Rapid transcriptional autoregulation of a yeast metalloregulatory transcription factor is essential for high-level copper detoxification

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Copper detoxification in the yeast *Candida glabrata* is carried out in large part by a family of metallothionein (MT) genes: a unique *MT-I* gene, a tandemly amplified *MT-IIa* gene, and a single unlinked *MT-IIb* gene. In response to elevated environmental copper levels, members of this MT gene family are transcriptionally activated by a copper-dependent, sequence-specific DNA-binding transcription factor, AMT1. AMT1 shares several structural and functional features with the *Saccharomyces cerevisiae* copper metalloregulatory transcription factor ACE1, which is constitutively expressed and poised for rapid transcriptional responses to the toxic metal copper. In this paper, we demonstrate that AMT1 is subject to positive transcriptional autoregulation, which is exerted through binding of copper-activated AMT1 to a single copper responsive element in the AMT1 promoter. A nonautoregulatory *amt1* mutant displayed a marked decrease in both copper tolerance and expression of the MT-II genes, which are critical for high-level copper detoxification in *Candida glabrata*. Kinetic analysis demonstrated the remarkably rapid AMT1 mRNA accumulation in the presence of copper, which is followed by increased expression of the metallothionein gene products. These results demonstrate that AMT1-positive autoregulation plays a critical role in metal detoxification and suggest that the rapid autoactivation of the AMT1 metalloregulatory transcription factor biosynthesis is essential for *C. glabrata* to quickly build up a cellular defense line to protect cells upon exposure to high environmental copper levels.

[Key Words: AMT1; autoregulation; *Candida glabrata*; copper; metalloregulatory transcription factor; metallothionein]

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A common response of cells to fluctuations in the levels of nutrients, toxins, or developmental signals is to reprogram the transcription of genes important for coping with the altered environment. In many cases, these genes encode both proteins that directly facilitate the adaptive or developmental response and regulatory genes encoding DNA-binding transcription regulation factors that control the expression of the structural genes (Angel et al. 1988). The metallothioneins (MTs) provide an important system to study the homeostatic processes that regulate cell responses to both essential and toxic metal ions and the mechanisms by which these signals are interpreted and transduced.

MTs are low-molecular-weight, cysteine-rich proteins that bind copper and other metal ions and therefore protect cells against metal toxicity (Kägi and Kojima 1987; Kägi and Schaffer 1988). Expression of MTs is activated, primarily at the level of gene transcription initiation, in

response to elevated levels of copper, zinc, mercury, and many other metals (for review, see Thiele 1992). The baker's yeast *Saccharomyces cerevisiae* contains a single MT gene, designated *CUP1* (Fogel and Welch 1982; Butt et al. 1984; Karin et al. 1984). In response to elevated copper concentrations, the *CUP1* gene is transcriptionally induced by a copper-activated sequence-specific DNA-binding transcription factor denoted ACE1 (Thiele 1988, 1992). Upon the binding of copper through its amino-terminal cysteine-rich metal-binding domain, ACE1 is thought to undergo a conformational change, which acts as a "functional switch" from apo-ACE1 to an active protein competent for specific DNA-binding to four independent sites within the *CUP1* upstream activation sequence (UAS_{CUP1}) (Füerst et al. 1988; Huibregste et al. 1989; Szczypka and Thiele 1989; Buchman et al. 1990; Evans et al. 1990; Hu et al. 1990; Thiele 1992). Previous experiments demonstrated that ACE1 exists as a single-copy gene in *S. cerevisiae* strains harboring either one copy (*cup1^s*) or multiple tandem copies (*CUP1^R*) of *CUP1* (Thiele 1988). Besides *CUP1*, ACE1 directly

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activates transcription of another gene, denoted *SOD1*, which encodes the copper–zinc superoxide dismutase; this protects cells from oxygen toxicity (Gralla et al. 1991). Furthermore, ACE1 biosynthesis is independent of copper, because constitutive levels of ACE1 are detected in *S. cerevisiae* cells growing either in the absence or presence of exogenous copper ions (Szczypka and Thiele 1989).

The opportunistic pathogenic yeast *Candida glabrata* provides an excellent system to study copper-dependent MT gene transcription. Like humans and other higher eukaryotes, this yeast harbors a family of MT genes that is divided into classes: a unique *MT-I* gene and two distinct *MT-II* genes, composed of a tandemly amplified *MT-IIa* gene and a single unlinked *MT-IIb* gene (Mehra et al. 1989, 1992). Both the *MT-I* and *MT-II* genes are transcriptionally activated by copper and its electronic analog, silver (Mehra et al. 1989). Recently, we isolated a *C. glabrata* gene encoding a copper-dependent transcription factor, denoted AMT1, which bears many structural and functional features of the ACE1 protein in *S. cerevisiae* (Zhou and Thiele 1991). AMT1 is composed of 265 amino acids organized to form two distinct domains; the amino-terminal domain (amino acid residues 1–115) is positively charged and contains the copper-activated DNA-binding function, which delivers monomeric AMT1 to binding sites in the MT target gene promoters. The carboxy-terminal region (residues 116–265) is negatively charged and, by analogy with ACE1, is likely to harbor the *trans*-activation function (Zhou and Thiele 1991; Zhou et al. 1992). Upon copper treatment, AMT1 binds to two and six binding sites in the *MT-I* and *MT-IIa* promoters, respectively, and activates target gene transcription in a copper concentration-dependent fashion (this paper; Zhou et al. 1992). Of the two MT classes in *C. glabrata*, the *MT-II* gene family was shown to play a major role in copper detoxification (Mehra et al. 1992). The structural and functional characteristics of AMT1 demonstrate that this protein has dual functions, acting both as a copper sensory molecule and as a transcription activator: It senses the elevated copper levels and directly initiates a genomic response to this signal by activating the transcription of copper detoxification genes (MTs).

To begin to investigate the mechanisms by which metalloregulatory transcription factors activate MT gene families, we analyzed the physiological factors that modulate expression of the *AMT1* gene. In contrast to its functional analog ACE1, which is constitutively expressed both at the level of transcription and translation (Szczypka and Thiele 1989), *AMT1* mRNA levels are dramatically increased in response to copper or silver, the same metal ions that foster the AMT1-dependent *MT-I* and *MT-II* gene transcription. In this report we demonstrate that *AMT1* is rapidly transcriptionally activated by its own gene product through a single binding site in the *AMT1* promoter. Furthermore, through the generation of yeast cells in which the sole functional *AMT1* gene is incapable of positive transcriptional autoregulation, we have demonstrated that this autoregulatory

mechanism is critical for cell survival in the presence of toxic environmental copper concentrations.

