superoxide dismutase, lysyl oxidase, cytochrome oxidase and dopamine β-hydroxylase (Linder, 1991), also render Cu a powerful source of cytotoxic reactive oxygen species (Halliwell, 1984). Detoxification of Cu in eukaryotic organisms is primarily achieved through the chelation of excess intracellular Cu by small cysteine-rich proteins known as metallothioneins (Kagi and Schaffer, 1988). In the opportunistic pathogenic yeast, *Candida glabrata*, three metallothionein genes are transcriptionally activated in response to excess Cu (*MT-I*, *MT-IIa and MT-IIb*) (Mehra *et al*., 1989). The Amt1 transcriptional activator is responsible for sensing the cytotoxic Cu threat and responding through the activation of these metallothionein genes. Amt1 has an amino-terminal DNA-binding domain that contains a zinc (Zn) atom and up to four Cu atoms bound in a polynuclear cluster in the activated state (Thorvaldsen *et al*., 1994). However, the Zn atom is not required for DNA binding and, accordingly, Zn does not stimulate transcriptional activation by Amt1. The carboxy-terminus of Amt1 harbours an 'acidic' class transcriptional activation domain.

The Cu-dependent transcriptional autoregulation of the *AMT1* gene is a critical step in the *C. glabrata* Cu detoxification response (Zhou *et al*., 1992). Upon chelating Cu, Amt1 binds to a single metal response element (MRE) within its own promoter to foster rapid and robust transcription autoactivation (Zhou and Thiele, 1993). *C. glabrata* cells that are incapable of autoregulating the *AMT1* gene are hypersensitive to exogenous Cu administration (Zhou and Thiele, 1993). Importantly, the single MRE within the *AMT1* promoter is located in a stably positioned nucleosome, and access to this nucleosomal MRE is facilitated by an adjacent 16 bp homopolymeric dA:dT DNA structural element (A16 element) (Zhu and Thiele, 1996). The A16 element is thought to provide nucleosomal access to the MRE by virtue of its inherent rod-like structure weakening the histone–DNA interactions adjacent to either of its ends (Zhu and Thiele, 1996). We have shown previously that the *C. glabrata AMT1* gene is appropriately autoregulated in response to Cu in *Saccharomyces cerevisiae* (Koch and Thiele, 1999), indicating that any cellular components required for *AMT1* gene autoregulation are conserved between these two yeasts. Using *S. cerevisiae*, we have also shown that the only known yeast poly dA:dT-binding

Accepted 22 March, 2001. *For correspondence. E-mail dthiele@umich.edu; Tel. (+1) 734 763 5717; Fax (+1) 734 763 4581. †Present address: Department of Molecular and Cell Biology, University of California-Berkeley, Berkeley, CA 94720-3206, USA.

protein, Dat1, does not function in *AMT1* autoregulation and that the nucleosomal access provided by the A16 element is not exclusive to Amt1, as it can also provide access to a mammalian transcription factor (glucocorticoid receptor) expressed in yeast (Koch and Thiele, 1999).

In this study, we have investigated whether cellular factors that are known to be involved in activated transcription are important for *AMT1* gene autoregulation. We demonstrate that the yeast histone acetyltransferase Gcn5 and the Swi/Snf chromatin remodelling complex are essential for Cu-dependent *AMT1* gene autoregulation. We also show that the requirement for these factors is target gene specific. We provide *in vitro* evidence that histone acetylation can influence the structure of the A16 element, and that histone acetylation and Swi/Snf increase nucleosomal *AMT1* MRE accessibility. Interestingly, we observed an absolute requirement for Gcn5 and Swi/Snf in Cu-dependent transcriptional activation that is not correspondingly observed for MRE accessibility *in vivo* in the absence of Cu. These data suggest that Swi/Snf and Gcn5 function at multiple levels in Cu-dependent gene activation.

Results

Histone acetyltransferase activity is essential for AMT1 *autoactivation* in vivo

To ascertain whether rapid Cu-induced transcriptional autoactivation from the *C. glabrata AMT1* gene requires the action of histone acetyltransferases *in vivo*, the kinetics and magnitude of Cu-activated *AMT1* gene expression were quantified in wild type and histone acetyltransferase mutants of *S. cerevisiae*. These experiments took advantage of our previous observation that the *AMT1* gene is rapidly and robustly activated in *S. cerevisiae* cells in response to Cu in an A16-dependent manner (Koch and Thiele, 1999). Full-length wild-type (A16) and S16 mutant *AMT1* genes were introduced into wild-type *S. cerevisiae* cells and isogenic strains bearing non-functional *GCN5* or *HAT1* histone acetyltransferase genes. The S16 *AMT1* mutant contains a version of the *AMT1* promoter in which the A16 element is replaced by a defined 16 bp non-homopolymeric stretch of DNA (Zhu and Thiele, 1996). *AMT1* expression was measured by RNase protection assays over a time course of induction by Cu. The results shown in Fig. 1 demonstrate that *AMT1* autoactivation in *S. cerevisiae* exhibits a Cu and A16 element dependency reminiscent of that observed in *C. glabrata* cells. Moreover, *gcn5Δ* strains exhibit a severe defect in both the rate and the magnitude of autoactivation from the wild-type *AMT1* promoter, as well as the residual low-level autoactivation that occurs with delayed kinetics from the *AMT1* S16 promoter (Fig. 1A and B). Histone acetyltransferases have been classified as type A (transcription associated) or type B (involved in chromatin maturation) (for a review, see Brown *et al*., 2000). Given that Gcn5 is a type A histone acetyltransferase (Brownell *et al*., 1996), we wanted to ascertain whether the requirement for histone acetylation in *AMT1* gene autoactivation was specific. We therefore determined whether the yeast type B histone acetyltransferase, Hat1 (Kleff *et al*., 1995), was required for *AMT1* gene autoregulation in *S. cerevisiae*. The analysis of *AMT1* autoactivation in *hat1Δ* cells (Fig. 1C and D) demonstrates no defect, suggesting a role for specific histone acetylation by Gcn5 or its functional homologue in *C. glabrata*.

The Swi/Snf nucleosome remodelling complex is required for AMT1 *autoactivation*

Previous investigations have demonstrated that many genes that require the *S. cerevisiae* Gcn5 histone acetyltransferase also exhibit a co-dependence on the Swi/Snf chromatin remodelling complex (Winston and Carlson, 1992; Pollard and Peterson, 1997; 1998) and also that the functions of Swi/Snf and Gcn5 are partially redundant (Roberts and Winston, 1996; Sudarsanam *et al*., 1999). To ascertain whether *AMT1* autoactivation is dependent on Swi/Snf, we quantified *AMT1* expression in isogenic wild-type and *swi1Δ* strains over a time course of Cu induction by RNase protection assays. As shown in Fig. 2A and quantified in Fig. 2B, the A16 element containing the *AMT1* promoter is rapidly activated in the wild-type strain, with maximal steady-state mRNA levels reached within 10–20 min after Cu addition. As observed previously, the S16 *AMT1* mutant is activated slowly after Cu administration, but reaches a steady-state RNA level approximately half that of the wild type after 60 min. In contrast, Cu-inducible expression of both the A16 and the S16 *AMT1* genes is severely impaired in response to Cu administration in the isogenic *swi1Δ* strain. These results clearly demonstrate a co-requirement for the Swi/Snf chromatin remodelling complex and Gcn5 histone acetyltransferase in Cu-activated transcription of the *AMT1* gene in *S. cerevisiae*.