Results

Metal-induced AMT1 expression and binding to the AMT1 promoter

The biosynthesis of the ACE1 metalloregulatory transcription factor from the baker's yeast *S. cerevisiae* is unaltered at the level of transcription or translation by exogenous copper ions (Szczypka and Thiele 1989). This presumably ensures the presence of adequate steady-state levels of existing ACE1 to rapidly respond to toxic copper ions by directly activating *CUP1* transcription. To determine whether the gene encoding the functionally analogous transcription factor from *C. glabrata*, *AMT1*, has a similar pattern of expression, we carried out RNA blotting experiments to detect *AMT1* mRNA from untreated cells or from *C. glabrata* cells incubated in the presence of metal ions. Figure 1 demonstrates that low steady-state levels of an mRNA species of ~1.0 kb are detected from untreated cells, the size of which is consistent with a mRNA encoding the 265-amino-acid AMT1 polypeptide. However, in cells treated with either copper or silver, steady-state levels of the *AMT1* mRNA were elevated 16-fold over levels detected in untreated cultures as determined by scanning densitometry. The addition of cadmium salts at the same final concentrations had no discernible effect on *AMT1* mRNA levels. Confirming our previous results for ACE1 in *S. cerevisiae*, readily detectable mRNA levels were observed from untreated cultures, which were not significantly affected by the addition of exogenous metals (Fig. 1, bottom). These observations demonstrate that copper and silver, the same metals that activate AMT1 to bind to and activate transcription of the *C. glabrata* *MT-I* and *MT-IIa* genes (Zhou and Thiele 1991; Zhou et al. 1992),

Figure 1. Increased accumulation of the *AMT1* mRNA in response to copper (Cu) and silver (Ag). Total RNA was prepared from logarithmic phase cultures of *C. glabrata* 85/038 and *S. cerevisiae* DTY7 cells untreated or incubated in the presence of the indicated metal, and concentrations are indicated at the top of each lane. The *AMT1* or *ACE1* mRNA species were detected by RNA blotting and hybridization with *AMT1*- or *ACE1*-specific DNA probe, and are indicated by arrowheads at left.

also induce accumulation of the *AMT1* mRNA steady-state levels in vivo.

Mapping of the *AMT1*-binding site in the *AMT1* promoter

Recently, we demonstrated that *AMT1* is essential for copper-responsive transcription of the *MT* gene family in *C. glabrata* through metal-activated DNA-binding to *MT* gene promoters (Zhou et al. 1992). This finding, in conjunction with the observation that *AMT1* mRNA levels are dramatically induced in response to copper or silver, suggests that transcription of the *AMT1* gene itself is also subject, directly or indirectly, to regulation by copper. To test whether the *AMT1* gene is a direct target for binding of copper-activated *AMT1*, we carried out DNA-binding studies by electrophoretic mobility shift assays. A 326-bp *Bgl*III-*Ssp*I DNA restriction fragment (–457 to –130 relative to the *AMT1* transcription initiation site) was radiolabeled and used as a probe in electrophoretic mobility shift assays using partially purified *AMT1* protein produced in *E. coli* (Zhou et al. 1992). We detected the formation of a single copper- or silver-dependent DNA–protein complex and no further complexes, even at high extract concentrations (data not shown). Cadmium, however, failed to induce any DNA–

protein complex formation. This experiment demonstrated that copper- or silver-activated *AMT1* directly interacts with a sequence in the *AMT1* promoter, consistent with direct *AMT1* metal-dependent positive autoregulation. Furthermore, a copper (Cu)–*AMT1* complex failed to form with a ^{32} P-labeled 169-bp *Ssp*I-*Rsa*I DNA restriction fragment (Zhou and Thiele 1991), which encompasses the downstream *AMT1* promoter fragment (–131 to +39), including the putative TATA box (–84 to –79) (data not shown). To specifically locate the *AMT1*-binding site in the *AMT1* promoter, we employed DNase I footprinting assays using partially purified *AMT1* protein produced in *Escherichia coli* (Zhou et al. 1992). As shown in Figure 2A, copper-activated *AMT1* protein protected a major region, from –195 to –180 on the coding strand and from –200 to –180 on the noncoding strand of the *AMT1* promoter against DNase I cleavage. This *AMT1* recognition sequence is highly similar to a consensus *AMT1*-binding site derived from in vitro DNase I footprinting assays of the *MT-I* and *MT-IIa* promoters, which contains a conserved “GCTG” core sequence preceded by an A/T-rich region (Zhou et al. 1992; Fig. 3A). Furthermore, two nucleotides (G-205 and A-204) on the coding strand and one nucleotide (T-207) on the noncoding strand were also protected by copper-activated *AMT1* (Fig. 2A). Interestingly, 3 bp up-

Figure 2. Copper-activated *AMT1* binds to a single site in the *AMT1* promoter. (A) DNase I footprinting of the *AMT1* promoter region. Footprinting reactions were carried out with either the ^{32} P-labeled coding strand (CODING) or noncoding strand (NONCODING) of the *AMT1* promoter fragments and the indicated amounts of the *AMT1* extract (in micrograms) at the top of each lane. Binding reactions were carried out in the absence (–) or presence (+) of 50 μ M CuSO_4 at room temperature for 10 min. G/A is the chemical sequencing reaction displaying adenine and guanine residues using the coding or noncoding probe fragments as templates. Solid vertical lines and numbers indicate the positions of the Cu–*AMT1*-protected regions relative to the *AMT1* transcription initiation site. (B) Methylation interference analysis of the Cu–*AMT1* complex and the *AMT1* promoter. The coding and noncoding *AMT1* probe fragments, either free (F) or bound with Cu–*AMT1* (C), were partially methylated with dimethylsulfate, isolated on electrophoretic mobility shift assay gels, cleaved with piperidine, and analyzed on a 6% sequencing gel. The numbers and arrowheads indicate positions of critical G or A residues which, when methylated, interfere with Cu–*AMT1* binding.

Figure 3. Copper-activated AMT1 fails to bind to the *AMT1(m)* promoter. (A) Sequence of the AMT1-binding site in the *AMT1* promoter. Base pairs are numbered relative to the *AMT1* transcription start site. Solid horizontal bars indicate the regions within the *AMT1* promoter protected by Cu-AMT1 from DNase I cleavage. Boxed residues are guanines and adenines, which, when methylated, interfere with copper-activated AMT1-binding in vitro. The two brackets indicate the two conserved domains within the AMT1 recognition sequence that are derived from comparison of the AMT1-binding sites in the *AMT1*, *MT-I*, and *MT-IIa* promoters (Zhou et al. 1992). The two A's above the arrows represent the two point mutations introduced to the AMT1-binding site by site-directed mutagenesis to derive the *AMT1(m)* promoter fragment. (B) Electrophoretic mobility shift assays using ³²P-labeled *AMT1* promoter fragments containing either the wild-type (*AMT1*) or mutant [*AMT1(m)*] AMT1-binding sites. The amount of AMT1 extract, (indicated in μg) was used in binding reactions with (+) or without (-) 100 μM CuSO₄. (F) Free probe DNA; (C) copper-activated AMT1 complexed to the wild-type *AMT1* promoter.

stream of the 5' extreme region of the AMT1-binding site lies a nucleotide sequence (-225 to -210) composed exclusively of 16 adenine residues. This region is completely resistant to DNase I cleavage even in the absence of Cu-AMT1. Whether this poly(A) stretch plays any functional role with respect to *AMT1* promoter activity is not currently known.