The requirement for Gcn5 and Swi/Snf by Amt1 is target gene specific

The observation that the Swi/Snf chromatin remodelling complex is required for *AMT1* autoactivation is somewhat surprising, given that our previous nuclease sensitivity studies demonstrated that the *AMT1* promoter nucleosome containing the A16 element and MRE is not detectably altered during gene activation (Zhu and Thiele,

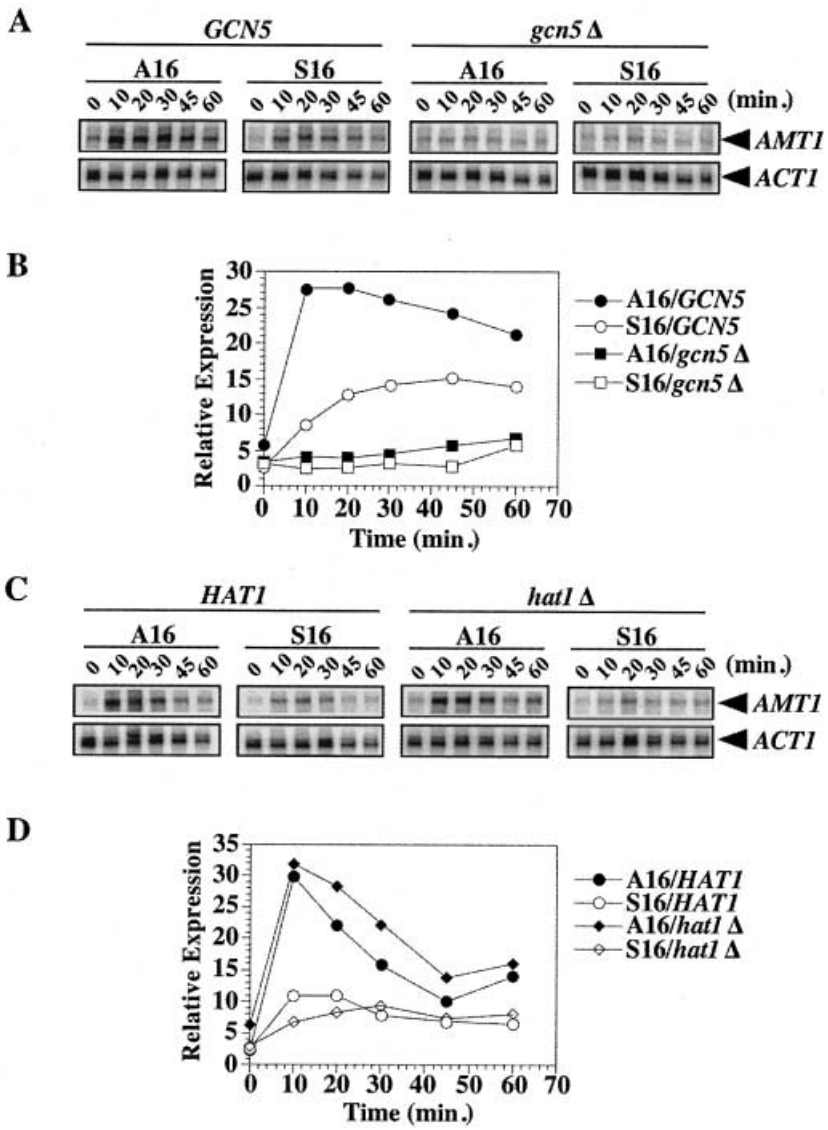


Fig. 1. Histone acetyltransferase activity is required for *AMT1* gene autoregulation.
RNase protection analysis of Cu-induced *AMT1* gene induction for wild-type (A16) and S16-containing *AMT1* genes in *S. cerevisiae*.
A. *S. cerevisiae* strains KKY9 (*GCN5*) and KKY10 (*gcn5Æ*) harbouring the above *AMT1* plasmids were grown to log phase in SC-ura medium treated with 100 μM $CuSO_4$; samples were taken at 0, 10, 20, 30, 45 and 60 min after the addition of $CuSO_4$ and total RNA extracted. Fifteen micrograms of RNA from each sample was analysed by RNase protection assay.
B. Quantification of *AMT1* mRNA expression in response to Cu. All values indicated are normalized to *ACT1* mRNA levels as an internal control. Arrowheads show *AMT1* mRNA and *ACT1* mRNA.
C. *S. cerevisiae* strains KKY7 (*HAT1*) and KKY8 (*hat1Δ*) harbouring the above *AMT1* plasmids were grown to log phase in SC-ura medium. Copper treatment and RNA analysis were performed as described in (A), and the quantified data are represented in (D).

1996). This suggests that Swi/Snf and Gcn5 are required to maintain features of the *AMT1* promoter nucleosome that facilitate the access of Cu-activated Amt1 *in vivo* and/or they function during the transcriptional activation of the *AMT1* gene at a step subsequent to Amt1 binding. We have demonstrated previously the presence of additional nucleosomes within the *AMT1* locus that extend beyond the transcriptional start (Koch and Thiele, 1999), which may provide the basis for a role in nucleosome remodelling during gene activation. Alternatively, there may be a general requirement by Amt1 for Gcn5 and Swi/Snf for transcriptional activation, which is independent of the chromatin context of its target genes. To investigate this latter possibility, we took advantage of a homologous Cu-dependent activator from *S. cerevisiae*, Ace1, which binds to, and activates transcription from, four nearly identical DNA sequences to Amt1 in the *CUP1* metallothionein gene promoter. In contrast to the *AMT1* promoter, our indirect end-labelling experiments (data not shown) and work by others (R. Simpson and L. Bergman, personal communication; Shen *et al*., 2001) demonstrate the absence of detectable stably positioned nucleosomes in the *CUP1* promoter using classical methods. In a strain devoid of endogenous Ace1, the *AMT1* gene and, independently, the *ACE1* gene were expressed constitutively from the Swi/Snf-independent *CYC1* promoter (Cairns *et al*., 1994). The ability of these Cu-activated transcription factors to activate *CUP1* expression in a Swi/Snf- and Gcn5-dependent manner was assayed by RNase protection experiments. Given that the *S. cerevisiae* heat shock transcription factor (HSF) also activates *CUP1* expression in response to several stresses, via an HSE element embedded within the four Ace1 binding sites, the dependence of heat shock induction of *CUP1* on