To determine the nucleotides in the single AMT1-binding site important for stable AMT1-*AMT1* promoter-DNA interactions, we carried out methylation interference assays using the same ³²P-labeled *AMT1* promoter fragments used for the DNase I footprinting analysis (Fig. 2B). Methylation of the guanine residues in the GCTG core-binding sequence (G-191 and G-188 on the coding strand and G-190 on the noncoding strand) strongly interfered with Cu-AMT1 binding to its target sequence. Formation of this complex was also inhibited by methylation of A-195, A-194, and A-193 on the noncoding strand. Because the positions for methylation by dimethylsulfate are N-7 on guanine and N-3 on adenine,

which are located in the major and minor grooves of B-form double helical DNA, respectively, this methylation interference assay also provided information on which regions of the binding site are in close proximity to bound copper-activated AMT1 protein (Thanos and Maniatis 1992). The observation that all of the critical G or A residues, as ascertained by methylation interference, are found within an 8-bp region suggests that Cu-AMT1 interacts with adjacent major and minor groove DNA, representing both core and A/T-rich domains of the AMT1-binding site, respectively (Fig. 3A).

AMT1-positive autoregulation is mediated by the single AMT1-binding site

To examine the functional significance of the AMT1-binding site in the *AMT1* promoter, we converted residues G-191 and G-188 in the core-binding domain, the integrity of which is critical for binding as demonstrated by methylation interference analysis, to adenines by site-directed mutagenesis (Fig. 3A; Ausubel et al. 1987). The 326-bp *AMT1* promoter fragments containing either the mutant or wild-type AMT1-binding sites, designated *AMT1(m)* or *AMT1*, respectively, were radiolabeled and subjected to electrophoretic mobility shift assays using the partially purified AMT1 protein produced in *E. coli*. As shown in Figure 3B, mutation of the critical guanine residues in the core domain of the AMT1-binding site abolished Cu-AMT1 binding to the *AMT1(m)* promoter fragment. The wild-type *AMT1* promoter fragment gave rise to a single Cu-AMT1-dependent complex.

To ascertain the role of this binding site in *AMT1* metal-responsive transcriptional autoregulation, we constructed reporter gene fusions between a 613-bp *Bgl*II-*Syl*I DNA restriction fragment containing the *AMT1* wild-type or *AMT1(m)* promoters and a portion of the *AMT1*-coding region, in-frame with the *E. coli lacZ* gene, encoding β-galactosidase (Fig. 4). These two reporter constructs were independently inserted into a *S. cerevisiae* plasmid, pRS316 (Sikorski and Hieter 1989), which is episomally maintained in multiple copies in *C. glabrata* (P. Zhou, M. Szczypka, R. Young and D.J. Thiele, in prep.). The resulting plasmids were then transformed into a *C. glabrata* strain harboring a *ura3* gene mutation for plasmid selection and maintenance, but which has a wild-type *AMT1* locus. Total RNA was prepared from the recipient cells either untreated or induced with 100 μM CuSO₄, and steady-state mRNA levels driven from the *AMT1-lacZ* and *AMT1(m)-lacZ* fusions were detected by primer extension assays using an oligonucleotide primer complementary to the *lacZ* gene-coding strand. Copper-induced *AMT1-lacZ* mRNA accumulation was observed in recipient cells harboring the wild-type *AMT1-lacZ* fusion and yielded two clusters of *AMT1-lacZ* mRNA start sites, which correspond precisely to authentic AMT1 mRNA start sites beginning at -54 and -79 relative to the ATG translation initiation codon (Fig. 4). However, no copper-dependent mRNA accumulation was observed to be driven from the *AMT1(m)-lacZ* fusion gene. These results demonstrate

Figure 4. A functional *AMT1*-binding site is required for copper-activated transcription driven by the *AMT1* promoter. (Left) Schematic representation of the *AMT1* or *AMT1(m)* promoters fused in-frame to the *E. coli* β -galactosidase gene (*lacZ*). pRS316 is the *C. glabrata* episomal plasmid that was used as a vector to introduce the *AMT1-lacZ* or *AMT1(m)-lacZ* fusion genes into *C. glabrata* Q cells. (Right) The *AMT1(m)* promoter is not responsive to copper. Total RNA was prepared from *C. glabrata* Q recipient cells carrying the p*AMT1-lacZ*, p*AMT1(m)-lacZ*, and pRS316 plasmids either untreated (–) or treated (+) with 100 μ M CuSO_4 . Twenty micrograms of each RNA was analyzed by primer extension using a synthetic oligonucleotide complementary to the *lacZ* mRNA. The two clusters of copper-inducible mRNA species marked by two brackets at left, represent the primer extension products due to the existence of two groups of initiation sites for *AMT1* transcription. Dideoxy sequencing reactions (lanes G,A,T,C) were carried out using p*AMT1-lacZ* plasmid DNA and the *lacZ*-specific primer, and were used to map the primer extension products.

that the *AMT1* gene is subject to direct metal-dependent transcriptional autoregulation, and this activation is mediated by the single *AMT1*-binding site in the *AMT1* promoter. Furthermore, copper-induced mRNA accumulation from the *AMT1-lacZ* fusion gene was dependent on a functional endogenous *AMT1* gene [data not shown].

Kinetics of accumulation of *AMT1*, *MT-I*, and *MT-II* mRNAs in response to copper

The known potent toxicity of copper (Kägi and Kojima 1987) dictates that copper detoxification genes must be functionally or biosynthetically activated quickly in response to copper exposure. Because *ACE1* is constitutively expressed in *S. cerevisiae*, but *AMT1* is highly inducible by copper in *C. glabrata*, we examined the kinetics of accumulation of *AMT1* mRNA and that of its target genes *MT-I* and *MT-II*, following copper exposure. Wild-type *C. glabrata* cells were grown in synthetic complete (sc) medium to log phase, induced with 100 μ M CuSO_4 , and harvested several times following copper addition; total RNA was prepared, and RNA blotting assays were performed to measure the steady-state levels of *AMT1*, *MT-I*, and *MT-II* mRNAs during the course of copper treatment. The *C. glabrata* *URA3* mRNA levels were also evaluated as a control for the amount and integrity of RNA loaded onto each lane of the RNA gel. As shown in Figure 5, *AMT1* mRNA accumulated to maximum levels extremely rapidly after ~5.5 min of exposure of cells to copper (a 5-min centrifugation was employed to harvest cells). The *MT-I* mRNA also reached very high levels quickly, although maximal steady-state levels of *MT-I* mRNA were not detected until ~10 min after copper exposure. However, high-level accumulation of the *MT-II* mRNA was only observed 1 hr after copper addition. These results demonstrated that *AMT1* gene expression is very rapidly autoactivated upon copper induction. The *MT-I* gene is also rapidly expressed at

high levels followed by an increase in *MT-II* mRNA accumulation under conditions of 100 μ M CuSO_4 treatment. Because the *MT-II* probe used in this experiment hybridizes to both the *MT-IIa* and *MT-IIb* mRNAs, the level of the *MT-II* signal that we observed is a combination of the two *MT-II* mRNA transcripts and, therefore, did not allow us to distinguish between the accumulation of these mRNA species. Because mutation of the *AMT1*-binding site abolishes metal-dependent *AMT1* mRNA accumulation (Fig. 4), we tested the function of a DNA fragment (–457 to +161) encompassing the *AMT1* promoter in fostering the rapid accumulation of *AMT1* mRNA. The *AMT1-lacZ* fusion gene in the p*AMT1-lacZ* recipient cells was also rapidly induced with kinetics identical to that of the authentic endogenous *AMT1*