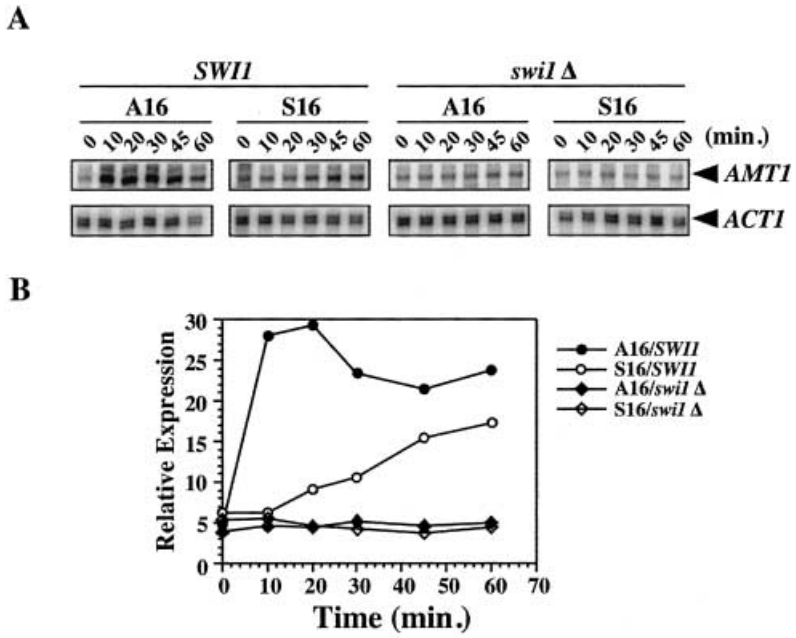


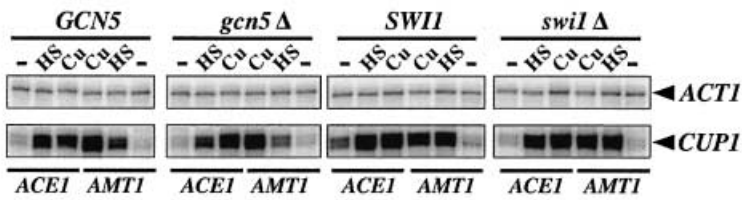
Fig. 2. The Swi/Snf nucleosome remodelling complex is required for *AMT1* gene autoregulation.
A. *S. cerevisiae* strains KKY17 (*SWI1*) and KKY18 (*swi1Æ*) harbouring plasmids containing the A16 and S16 *AMT1* genes were grown to log phase in SC-ura medium. Copper treatment and RNA analysis were performed as described in the legend to Fig. 1, and the quantified data are represented in (B).

Swi/Snf and Gcn5 was also ascertained. The data shown in Fig. 3A and summarized in Fig. 3B clearly demonstrate that Amt1 and Ace1, as well as HSF, function in activated transcription from the *CUP1* promoter in a Swi/Snf- and Gcn5-independent manner. Given that the *CUP1* promoter harbours no detectable stably positioned nucleosomes, these data suggest that the requirement for Swi/Snf and Gcn5 for activation from the *AMT1* gene promoter depends, in part, on the chromatin context of target genes and is not a general requirement for transcriptional activation by Amt1.

Histone acetylation modulates the structure of the AMT1 *nucleosome* in vitro

Given that histone amino-terminal tail acetylation has been implicated in modulating the control that histones have over the DNA wrapped around them, the effects of histone acetylation on the structure of an *AMT1* nucleosome was assessed after reconstitution *in vitro*. Using a radiolabelled *AMT1* promoter fragment encompassing the boundaries of the stably positioned nucleosomal region defined previously in yeast chromatin (Zhu and Thiele, 1996), nucleosomes were reconstituted *in vitro* with either wild-type (A16) or mutated (S16) element promoter derivatives. Human HeLa cell oligonucleosomes from control cells and from cells propagated in the presence of sodium butyrate, a histone deacetylase inhibitor that results in the accumulation of hyperacetylated histones, were used to generate *AMT1* promoter mononucleosomes by high-salt octamer transfer (see *Experimental procedures*). Figure 4A shows the results of *AMT1* mononucleosome reconstitution resolved on a native polyacrylamide gel using A16 and S16 *AMT1* promoter DNA fragments and control or hyperacetylated histones. Quantification revealed that $>95\%$ of the wild-type or mutant *AMT1* promoter DNA was reconstituted into mononucleosomes using both sources of core histones. Partial DNase I cleavage was used to probe the structures of these reconstituted mononucleosomes and demonstrated the appearance of nearly identical 10 bp ladders near the termini of the DNA, characteristic of stably positioned nucleosomes in which DNase I accesses the minor groove at every helical turn. The similar DNase I digestion patterns suggest that the A16 and S16 mononucleosomes have the same rotational phase (Fig. 4B). Moreover, the sites of DNase I cleavage for the nucleosomes reconstituted *in vitro* correlate closely with the sites of DNase I cleavage observed previously in *C. glabrata* chromatin (Zhu and Thiele, 1996), suggesting that the mononucleosomes formed *in vitro* have a very similar rotational phase to that observed in yeast chromatin. Interestingly, mononucleosomes reconstituted from the wild-type *AMT1* promoter (A16) and hyperacetylated histones exhibit clear sites of DNase I hypersensitivity that appear directly adjacent to the 3′ end of the A16 tract (Fig. 4B), positions of DNase I hypersensitivity similar to those observed in *C. glabrata* chromatin with the wild-type, but not the S16, promoter (Zhu and Thiele, 1996). This DNase I hypersensitivity is dramatically elevated in the A16 mononucleosomes compared with cleavage at these sites observed in nucleosomes reconstituted with the *AMT1* S16 derivative and either hyperacetylated or control histones. A novel region of DNase I cleavages also occurs near the middle of the A16 tract in the nucleosomes assembled *in vitro* with

A



B

Activator	Amt1p	Amt1p	Ace1p	HSF
Stimulus	Cu	Cu	Cu	Heat
Target gene	*AMT1*	*CUP1*	*CUP1*	*CUP1*
GCN5	+	+	+	+
gcn5 Δ	-	+	+	+
SWI1	+	+	+	+
swi1 Δ	-	+	+	+

Fig. 3. Swi/Snf and Gcn5p are not required for *CUP1* gene activation by Amt1, Ace1 or HSF.
A. *S. cerevisiae* strains KKY9 (*GCN5*), KKY10 (*gcn5Δ*), KKY17 (*SWI1*) and KKY18 (*swi1Δ*) were transformed with either p316:ACE1 (*ACE1*) or p416CYC1-AMT1 (*AMT1*), and *CUP1* gene induction by either copper or heat stress was analysed by RNase protection. Log-phase growing cells were treated with 100 μM $CuSO_4$ for 30 min or subjected to heat stress at 39°C for 20 min. RNA isolation and analysis was performed as indicated in the legend to Fig. 1, with the exception that *CUP1* and *ACT1* mRNA levels were analysed.
B. Summary of the requirements for Swi/Snf and Gcn5 by Amt1, Ace1 and HSF activating *CUP1* gene expression in response to Cu or heat stress, compared with the requirements for both Swi/Snf and Gcn5 by Amt1 for the activation of the *AMT1* promoter in response to copper.

hyperacetylated histones that has not been observed in *C. glabrata* chromatin, presumably because of a structural perturbation of the A16 tract. This may reflect differences in the DNase I digestion conditions using chromatin versus a relatively purified system of *in vitro*-reconstituted nucleosomes. Alternatively, this could represent differences in sites of histone acetylation from the donor HeLa oligonucleosomes compared with histone acetylation within the *AMT1* nucleosome in *C. glabrata* cells.