Figure 5. Kinetics of accumulation of the *AMT1*, *MT-I*, *MT-IIa*, and *MT-IIb* mRNAs in response to copper exposure. Wild-type *C. glabrata* 85/038 cells were grown to early logarithmic phase and treated with 100 μ M CuSO_4 for the time (in min) indicated at the top of each lane. Total RNA was prepared, and 30 μ g was used in RNA blotting assays. *AMT1*, *MT-I*, *MT-II*, and *URA3* (internal control) mRNA species were detected by the corresponding probes. Positions corresponding to each mRNA species are indicated by arrows at right.

gene as determined by primer extension assays (data not shown). These results strongly suggest that rapid *AMT1* metal-dependent autoregulation is largely mediated through the *AMT1* promoter-binding site and at the level of transcription initiation.

AMT1 metal-dependent autoregulation is critical for normal copper homeostasis

Due to experimental complexities, the physiological significance of transcription factor gene autoregulation in higher eukaryotes has not been directly tested in the complete absence of an endogenous functional allele. To determine whether *AMT1* transcriptional autoregulation is critical for cellular copper detoxification in *C. glabrata*, we uncoupled copper-dependent autoactivation from the *AMT1* promoter in the context of an intact, single endogenous functional *AMT1* gene. This was achieved by introducing the two point mutations in the *AMT1*-binding site, described in Figure 3A, in the complete *AMT1* gene; therefore, this gene is identical to the wild-type *AMT1* gene, with the exception of a nonfunctional *AMT1*-binding site in the *AMT1* gene promoter. The wild-type or nonautoregulatory mutant *AMT1* genes [*AMT1* and *AMT1(m)*, respectively] were subcloned into a *C. glabrata* integrative plasmid, U1(b), carrying the *C. glabrata* *URA3* gene (P. Zhou, M. Szczypka, R. Young, and D. Thiele, in prep.) and inserted at the *C. glabrata* *ura3* locus within a strain in which the endogenous *AMT1* gene had been insertionally inactivated (Zhou et al. 1992). The targeted single-copy integration was verified by Southern blotting (data not shown). The resultant isogenic strains are designated *AMT1::URA3* and *AMT1(m)::URA3*, respectively.

We then asked whether *AMT1* gene transcriptional autoregulation is critical for the protection of yeast cells in the presence of high environmental copper levels. The copper-resistant phenotype of the isogenic *AMT1::URA3* and *AMT1(m)::URA3* strains, as well as their parental strains *C. glabrata* 85/038 (wild-type) and *amt1-1* (*amt1* gene insertionally inactivated), was tested for copper resistance. As shown in Figure 6, the parental wild-type and *AMT1::URA3* cells are indistinguishable in their resistance to copper and grow on agar containing up to 1.5 μM CuSO_4 . These observations demonstrate that integration of the wild-type *AMT1* gene at the *ura3* chromosomal locus does not affect its expression or function. The *AMT1(m)::URA3* strain, however, gave rise to a maximal copper resistance level at 400 CuSO_4 , a three- to fourfold decrease compared with the wild-type or *AMT1::URA3* strains. As demonstrated previously, the *amt1-1* strain is hypersensitive to copper and fails to grow even in the presence of 25 μM CuSO_4 (Zhou et al. 1992). In these experiments, copper-sensitive cells arrested at the single-cell stage when challenged with toxic concentrations of CuSO_4 (data not shown). These results clearly demonstrate that *AMT1* gene-positive autoregulation is essential for *C. glabrata* resistance to high environmental copper levels. The nonautoregulatory *AMT1* allele [*AMT1(m)::URA3*], survives on media con-

Figure 6. *AMT1* autoregulation is essential for *C. glabrata* cells to survive on high copper concentrations. The nonautoregulatory *AMT1(m)::URA3* allele fails to confer wild-type copper resistance to *C. glabrata*. Isogenic *C. glabrata* strains 85/038, *amt1-1*, *AMT1::URA3*, and *AMT1(m)::URA3* were streaked onto SC agar and SC agar containing the concentrations of CuSO_4 as indicated. Plates were incubated at 30°C for 2 days. The grid indicates the relevant genotypes of the four individual strains streaked onto each sector of the plates.

taining ~ 10 times the concentration of exogenous copper as the *amt1-1* strain. Although the mutation in the *AMT1* promoter abolishes *AMT1* binding and autoactivation, the *AMT1(m)::URA3* allele drives the expression of the *AMT1* mRNA and, presumably, protein, owing to *AMT1*-independent mechanisms of expression of this gene (see Fig. 7). Consistent with this possibility, we detected the formation of *AMT1*-independent DNA-protein complexes using whole cell extracts from the *amt1-1* strain and the 326-bp *AMT1* promoter fragment described in Figure 2. Furthermore, the formation of these complexes was not affected by guanine mutations of the core *AMT1*-binding sequence [*AMT1(m)* probe; data not shown].

Copper sensitivity of cells unable to autoactivate AMT1 correlates with a defect in MT mRNA accumulation

Previous studies suggested that because the *C. glabrata* *MT-I* and *MT-II* genes are transcriptionally coinduced by copper in a concentration-dependent manner, the copper-responsive transcriptional activator *AMT1* is not limiting in its abundance (Mehra et al. 1989, 1992). Our demonstrations that *AMT1* is both transcriptionally positively autoregulated and that this is essential for normal copper resistance suggest that positive autoregulation

Figure 7. Accumulation of the *AMT1*, *MT-I*, and *MT-IIa*, and *MT-IIb* mRNAs in response to increasing copper levels. *AMT1::URA3* and *AMT1(m)::URA3* cells were grown in SC medium lacking uracil (SC – ura) to logarithmic phase. Parallel cultures were untreated or treated for 10 min with the copper concentrations indicated at the top of each lane. Specific *AMT1*, *AMT1::hisG*, *MT-I*, and *MT-II* mRNAs were detected by RNA blotting (left). As an internal control for the loading of RNA, *C. glabrata* actin mRNA levels were analyzed using a hybridization probe derived from the cloned *S. cerevisiae* actin gene, which hybridizes to the *C. glabrata* actin gene (Mason et al. 1987). The radioactivity (cpm) associated with each individual mRNA species was quantitated by counting the dried nitrocellulose membranes with a Beta-gen scanner. The amounts of each mRNA species were normalized to the corresponding actin mRNA level and plotted against copper concentration (in μM). (Right) The three graphs indicate the steady-state mRNA levels from each gene in response to the range of copper levels. Each mRNA species is represented by the symbol shown at the right of each graph.