Histone acetylation and Swi/Snf facilitate Amt1 nucleosomal access in vitro

The results of our experiments demonstrate that the Gcn5 histone acetyltransferase is important for rapid autoactivation of *AMT1* gene transcription *in vivo* and that hyperacetylated histones are important for the formation of regions of DNase I hypersensitivity that flank the A16 tract on nucleosomes formed *in vitro*. Moreover, we have also demonstrated an absolute requirement for Swi/Snf for Cu-activated *AMT1* gene autoregulation. We therefore ascertained whether histone acetylation and/or the Swi/Snf complex facilitate nucleosomal access in an A16-dependent manner *in vitro*. Wild-type (A16) and S16-containing *AMT1* promoter fragments were reconstituted into mononucleosomes *in vitro*, and nucleosomal access was monitored by cleavage at a naturally occurring nucleosomal *Bsp*HI restriction site that is directly adjacent to the A16/S16 DNA elements (see Fig. 6A for model). The results of the nucleosomal accessibility experiments are shown in Fig. 5. When reconstituted with hyperacetylated histones and in the absence of Swi/Snf, there is a clear enhancement of nucleosomal access in the A16 nucleosome (lane 3, 17% cleavage; and lane 5, 40% cleavage) that is not observed in the S16 nucleosome (lane 9, 18% cleavage; and lane 11, 9% cleavage). These results indicate that the A16 element and histone acetylation co-operate to enhance nucleosome accessibility. In contrast, increased nucleosomal access is observed for both A16 (lane 3, 17% cleavage; and lane 4, 35% cleavage) and S16 (lane 9, 18% cleavage; and lane 10, 29% cleavage) in the presence of Swi/Snf and in the absence of histone hyperacetylation, which indicates that the nucleosome accessibility provided by Swi/Snf is more general in nature and not specific to the A16 element. It is important to note that the amount of Swi/Snf complex used in these experiments is substoichiometric, with higher amounts stimulating *Bsp*HI cleavage of 100% of the molecules on both A16 and S16 nucleosomes (data not shown). The combination of the A16 element, histone hyperacetylation and Swi/Snf treatment resulted in nucleosomes that were the most accessible to cleavage with *Bsp*HI (compare lane 6, 45% cleavage and lane 12, 21% cleavage), indicating that each of these three modes of enhancing nucleosomal access can function together.

Gcn5 and Swi/Snf are important for AMT1 *promoter accessibility* in vivo

The data presented here suggest that histone acetylation and Swi/Snf serve important functions in modulating the structure of the *AMT1* nucleosome *in vitro* and in providing increased access to Amt1 protein on nucleosomes formed *in vitro*. Because Amt1 is rapidly bound to

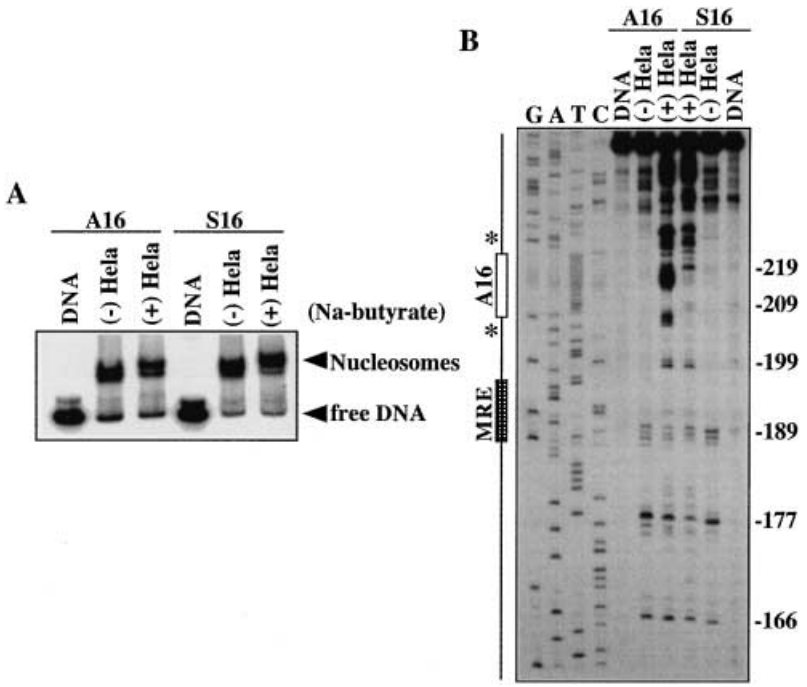


Fig. 4. Histone acetylation modulates the A16 element structure. Nucleosomes were reconstituted *in vitro* onto 146 bp *AMT1* promoter fragments, corresponding to the boundaries of the positioned *AMT1* promoter nucleosome *in vivo*, derived from the wild-type (A16) and S16-containing *AMT1* genes. Reconstituted nucleosomes were prepared by octamer transfer using oligonucleosome samples from untreated HeLa cells (–) or sodium butyrate-treated HeLa cells (+) as the source of histones.
A. Native PAGE analysis of the reconstituted nucleosomes.
B. DNase I cleavage analysis of reconstituted nucleosomes labelled with ^{32}P on the non-coding strand. G, A, T and C represent dideoxy DNA sequencing reactions used for identifying the positions of DNase I cleavage. Numbers indicated to the right represent the *AMT1* promoter position relative to the start of transcription. DNA, naked DNA cut with DNase I; asterisk, sites of DNase I hypersensitivity observed *in vivo*; MRE, metal response element.