may ensure an adequate supply of the *AMT1* transcription factor to activate the transcription of *C. glabrata* copper homeostasis genes. Because free intracellular copper could cause rapid macromolecular damage through redox chemistry (Halliwell and Gutteridge 1984), and *AMT1* is expressed at very low levels in the absence of exogenous copper (Figs. 1 and 7), perhaps rapid *AMT1* autoregulation is essential for cells to synthesize sufficient *AMT1* protein to boost the synthesis of metallothioneins promptly before cells are irreversibly dam-

aged by sudden increases in intracellular copper concentrations. To test this hypothesis, we carried out RNA blotting assays to determine the steady-state mRNA levels of the *AMT1*, *MT-I*, and *MT-II* genes after treating the *AMT1::URA3* and *AMT1(m)::URA3* cells with increasing doses of copper for a brief period of time (10-min induction plus 5-min centrifugation) (Fig. 7, left). The *S. cerevisiae* actin gene, which hybridizes to a single mRNA species in *C. glabrata*, was used as a control for the amount and integrity of RNA loaded onto each lane

of the gel in this experiment. Figure 7 (right) shows a quantitation of the steady-state mRNA levels of *AMT1*, *AMT1::hisG*, *MT-I*, and *MT-II* in *AMT1::URA3* and *AMT1(m)::URA3* cells exposed to the range of copper concentrations. The wild-type *AMT1* gene integrated at the *C. glabrata ura3* locus was highly induced when cells were exposed to copper, reaching a maximal eightfold induction after a 10-min exposure to 100 μ M CuSO_4 . The endogenous *amt1-1* locus, which carries an insertion of the *Salmonella hisG* gene within the *AMT1* open reading frame, synthesized a hybrid *AMT1::hisG* mRNA whose identity was confirmed by its hybridization to a ^{32}P -labeled *hisG* probe by RNA blotting (data not shown). The expression of this hybrid gene was also induced by copper-activated *AMT1* with a similar dose response and a 4.2-fold induction, presumably mediated through the intact *AMT1*-binding site within the promoter of the *hisG* insertionally inactivated *amt1-1* locus. In contrast, no copper-inducible mRNA accumulation was observed for the *AMT1(m)* gene in the *AMT1(m)::URA3* strain. However, mRNA driven from the *AMT1::hisG* hybrid gene accumulated in response to exogenous copper, although there was an induction of only ~ 1.6 -fold. Low steady-state levels of the *AMT1::hisG* mRNA were also detected in the *amt1-1* strain by RNA blotting upon prolonged exposure and were not altered upon copper treatment (data not shown). These findings demonstrate that copper-induced transcription driven by the *AMT1* promoter is dependent on both the presence of a functional *AMT1*-binding site and a functional *AMT1* protein. The *AMT1* or *AMT1::hisG* mRNA levels in the *AMT1::URA3* cells were highest when cells were treated with 100 μ M copper and slightly decreased at higher copper concentrations.

RNA blotting with the *MT-I* probe showed that the *MT-I* gene was expressed at maximum levels even at low copper concentrations (100 μ M). The overall level of the *MT-I* mRNA in the *AMT1(m)::URA3* strain is ~ 1.4 -fold lower than that in the *AMT1::URA3* strain. The *MT-II* mRNA levels (*MT-IIa* and *MT-IIb*) in these two isogenic strains are similar upon addition of sublethal concentrations of copper, which are 100 and 300 μ M of CuSO_4 . A reproducible twofold difference of the *MT-II* mRNA level was observed when the strains were treated with lethal doses of copper for *AMT1(m)::URA3* cells (500 μ M and 1 mM). It should be noted that 10-min exposure of *AMT1(m)::URA3* with 500 μ M or 1 mM copper did not immediately impair cell growth, as judged by similar increases of OD_{600} as well as similar levels of actin mRNA observed between *AMT1::URA3* and *AMT1(m)::URA3* cells following copper treatment. Taken together, these results demonstrate that the *MT-I* and *MT-II* mRNA steady-state levels were significantly compromised in the nonautoregulatory *AMT1(m)::URA3* allele.

MT-I and *MT-II* protein levels are correspondingly reduced in *AMT1* nonautoregulatory cells

The steady-state levels of *MT-I* and *MT-II* protein under copper-induced conditions were analyzed by pulse-label-

ing proteins in *AMT1::URA3* and *AMT1(m)::URA3* cells with [^{35}S]cysteine, followed by fluorographic analysis on a 25% native polyacrylamide gel (Fig. 8). This experiment demonstrates that the *MT-I* and *MT-II* proteins were undetectable in both *AMT1::URA3* and *AMT1(m)::URA3* cells grown in the absence of copper. Addition of increasing doses of copper resulted in the elevated synthesis of the *MT-II* protein with a significantly higher *MT-II* level observed in the *AMT1::URA3* cells treated with all copper concentrations than that in the *AMT1(m)::URA3* cells. The *MT-I* protein reached similar maximum levels upon treatment with low copper (100 μ M) in both strains, and this level was similar when the strains were induced with higher concentrations of copper. These results parallel the steady-state *MT-I* and *MT-II* mRNA levels that we obtained for *AMT1::URA3* and *AMT1(m)::URA3* cells under the same copper-induced conditions (Fig. 7).

Discussion

An interesting and important question in gene regulation is how transcription factor genes themselves are regulated, and does this regulation play a key role in the function of the encoded protein? In prokaryotes, autogenous regulation has been demonstrated for several transcriptional repressors and often results in down-regulation of genes encoding these DNA-binding proteins (for review, see Maloy and Stewart 1993). In eukaryotes, positive transcriptional autoregulation has been observed for many transcription factors and is suggested to be an important regulatory mechanism in a large number of biological processes such as cell growth, differentiation, development, and others (for review, see Serfling 1989; Falvey and Schibler 1991). However, efforts to precisely delineate the physiological role of transcription factor autoregulation in these systems have been impeded by the fact that multiple regulatory mechanisms function through the upstream regulatory regions at the same dif-

Figure 8. The *MT-I* and *MT-II* protein levels in *AMT1::URA3* and *AMT1(m)::URA3* cells in response to copper. [^{35}S]Cysteine-labeled total soluble proteins were prepared from control or copper-treated *AMT1::URA3* and *AMT1(m)::URA3* cells at the concentrations indicated at the top of each lane. Each protein extract (1.0 μ g) was subjected to electrophoresis on a 25% non-denaturing polyacrylamide gel. The arrowheads at left indicate the *MT-I* and *MT-II* protein species.

ferentiation or developmental stage, and by the difficulty of genetic manipulations in higher eukaryotes, such as gene knockouts and homologous recombination.

As a unicellular eukaryotic microorganism, yeast provides a powerful model system to study gene regulation and other biochemical processes, largely owing to the ease of genetic manipulations. In this work we demonstrated that the AMT1 metalloregulatory transcription factor of *C. glabrata* functions in a positive autoregulatory loop, which is distinct from the constitutively expressed *ACE1* gene of *S. cerevisiae* (Szczypka and Thiele 1989). *AMT1* is among the first reported yeast transcription factors that directly participates in its own transcription, and the physiological significance of this autoregulation has been established with respect to its role in activating the transcription of target genes that play a critical role in cell defense mechanisms against metal toxicity. In this paper, we demonstrated that *AMT1* autoregulation is mediated by the single AMT1-binding site in the *AMT1* promoter and requires functional AMT1 protein.

On the basis of previous studies (Mehra et al. 1989, 1992; Zhou and Thiele 1991; Zhou et al. 1992) and results obtained in this work, we propose the following autoregulatory model for *AMT1* expression and the copper-induced transcriptional response in *C. glabrata* cells. Under normal growth conditions and low environmental copper concentrations, *C. glabrata* cells synthesize low basal levels of the copper sensory molecule AMT1, as well as MTs and other putative copper homeostatic proteins. MTs may sequester the low concentration of biologically available cellular copper ions preferentially over AMT1, thereby preventing futile transcription of *AMT1*, *MT-I*, *MT-II*, and potentially other, as yet unidentified, AMT1-dependent copper-regulated genes. High environmental copper concentrations lead to an increased copper accumulation, and intracellular copper ions would be available to apo-AMT1 protein produced from basal level transcription of this gene. The coordination of copper by the AMT1 amino-terminal DNA-binding domain activates sequence-specific DNA binding and, once deposited on target gene promoters, first activates transcription of the *AMT1* and *MT-I* genes, followed by the *MT-II* isoform genes. The specific properties of the *AMT1* promoter, either a high-affinity AMT1-binding site or interactions with other transcription factors, may enhance the specific affinity of Cu-AMT1 or its transcriptional potency and contribute to the rapid autoactivation of the *AMT1* gene. Although the relative *in vivo* affinity of Cu-AMT1 for the *AMT1*, *MT-I*, and *MT-II* promoters has not yet been determined, the results that we observed for *AMT1*, *MT-I*, and *MT-II* mRNA levels suggest that even at low copper concentrations (100 μ M), the *AMT1* and *MT-I* genes are rapidly activated to very high levels while the *MT-II* genes are only partially activated. This observation suggests a possible role of *MT-I* as a first line of defense to respond to low levels of copper in the environment. Increasing doses of copper treatment result in the elevated synthesis of the *MT-II* mRNA and protein presumably because

the MT-I protein alone is insufficient for sequestering intracellular copper ions. This high-level *MT-II* gene expression is dependent on two factors: One is the *AMT1* autoregulation that produces sufficient apo-AMT1 protein; second is the increasing amount of copper that switches more apo-AMT1 into the transcriptionally competent Cu-AMT1 available to the *C. glabrata* MTs, especially the tandemly amplified *MT-IIa* gene. Although the MT-I and MT-II proteins bind copper efficiently, we cannot exclude the possibility that MT-I, MT-II, or AMT1 proteins, or other proteins encoded by AMT1-dependent genes, have additional activities that protect cells from copper toxicity. Consistent with this possibility, Tamai et al. (1993) have recently demonstrated that *S. cerevisiae* Cu-MT protein has potent antioxidant activity. Furthermore, because Cu-ACE1 activates *SOD1* transcription (Gralla et al. 1991), it is possible that Cu-AMT1 may also activate other target genes, and the decreased tolerance of the nonautoregulatory *AMT1* mutant strain to copper may result from defective expression of several copper homeostatic genes regulated by AMT1. Further experiments will determine the relative affinity of Cu-AMT1 for the *AMT1*, *MT-I*, and *MT-II* promoters and the precise mechanism by which *AMT1* is rapidly transcriptionally autoactivated.

As a copper sensory molecule, AMT1 responds quickly to the increase in intracellular copper levels by an amplification of its own gene product. Although the mechanisms for copper transport and distribution in *C. glabrata* and other organisms have not been elucidated in detail, copper uptake in *S. cerevisiae* has been demonstrated to be a rapid process, and the rate of intracellular copper accumulation is proportional to the medium copper concentrations (Lin and Kosman 1990). The rapid copper-dependent *AMT1* and *MT-I* transcription suggests that copper uptake in *C. glabrata* is not rate limiting; therefore, the cells may be immediately subjected to copper toxicity upon addition of copper to the growth media. The rapid *AMT1* autoregulation ensures that AMT1 proteins are made promptly and at a concentration sufficient for its function as a copper-dependent transcription factor to stimulate high-level expression of the multiple *C. glabrata* MT genes before extensive copper-induced cellular damage occurs. Recently, it has been observed that pretreatment of human HeLa H454 cells with a low dose of cadmium results in superinduction of the human MT-II_A gene in response to subsequent cadmium administration (A. Leone, pers. comm.). This may reflect a similar autoregulatory expression of the gene, or genes, encoding putative metal response element (MRE)-binding proteins in humans and other organisms bearing an MT gene family.

In contrast to *S. cerevisiae*, in which the gene encoding the ACE1 copper metalloregulatory transcription factor is constitutively expressed (Szczypka and Thiele 1989), *AMT1* autoregulation reflects another level of regulatory complexity for copper homeostatic genes in *C. glabrata*. Although the opportunistic pathogenic yeast *C. glabrata* and the baker's yeast *S. cerevisiae* are evolutionarily related (Barns et al. 1991), the fact that *C. glabrata* cells are

found in a wide variety of habitats including soil, water, on animals and human tissues or organs suggests that this yeast encounters many environmental elements with quite different copper contents (Sinnott et al. 1987). As a unicellular eukaryotic microorganism, a yeast cell is highly accessible to environmental changes in metal concentrations. The development of a rapid positive autoregulatory mechanism for a metal sensing switch, *AMT1*, could allow *C. glabrata* to accumulate sufficient amounts of copper when available concentrations are low but respond rapidly when challenged with toxic environmental copper concentrations.

Materials and methods

Strains, plasmids, and growth conditions

The parental wild-type *C. glabrata* strain 85/038 was a gift of P. Magee (University of Minnesota, St. Paul). The *ura3⁻* (Q) strain and the *AMT1* disruption strain *amt1-1* were constructed as described in detail (Zhou et al. 1992) and were used as recipient hosts for transformation by *C. glabrata* episomal or integrative plasmids described in the following sections. The *amt1-1* strain is the parental strain for the integration of wild-type (*AMT1*) or mutant [*AMT1(m)*] genes at the *ura3* locus to construct isogenic *AMT1::URA3* and *AMT1(m)::URA3* strains by transformation and homologous recombination (Aubusel et al. 1987; P. Zhou, M. Szczypka, R. Young, and D. Theile, in prep.). Yeast cells were grown in rich (YPD) or SC medium lacking uracil (Ausbuel et al. 1987). Copper resistance tests, copper treatment, and the length of incubation are described in the respective figure legends. The *S. cerevisiae* strain DTY7 (Szczypka and Thiele 1989), which contains the wild-type *ACE1* and three copies of the tandemly amplified *CUP1* genes, was used to examine the mRNA levels from the *ACE1* gene under control or metal-induced conditions. *E. coli* strain XL-1 blue (Stratagene) was employed for the construction and maintenance of plasmids using standard techniques (Ausbuel et al. 1987).