the *AMT1* MRE *in vivo* as a consequence of Cu induction, it is critical that the nucleosomal MRE be in an accessible state before Amt1 activation. We therefore tested the potential role of Swi/Snf and Gcn5 in establishing the *AMT1* promoter nucleosome harbouring the MRE and A16 element in an accessible architecture *in vivo*. The ability of the restriction endonuclease *Bsp*HI to cleave at a naturally occurring recognition sequence located immediately downstream of the A16 tract (Fig. 6A) in yeast chromatin isolated from isogenic wild-type, *swi1Δ* and *gcn5Δ* strains was measured. Importantly, the chromatin used for the *Bsp*HI accessibility experiment was obtained from cells that were not treated with Cu and therefore represents the state of the *AMT1* promoter before gene activation. Cleavage at the *Bsp*HI site was assessed by Southern blotting with an *AMT1* promoter probe, after cleaving at a second restriction site, giving rise to a 1.38 kb DNA fragment. The results in Fig. 6B show that, after a 10 min restriction digest, 67% of the wild-type *AMT1* promoter (A16) DNA is cleaved with *Bsp*HI, whereas in the *gcn5Δ* strain, only 28% of the *Bsp*HI sites in the wild-type *AMT1* promoter were cleaved at the same time point. Similarly, in the *SWI1* wild-type strain, 60% of the *Bsp*HI sites are cleaved in 10 min, whereas only 35% are cleaved in the isogenic *swi1Δ* strain. Consistent with the requirement for the A16 element, only 35% of the *Bsp*HI sites were cleaved in the S16 promoter derivative in the wild-type background. It is important to note that cleavage by *Bsp*HI does not increase significantly between 10 and 20 min (data not shown), indicating that *Bsp*HI enzyme is not limiting under these assay conditions and that the digestions were performed to completion. The reduced nucleosome accessibility observed in chromatin from the *gcn5Δ* and *swi1Δ* strains, combined with the data from nucleosomes reconstituted *in vitro*, demonstrate that the Gcn5 histone acetyltransferase and the Swi/Snf chromatin remodelling complex contribute to establishing an accessible *AMT1* promoter. It is important to note, however, that the absolute requirement for Swi/Snf and Gcn5 in transcriptional autoregulation is not correspondingly reflected in the level of nucleosome accessibility provided by these factors. It therefore appears evident that Swi/Snf and Gcn5 may function at additional levels of *AMT1* gene autoregulation.

Discussion

The Cu-metalloregulatory transcription factors, Amt1 in *C. glabrata* and Ace1 in *S. cerevisiae*, are members of a unique family of transcription factors that are activated through the binding of Cu within their DNA-binding domains (Fürst *et al*., 1988; Hu *et al*., 1990; Zhou and Thiele, 1991). These proteins are highly homologous to each other and share very similar DNA binding sites (Huibregtse *et al*., 1989; Zhou *et al*., 1992; Koch and Thiele, 1996). It is clear, however, that they differ somewhat in their mechanisms for transcriptional activation. Heterologously expressed Amt1 is capable of inducing the *S. cerevisiae CUP1* metallothionein gene, as shown in Fig. 3 and by Koch and Thiele (1999), whereas Ace1 is incapable of inducing the *C. glabrata*

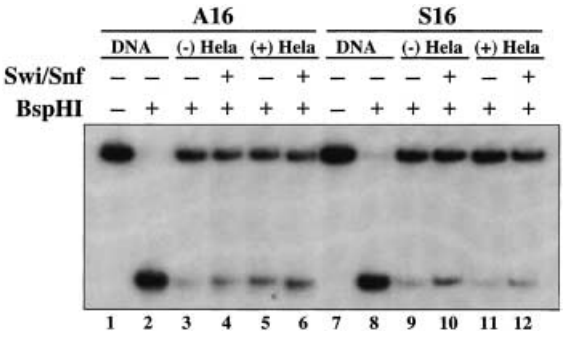


Fig. 5. Histone acetylation, Swi/Snf and the A16 element facilitate nucleosomal access *in vitro*. Wild-type (A16) (lanes 1–6) and mutant (S16) (lanes 7–12) *AMT1* promoter fragments radiolabelled with ^{32}P were reconstituted into nucleosomes *in vitro* using control [(–)HeLa] (lanes 3, 4, 9 and 10) or hyperacetylated [(+)HeLa] (lanes 5, 6, 11 and 12) HeLa cell core histones in the presence of carrier DNA. Nucleosomes (25 nM total) were incubated in the absence (lanes 3, 5, 9 and 11) or presence (lanes 4, 6, 10 and 12) of 0.75 nM Swi/Snf for 30 min at 30°C. Nucleosomal DNA accessibility was then monitored by cleavage with *Bsp*HI restriction endonuclease followed by native PAGE. Cleavage of full-length (154 bp) naked DNA with *Bsp*HI yields a 57 bp cleavage product (lanes 1, 2, 7 and 8).

metallothionein genes in *S. cerevisiae* (Thorvaldsen *et al*., 1993). We have focused our studies on the molecular details of *AMT1* gene autoregulation to understand the mechanisms of rapid transcriptional activation in response to the potentially toxic metal Cu.

Our previous studies have shown that the *AMT1* gene is rapidly autoregulated upon exposure to elevated levels of exogenous Cu (Zhou and Thiele, 1993). This autoregulatory response is critical for the survival of *C. glabrata* cells and occurs through a single MRE in the *AMT1* promoter (Zhou and Thiele, 1993). We have demonstrated that this MRE is located in a stably positioned nucleosome *in vivo* and that a 16 bp homopolymeric dA:dT element (A16 element) located directly adjacent to the MRE, within the nucleosome, fosters Amt1 access to the nucleosomal MRE (Zhu and Thiele, 1996). The nucleosomal accessibility provided by the A16 element is thought to be derived from its rigid, rod-like structure weakening the interactions of the histones with the nucleosomal DNA (Nelson *et al*., 1987; Zhu and Thiele, 1996). Consistent with this hypothesis, we have demonstrated previously that the A16 element does not require the poly dA:dT binding protein, Dat1, to function in *AMT1* gene autoregulation (Koch and Thiele, 1999). We have also shown that the position and length of the homopolymeric dA:dT element is important for *AMT1* gene autoregulation, and that the A16 element can function independently of Amt1 to provide nucleosomal access to the rat glucocorticoid receptor (Koch and Thiele, 1999).

In this study, we have investigated the role of two other cellular factors, Swi/Snf and Gcn5, in the Cu-dependent transcriptional autoregulation of the *AMT1* gene. There is an absolute dependency on both Swi/Snf and Gcn5 for *AMT1* gene autoregulation (Figs 1 and 2) in *S. cerevisiae*, which would imply an equivalent requirement for the homologous proteins in *C. glabrata*. We have also established that the requirement for Swi/Snf and Gcn5 is target gene specific (Fig. 3), as they are not required for the Cu-dependent activation of the *S. cerevisiae CUP1* metallothionein gene by Amt1. Stably positioned nucleosomes are not detectable on the *CUP1* promoter using classical nucleosome mapping methods (data not shown; R. Simpson and L. Bergman, personal communication; Shen *et al*., 2001). However, a novel approach to nucleosome mapping, known as monomer extension, was used recently to study the chromatin organization of

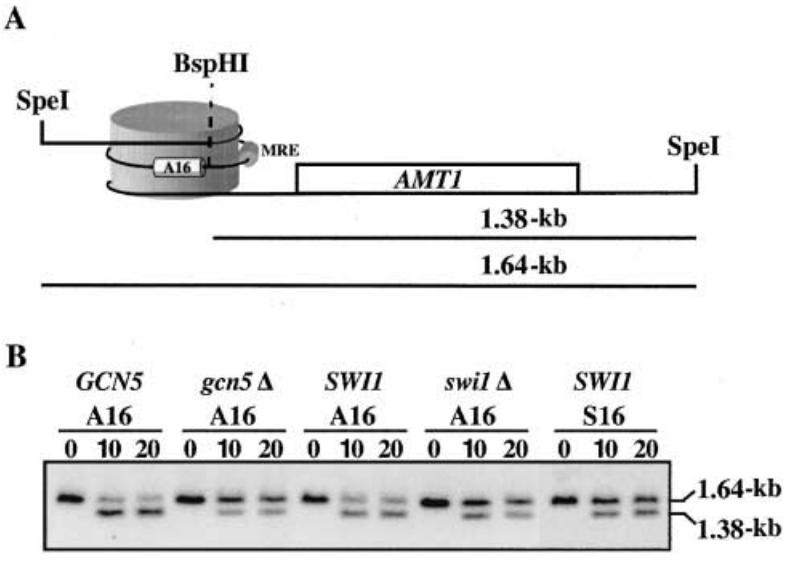


Fig. 6. Swi/Snf and Gcn5p are important for *AMT1* promoter accessibility *in vivo*.
A. Schematic representation of the 1.64 kb *AMT1 Spe*I fragment, with the position of the *Bsp*HI restriction endonuclease cleavage site adjacent to the A16 element (white box) and the downstream MRE (grey box). Cleavage by *Bsp*HI releases a 1.38 kb fragment that was used to determine accessibility and a smaller 260 bp fragment that was not analysed for (B).
B. The results of *Bsp*HI accessibility on the *AMT1* promoter adjacent to the A16 element. Nuclear samples from wild-type and isogenic *gcn5Δ and swi1Δ* strains were digested with *Bsp*HI for 10 min or 20 min before terminating digestion by snap-freezing the samples in liquid nitrogen. After secondary digestion with *Spe*I, resolving the DNAs by agarose gel electrophoresis and Southern blotting, the blot was probed with a radiolabelled 1.64 kb *AMT1 Spe*I fragment.

the *CUP1* promoter (Shen *et al*., 2001). These studies reveal that the chromatin architecture of the *CUP1* promoter is very complex, containing an array of overlapping nucleosome positions. Interestingly, they also observed Ace1-dependent chromatin remodelling of the *CUP1* promoter. Given that we have demonstrated (Fig. 5) that Swi/Snf and Gcn5 are not required for *CUP1* gene activation by Ace1, this would imply that Ace1 recruits a different chromatin remodelling activity to the *CUP1* promoter. The identification of this chromatin remodelling activity and the mechanism by which it is used on the *CUP1* promoter would be of great interest. It is important to note that, in addition to having a complex chromatin architecture, the *CUP1* gene is unusual in that it does not require the Srb4 mediator and RNA pol II CTD (McNeil *et al*., 1998), TFIIA (Ozer *et al*., 1998; Liu *et al*., 1999), TFIIE (Sakurai and Fukasawa, 1999) or TFIIH (Lee and Lis, 1998). It may therefore be formally possible that these unusual attributes of the *CUP1* gene are responsible for the lack of a Swi/Snf and Gcn5 requirement. The requirement for these factors in *AMT1* gene autoregulation has not been tested, and we therefore cannot speculate as to whether the Cu-dependent activation of the *CUP1* and *AMT1* genes differ in this respect.

The level at which Swi/Snf and Gcn5 play a role in *AMT1* gene autoregulation is an intriguing question. Our *in vitro*-reconstituted *AMT1* promoter mononucleosomes exhibit a clear elevation in DNase I hypersensitivity at the ends of the A16 element (Fig. 4) that are reminiscent of what has been observed *in vivo* (Zhu and Thiele, 1996). Hypersensitivity to nuclease cleavage at the ends of the A16 element has been attributed (Zhu and Thiele, 1996) to the A16 element adopting the rod-like structural characteristics that it is known to have in solution (Nelson *et al*., 1987). As we have already established that the A16 element is critical for providing nucleosomal access to Cu-activated Amt1 (Zhu and Thiele, 1996), these data would suggest a role for histone acetylation before Amt1 binding and gene activation. Furthermore, the *in vitro* chromatin accessibility measurements demonstrate that maximal nucleosome accessibility is achieved with a combination of histone acetylation, the A16 element and Swi/Snf. However, the accessibility provided by Swi/Snf appears to be independent of the A16 element and histone acetylation. Given the absolute requirement for Swi/Snf in *AMT1* gene autoregulation, it would appear evident that the role of Swi/Snf is more general than that of histone acetylation. An important caveat to the above supposition is that, in this study, our *in vitro* studies were performed with mononucleosomes. The effects of Swi/Snf and acetylation on nucleosome accessibility may prove to be more profound with nucleosomal arrays. Differences between the *in vitro* results obtained using mononucleosomes and nucleosome arrays have been documented (Herrera *et al*., 2000). We have therefore ascertained the effects of Gcn5 and Swi/Snf on nucleosome accessibility *in vivo*, using chromatin isolated from *gcn5Δ* and *swi1Δ* genetic backgrounds. Our results show that, in the absence of added Cu, a *Bsp*HI restriction site that is between the A16 element and the MRE is less accessible in strains harbouring the *gcn5Δ* and *swi1Δ* alleles than their wild-type counterparts. These results suggest that Swi/Snf and Gcn5 aid in the formation of an accessible *AMT1* promoter nucleosome. The observation that these effects are less dramatic than the 'all-or-none' effect seen in Cu-dependent *AMT1* gene transcriptional autoregulation may imply that Gcn5 and Swi/Snf function at other levels of *AMT1* gene activation. We have demonstrated previously the presence of additional nucleosomes on the *AMT1* promoter (Koch and Thiele, 1999), which may provide the basis for an addition role for these chromatin remodelling factors. Consistent with this hypothesis, previous studies have provided evidence that Swi/Snf functions *in vivo* at a step subsequent to transcription factor binding (Ryan *et al*., 1998; Biggar and Crabtree, 1999; Sudarsanam *et al*., 1999). The results presented in this study provide important insight into the molecular details of Cu-dependent transcriptional regulation by Amt1, a member of the unique family of yeast Cu-metalloregulatory transcription factors.

Experimental procedures

Yeast strains and plasmids

Saccharomyces cerevisiae strains W303-1a (*MATa ade2 ura3 leu2 trp1 his3 can1*), SK56 (isogenic to W303-1a plus *hat1-2::TRP1*), BWG1-7a (*MATa ura3-52 leu2-3112 his4-519 ade1-100*), 1-7a *gcn5Δ* (isogenic to BWG1-7a plus *gcn5Δ::hisG*), CY342 (*MATa ura3-Δ99 leu2-Δ1 his3-Δ200 ade2-101*), and CY258 (isogenic to CY342 plus *swi1-Δ::LEU2*) were used in these studies. The *S. cerevisiae ACE1* copper-metalloregulatory transcription factor gene was deleted from each of these strains as described previously (Butler and Thiele, 1991) to generate the new strains KKY7, KKY8, KKY9, KKY10, KKY17 and KKY18 respectively. Yeast strains were grown at 30°C in rich medium (YPD) or synthetic complete (SC) medium lacking the appropriate nutrient for plasmid selection. The *Escherichia coli* strain DH5αF′ was used for the construction and maintenance of plasmids by standard techniques.