To study the role that the single *AMT1*-binding site plays in *AMT1* autoregulation, two guanine residues (G-192 and G-189) in the GCTG core-binding sequence were changed to adenines by oligonucleotide-directed mutagenesis using the mutagenic primer 5'-CGCCCACTACTTTTAAAGTTAGTCAAATT-AGC-3', which hybridizes to *AMT1* promoter nucleotide positions -201 to -168 relative to the +1 transcription start site (this paper; Ausbuel et al. 1987; Zhou and Thiele 1991). The *in vivo* autoactivation of the *AMT1* gene was demonstrated using *AMT1-lacZ* or *AMT1(m)-lacZ* fusion genes containing either the wild-type or mutant *AMT1*-binding site, respectively. The 616-bp *BglII-StyI* DNA restriction fragment, which contains the *AMT1* or *AMT1(m)* promoter and the first 36 *AMT1* codons was first blunt-ended at the *StyI* site by the Klenow fragment of DNA polymerase I and then subcloned into the plasmid YEp356(R) to construct *AMT1-lacZ* or *AMT1(m)-lacZ* in-frame fusion genes (Myers et al. 1986). The 4.3-kb *HindIII-StuI* DNA restriction fragments containing the *AMT1-lacZ* or *AMT1(m)-lacZ* fusions were subcloned into the *C. glabrata* episomal plasmid pRS316 to make plasmids pAMT1-lacZ and pAMT1(m)-lacZ (Sikorski and Hieter 1989). These two pRS316-based plasmids can stably transform the strain Q (*ura3⁻*) and replicate in multiple copies in *C. glabrata* cells (P. Zhou, M. Szczypka, R. Young, and D. Theile, in prep.).

To characterize the biological role of *AMT1* autoregulation, two integrative plasmids were constructed as follows: A 4.0 kb genomic fragment containing the *C. glabrata URA3* gene was

inserted at the *BamHI* site of pRS425 to make plasmid U1(b) (Sikorski and Hieter 1989; P. Zhou, M. Szczypka, R. Young, and D. Theile, in prep.). The 1.6-kb *BglII-SpeI* DNA restriction fragments containing either the wild-type [*AMT1*] or mutant [*AMT1(m)*] *AMT1* genes were then subcloned into U1(b) to make integrative plasmids YIpAMT1::URA3 and YIpAMT1(m)::URA3. These two plasmids were digested to completion with *StuI* restriction enzyme, which has a unique site located in the *URA3* open reading frame to facilitate sequence-specific recombination. The linearized integrative plasmids were integrated in single copy at the *URA3* locus of the *amt1-1* strain, in which the authentic *AMT1* locus was disrupted by an insertion of the *Salmonella hisG* gene (Zhou and Thiele 1991).

Because our original *AMT1* genomic clone lacks the 3' untranslated sequences of this gene, which contains the *AMT1* transcription termination signal, we carried out inverse PCR (IPCR) and DNA cloning to isolate a 1.6-kb *BglII-SpeI* DNA restriction fragment that encompasses the entire *AMT1* mRNA transcript (Ochman et al. 1990; GenBank accession number M69146). This experiment also allowed us to identify a short extragenic sequence that was ligated 3' to the *AMT1*-coding sequence during our initial cloning; however, this did not alter the *AMT1* promoter or coding sequence.

RNA analysis

The steady-state levels of *AMT1*, *MT-I*, and *MT-II* mRNA before or after induction by copper or other metal ions were analyzed by either RNA blotting or primer extension assays as described (Ausbuel et al. 1987). *C. glabrata* wild-type 85/038, Q (*ura3⁻*), or *amt1-1* cells containing episomal or integrative plasmids were grown to logarithmic phase and induced with different copper concentrations and time periods as indicated in each figure legend. Specific *AMT1*, *MT-I*, *MT-II*, *URA3*, and actin mRNA species were detected using ³²P-labeled DNA fragments, respectively, as follows: 1.6-kb *BglII-SpeI* (this paper; Zhou and Thiele 1991); 0.7-kb *EcoRI-ApaI* (Mehra et al. 1989); 0.7-kb *EcoRI-SmaI* (Mehra et al. 1990); 1.5-kb *XhoI-PstI* (P. Zhou, M. Szczypka, R. Young, and D. Theile, in prep.); and 1.6-kb *HindIII* (Ng and Abelson 1980) DNA restriction fragments. It is noteworthy that the *MT-II* probe used in RNA blot hybridization detected both the *MT-IIa* and *MT-IIb* mRNAs. Deoxyoligonucleotides were synthesized for use as primers in extension reactions and were labeled at 5' termini with polynucleotide kinase and [γ -³²P]ATP; the *AMT1*-specific primer contained 26 nucleotides with the sequence 5'-GATTACTACCATGGTG-CAAATGTGTG-3', complementary to nucleotide positions +65 to +40 of the *AMT1* gene (Zhou and Thiele 1991) and was used to determine the *AMT1* transcription initiation sites. The *AMT1*-specific primer was also employed in the primer extension reactions using the *AMT1-lacZ* mRNA as a template. This experiment demonstrated that the transcription of the *AMT1-lacZ* fusion gene initiated at sites identical to those of the authentic *AMT1* gene (P. Zhou, unpubl.).

Analysis of DNA-protein interactions

AMT1 DNA-binding studies were carried out with partially purified *AMT1* protein expressed in *E. coli* using the T7 RNA polymerase system (Studier et al. 1990; Zhou and Thiele 1991; Zhou et al. 1992). Electrophoretic mobility shift assays were used to detect binding of the copper-activated *AMT1* protein to the ³²P-labeled 326-bp *BglII-SspI* DNA restriction fragments containing either the wild-type or mutant *AMT1*-binding site.

DNase I footprinting and methylation interference assays

were used to map the AMT1-binding site within the *AMT1* promoter. Two plasmids were constructed for the isolation of the *AMT1* promoter DNA restriction fragment probes. The 326-bp *BglII*–*SspI* fragment was inserted into the *Bam*HI–*EcoRV* sites of pBluescript SK(+) to make plasmid pAMT1(U). The 369-bp *MvaI*–*RsaI* DNA restriction fragment was end-repaired by the Klenow fragment of DNA polymerase I and subcloned into the *EcoRV* site of pBluescript SK(+) to make plasmid pAMT1(L). Probes labeled on the coding or noncoding strand of the *AMT1* promoter were prepared by digestion of pAMT1(U) and pAMT1(L) with appropriate restriction enzymes and then radiolabeled with [α - ^{32} P]dCTP and the Klenow fragment of DNA polymerase I (Ausubel et al. 1987). DNase I footprinting and methylation interference assays were carried out as described in detail (Ausubel et al. 1987; Zhou et al. 1992).