The plasmids pRSAMT1 and pRSS16 harbouring *AMT1* genes with the A16 or S16 element promoter arrangements, respectively, have been described previously (Zhu and Thiele, 1996). Plasmid p316:ACE1 was constructed by subcloning the 1.8 kb *Hin*dIII fragment from pRI-3, containing the entire *ACE1* structural gene, into the *Hin*dIII site of pRS316. To produce Amt1 at low constitutive levels from the *CYC1* promoter, the plasmid p416CYC1-AMT1 was constructed. p416CYC1-AMT1 contains the full *AMT1* open reading frame (ORF) cloned into the multiple cloning site of

the plasmid p416-CYC1, which places Amt1 expression under the control of the constitutive *CYC1* promoter whose basal activity is not regulated by Swi/Snf (Cairns *et al*., 1994).

AMT1 nucleosome reconstitution

pSK+−A16 or pSK+−S16 plasmids were digested with *Xba*I and labelled with Klenow using [α-^{32}P]-dCTP. Labelled fragments were digested with *Asp*718, purified on a native 8% polyacrylamide gel, and 500 000 c.p.m. of the resultant 153 bp fragment, encompassing the *AMT1* nucleosomal boundaries, including the A16 or S16 elements and the MRE, previously ascertained in *C. glabrata* chromatin, was incubated in the presence of 2 μg of carrier DNA, 1.5 μg of bovine serum albumin (BSA), 2.4 μg of core histones from HeLa cells grown under standard conditions or in the presence of the histone deacetylase inhibitor sodium butyrate (10 μM; Coté *et al*., 1995) as described and 2 M NaCl in a final volume of 10 μl at 37°C for 15 min. Samples were serially diluted to a buffer containing 50 mM HEPES, pH 7.5, 1 mM EDTA, 5 mM dithiothreitol (DTT) and 0.5 mM phenylmethylsulphonyl fluoride (PMSF) and 1.5 M, 1 M, 0.8 M, 0.7 M, 0.6 M, 0.5 M, 0.4 M, 0.25 M and 0.2 M NaCl and incubated for 15 min at 30°C. Samples were finally diluted to 0.1 M NaCl in a buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1% NP-40, 5 mM DTT, 0.5 mM PMSF, 20% glycerol and 100 μg ml^{-1} BSA and incubated at 30°C for 15 min.

DNase I mapping of mononucleosome structure

AMT1 promoter mononucleosomes used for *in vitro* DNase I structural analysis were reconstituted as described above with the exception that the ^{32}P-labelled *AMT1* promoter fragment was generated as described below. *AMT1* promoter fragments (146 bp) were polymerase chain reaction (PCR) amplified using the oligonucleotides 5′-CATTTTCTCAGTGG CACTCGGAGAC-3′ (Cod) and 5′-TTGCCTTTAAAGATCAA TATTATCC-3′ (Ncod) with the plasmids pRSAMT1 and pRSS16 as templates. Before PCR amplification, the Ncod oligonucleotide was end labelled using [γ-^{32}P]-ATP and T4 polynucleotide kinase. The resultant PCR products were reconstituted into mononucleosomes and treated with DNase I using standard conditions (Coté *et al*., 1995). DNase I cleavage products were resolved by denaturing polyacrylamide electrophoresis.

Restriction enzyme accessibility assays

The isolation and restriction endonuclease digestions of yeast chromatin were performed essentially as described by Almer and Horz (1986). For *Bsp*HI digestion of chromatin in the nuclear preparations, 100 units of *Bsp*HI was used, and 40 units of *Spe*I was used to digest the purified genomic DNA from each sample to completion. The samples were resolved on a 0.8% agarose gel and transferred to nitrocellulose using standard procedures. The 1.64 kb *AMT1 Spe*I fragment from plasmid pRSAMT1 was labelled using [α-^{32}P]-dCTP and the random prime labelling kit from Bethesda Research Laboratories and used to probe the nitrocellulose blot. Quantification of the results was performed using a PhosphorImager, and the calculated percentage accessibility refers to the percentage of the total DNA used in the assay that was cleaved by *Bsp*HI. *In vitro Bsp*HI accessibility assays were carried out using 5 ng (DNA probe plus carrier DNA) of naked or reconstituted (2.5 nM total nucleosome) DNA in the presence or absence of Swi/Snf in 20 mM HEPES, pH 7.5, 4 mM $MgCl_2$, 1 μM $ZnCl_2$, 2 mM DTT, 0.2 mM PMSF, 5% glycerol, 200 ng ml^{-1} BSA, 50 mM NaCl and 1 mM ATP in a final volume of 20 μl and incubated at 30°C for 30 min. *Bsp*HI (8 units) was added, and the samples were incubated at 30°C for 90 min. Reactions were terminated as for DNase I footprinting, and reaction products were fractionated by electrophoresis on a 6% native polyacrylamide gel. Restriction enzyme accessibility measurements for any given set of conditions were performed at least twice, and the data presented in Figs 5 and 6 are representative of the trend seen in each experiment.

Analysis of AMT1 *and* CUP1 *gene expression*

The yeast strains KKY7, KKY8, KKY9, KKY10, KKY17 and KKY18 were independently transformed with pRSAMT1, pRSS16, p416CYC1-AMT1 or p316:ACE1 using the protocol and plated onto SC-ura agar plates. Growth conditions, $CuSO_4$ induction of *AMT1* and *CUP1* genes and analysis of the *AMT1* and *CUP1* mRNA levels by RNase protection assays were carried out as described previously (Koch and Thiele, 1999). Analysis of gene expression for any given set of conditions was performed at least twice, and the data presented in Figs 1–3 are representative of the trend seen in each experiment.

Acknowledgements

We thank Marissa Vignali and Jerry Workman for the control and hyperacetylated HeLa cell core histones, and Leonard Guarente, Shelly Berger and Craig Peterson for yeast strains. This work was supported by a grant from the United States National Institutes of Health (GM41840) to D.J.T. and from the Medical Research Council of Canada (MRC) to J.C. J.C. is an MRC Scholar. K.A.K. was supported by the Cellular Biotechnology Training Program from the National Institutes of Health Grant (GM08353).

References

Almer, A., and Horz, W. (1986) Nuclease hypersensitive regions with adjacent positioned nucleosomes mark the gene boundaries of the PHO5/PHO3 locus in yeast. *EMBO J* **5**: 2681–2687.

Biggar, S.R., and Crabtree, G.R. (1999) Continuous and widespread roles for the Swi–Snf complex in transcription. *EMBO J* **18**: 2254–2264.

Brown, C.E., Lechner, T., Howe, L., and Workman, J.L. (2000) The many HATs of transcription coactivators. *Trends Biochem Sci* **25**: 15–19.

Brownell, J.E., Zhou, J., Ranalli, T., Kobayashi, R., Edmondson, D.G., Roth, S.Y., and Allis, C.D. (1996) Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p

linking histone acetylation to gene activation. *Cell* **84**: 843–851.

Butler, G., and Thiele, D.J. (1991) ACE2, an activator of yeast metallothionein expression which is homologous to SWI5. *Mol Cell Biol* **11**: 476–485.

Cairns, B.R., Kim, Y.J., Sayre, M.H., Laurent, B.C., and Kornberg, R.D. (1994) A multisubunit complex containing the SWI1/ADR6, SWI2/SNF2, SWI3, SNF5, and SNF6 gene products isolated from yeast. *Proc Natl Acad Sci USA* **91**: 1950–1954.

Coté, J., Utley, R.T., and Workman, J.L. (1995) Basic analysis of transcription factor binding to nucleosomes. *Methods Mol Genet* **6**: 101–108.

Fürst, P., Hu, S., Hackett, R., and Hamer, D. (1988) Copper activates metallothionein gene transcription by altering the conformation of a specific DNA binding protein. *Cell* **55**: 705–717.

Halliwell, B., and Gutteridge, J.M. (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem J* **219**: 1–14.

Herrera, J.E., Schiltz, R.L., and Bustin, M. (2000) The accessibility of histone H3 tails in chromatin modulates their acetylation by P300/CBP-associated factor. *J Biol Chem* **275**: 12994–12999.

Hu, S., Furst, P., and Hamer, D. (1990) The DNA and Cu binding functions of ACE1 are interdigitated within a single domain. *New Biol* **2**: 544–555.

Huibregtse, J.M., Engelke, D.R., and Thiele, D.J. (1989) Copper-induced binding of cellular factors to yeast metallothionein upstream activation sequences. *Proc Natl Acad Sci USA* **86**: 65–69.

Kagi, J.H., and Schaffer, A. (1988) Biochemistry of metallothionein. *Biochemistry* **27**: 8509–8515.

Kleff, S., Andrulis, E.D., Anderson, C.W., and Sternglanz, R. (1995) Identification of a gene encoding a yeast histone H4 acetyltransferase. *J Biol Chem* **270**: 24674–24677.

Koch, K., and Thiele, D.J. (1996) Autoactivation by a *Candida glabrata* copper metalloregulatory transcription factor requires critical minor groove interactions. *Mol Cell Biol* **16**: 724–734.

Koch, K.A., and Thiele, D.J. (1999) Functional analysis of a homopolymeric (dA–dT) element that provides nucleosomal access to yeast and mammalian transcription factors. *J Biol Chem* **274**: 23752–23760.

Lee, D., and Lis, J.T. (1998) Transcriptional activation independent of TFIIH kinase and RNA polymerase II mediator *in vivo*. *Nature* **393**: 389–392.

Linder, M.C. (1991) *Biochemistry of Copper.* New York: Plenum Press.

Liu, Q., Gabriel, S.E., Roinick, K.L., Ward, R.D., and Arndt, K.M. (1999) Analysis of TFIIA function *in vivo*: evidence for a role in TATA-binding protein recruitment and gene specific activation. *Mol Cell Biol* **19**: 8673–8685.

McNeil, J.B., Agah, H., and Bentley, D. (1998) Activated transcription independent of the RNA polymerase II holoenzyme in budding yeast. *Genes Dev* **12**: 2510–2521.

Mehra, R.K., Garey, J.R., Butt, T.R., Gray, W.R., and Winge, D.R. (1989) Candida glabrata metallothioneins. Cloning and sequence of the genes and characterization of proteins. *J Biol Chem* **264**: 19747–19753.

Nelson, H.C., Finch, J.T., Luisi, B.F., and Klug, A. (1987) The structure of an oligo (dA) oligo (dT) tract and its biological implications. *Nature* **330**: 221–226.

Ozer, J., Lezina, L.E., Ewing, J., Audi, S., and Lieberman, P.E. (1998) Association of transcription factor IIA with TATA binding protein is required for transcriptional activation of a subset of promoters and cell cycle progression in *Saccharomyces cerevisiae*. *Mol Cell Biol* **18**: 2559–2570.

Pollard, K.J., and Peterson, C.L. (1997) Role for ADA/GCN5 products in antagonizing chromatin-mediated transcriptional repression. *Mol Cell Biol* **17**: 6212–6222.

Pollard, K.J., and Peterson, C.L. (1998) Chromatin remodeling: a marriage between two families? *Bioessays* **20**: 771–780.

Roberts, S.M., and Winston, F. (1996) SPT20/ADA5 encodes a novel protein functionally related to the TATA-binding protein and important for transcription in *Saccharomyces cerevisiae*. *Mol Cell Biol* **16**: 3206–3213.

Ryan, M.P., Jones, R., and Morse, R.H. (1998) SWI-SNF complex participation in transcriptional activation at a step subsequent to activator binding. *Mol Cell Biol* **18**: 1774–1782.

Sakurai, H., and Fukasawa, T. (1999) Activator-specific requirement for the general transcription factor TFIIE in yeast. *Biochem Biophys Res Commun* **261**: 734–739.

Shen, C.H., Leblanc, B.P., Alfieri, J.A., and Clark, D.J. (2001) Remodeling of yeast CUP1 chromatin involves activator-dependent repositioning of nucleosomes over the entire gene and flanking sequences.. *Mol Cell Biol* **21**: 534–547.

Sudarsanam, P., Cao, Y., Wu, L., Laurent, B.C., and Winston, F. (1999) The nucleosome remodeling complex, Snf/Swi, is required for the maintenance of transcription *in vivo* and is partially redundant with the histone acetyltransferase, Gcn5. *EMBO J* **18**: 3101–3106.

Thorvaldsen, J.L., Sewell, A.K., McCowen, C.L., and Winge, D.R. (1993) Regulation of metallothionein genes by the ACE1 and AMT1 transcription factors. *J Biol Chem* **268**: 12512–12518.

Thorvaldsen, J.L., Sewell, A.K., Tanner, A.M., Peltier, J.M., Pickering, I.J., George, G.N., and Winge, D.R. (1994) Mixed Cu+ and Zn2+ coordination in the DNA-binding domain of the AMT1 transcription factor from Candida glabrata. *Biochemistry* **33**: 9566–9577.

Winston, F., and Carlson, M. (1992) Yeast SNF/SWI transcriptional activators and the SPT/SIN chromatin connection. *Trends Genet* **8**: 387–391.

Zhou, P.B., and Thiele, D.J. (1991) Isolation of a metal-activated transcription factor gene from *Candida glabrata* by complementation in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* **88**: 6112–6116.

Zhou, P., and Thiele, D.J. (1993) Rapid transcriptional autoregulation of a yeast metalloregulatory factor is essential for high-level copper detoxification. *Genes Dev* **7**: 1824–1835.

Zhou, P., Szczypka, M.S., Sosinowski, T., and Thiele, D.J. (1992) Expression of a yeast metallothionein gene family is activated by a single metalloregulatory transcription factor. *Mol Cell Biol* **12**: 3766–3775.

Zhu, Z., and Thiele, D.J. (1996) A specialized nucleosome modulates the kinetics of transcription factor binding to a *Candida glabrata* metal responsive element *in vivo*. *Cell* **87**: 459–470.