³⁵S-Labeling of the MTs and gel electrophoresis

To analyze the MT-I and MT-II protein levels in *C. glabrata* cells induced with copper, a 5-ml culture of the isogenic *AMT1::URA3* and *AMT1(m)::URA3* cells were grown to log phase in SC media lacking cysteine and methionine (SC – Cys – Met). Cells were pelleted and resuspended in 0.4 ml of SC – Cys – Met and incubated at 30°C for 10 min with shaking in the presence of 8.0 μ l of [^{35} S]cysteine (ICN, 227 μ Ci/ml) and CuSO₄ at concentrations indicated in Figure 8. Cells were then harvested, suspended in 400 μ l of lysis buffer (10.0 mM Tris-HCl at pH 7.8, 10 mM PMSF, 1 mM dithiothreitol), and 400 μ l of acid-washed glass beads, and lysed by vortexing at top speed four times for 1 min each at 4°C. The soluble proteins were isolated by centrifugation at 14,000 rpm for 15 min, and protein concentrations were determined by the Bradford assay (Ausubel et al. 1987). Protein (1.0 μ g) was treated with 500 μ M CuSO₄ at room temperature for 10 min. This step saturated MTs with copper and converted all MT-I and MT-II protein isoforms into the uniformly copper-coordinated conformations that migrate as distinct bands on native polyacrylamide gel (Fig. 8). Equal volumes of buffer (0.1 M Tris-HCl at pH 6.8, 25% glycerol, 0.35 M 2-mercaptoethanol, 0.1% bromphenol blue) were added to each protein extract. The samples were then loaded on 25% nondenaturing polyacrylamide gels and subjected to electrophoresis at 200 V until the bromphenol blue reached the bottom of the gel. The proteins were fixed in the gel with 10% acetic acid and 30% methanol for 1 hr and were fluorographed with EN³HANCE solution as described by the supplier (DuPont). The gel was then dried under vacuum and exposed to Kodak XAR-5 film with an intensifying screen at –80°C. [^{35} S]Cysteine-labeled protein extracts from *C. glabrata* 2001-L5 and RM4 (*MT-IIa* Δ , *MT-IIb* Δ) cells were coelectrophoresed on a 25% native polyacrylamide gel and served as controls to identify the MT-I and MT-II polypeptides. It should be noted that the *MT-IIa* and *MT-IIb* genes encode identical protein species, although their mRNA transcripts are slightly different in the 3' untranslated regions (Mehra et al. 1992).

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References

- Angel, P., K. Hattori, T. Smeal, and M. Karin. 1988. The *jun* proto-oncogene is positively autoregulated by its product, Jun/AP-1. *Cell* 55: 875–885.
- Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, eds. 1987. *Current protocols in molecular biology*. Greene/Wiley, New York.
- Barns, S.M., D.J. Lane, M.L. Sogin, C. Bibeau, and W.G. Weisburg. 1991. Evolutionary relationships among pathogenic *Candida* species and relatives. *J. Bacteriol.* 173: 2250–2255.
- Buchman, C., P. Skroch, W. Dixon, T.D. Tullius, and M. Karin. 1990. A single amino acid change in CUP2 alters its mode of DNA-binding. *Mol. Cell. Biol.* 10: 4778–4787.
- Butt, T.R., E. Sternberg, J. Herd, and S.T. Crooke. 1984. Cloning and expression of a yeast copper metallothionein gene. *Gene* 27: 23–33.
- Evans, C.F., D.R. Engelke, and D.J. Thiele. 1990. ACE1 transcription factor produced in *Escherichia coli* binds multiple regions within yeast metallothionein upstream activation sequences. *Mol. Cell. Biol.* 10: 426–429.
- Falvey, E. and U. Schibler. 1991. How are the regulators regulated? *FASEB J.* 5: 309–314.
- Fogel, S. and J. W. Welch. 1982. Tandem gene amplification mediates copper resistance in yeast. *Proc. Natl. Acad. Sci.* 79: 5342–5346.
- Fürst, P., S. Hu, R. Hackett, and D.H. Hamer. 1988. Copper activates metallothionein gene transcription by altering the conformation of a specific DNA-binding protein. *Cell* 55: 705–717.
- Gralla E.B., D.J. Thiele, P. Silar, and J.S. Valentine. 1991. ACE1, a copper-dependent transcription factor, activates expression of the yeast copper, zinc superoxide dismutase gene. *Proc. Natl. Acad. Sci.* 88: 8558–8562.
- Halliwell, B. and J.M.C. Gutteridge. 1984. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J.* 219: 1–14.
- Hu, S., P. Fürst, and D.H. Hamer. 1990. The DNA and copper binding function of ACE1 are interdigitated within a single domain. *New Biol.* 2: 1–13.
- Huibregste, J.M., D.R. Engelke, and D.J. Thiele. 1989. Copper-induced binding of cellular factors to yeast metallothionein upstream activation sequences. *Proc. Natl. Acad. Sci.* 86: 65–69.
- Kägi, J.H.R. and Y. Kojima. 1987. Chemistry and biochemistry of metallothionein. In *Metallothionein II* (ed. J.H.R. Kägi and Y. Kojima), pp. 25–61. Birkhäuser Verlag, Basel, Switzerland.
- Kägi, J.H. and A. Schaffer. 1988. Biochemistry of metallothionein. *Biochemistry* 27: 8509–8515.
- Karin, M., R. Najarian, A. Haslinger, P. Valenzuela, J.W. Welch, and S. Fogel. 1984. Primary structure and transcription of an amplified genetic locus: The *CUP1* locus of yeast. *Proc. Natl. Acad. Sci.* 81: 337–341.

- Lin, C.M. and D.J. Kosman. 1990. Copper uptake in wild-type and copper metallothionein-deficient *Saccharomyces cerevisiae*. *J. Biol. Chem.* **265**: 9194–9200.
- Maloy, S. and V. Stewart. 1993. Autogenous regulation of gene expression. *J. Bacteriol.* **175**: 307–316.
- Mason, M.M., B.A. Lasker, and W.S. Riggsby. 1987. Molecular probes for identification of medically important *Candida* species and *Torulopsis glabrata*. *J. Clin. Microbiol.* **25**: 563–566.
- Mehra, R.K., J.R. Garey, T.R. Butt, W.R. Gray, and D.R. Winge. 1989. *Candida glabrata* metallothioneins. *J. Biol. Chem.* **264**: 19747–19753.
- Mehra, R.K., J.R. Garey, and D.R. Winge. 1990. Selective and tandem amplification of a member of the metallothionein gene family in *Candida glabrata*. *J. Biol. Chem.* **265**: 6369–6375.
- Mehra, R.K., J.L. Thorvaldsen, I.G. Macreadie, and D.R. Winge. 1992. Disruption analysis of metallothionein-encoding genes in *Candida glabrata*. *Gene* **114**: 75–80.
- Myers, A.M., A. Tzagoloff, D.M. Kinney, and C.J. Lusty. 1986. Yeast shuttle and integrative vectors with multiple cloning sites suitable for construction of *lacZ* fusions. *Gene* **45**: 299–310.
- Ng, R. and J. Abelson. 1980. Isolation and sequence of the gene for actin in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci.* **77**: 3912–3916.
- Ochman, H., M.M. Medhora, D. Garza, and D.L. Hartl. 1990. Amplification of flanking sequences by inverse PCR. In *PCR protocols, a guide to methods and applications* (ed. M.A. Innis, D.H. Gelfand, J.J. Sininsky, and T.J. White), pp. 219–227. Academic Press, San Diego, CA.
- Serfling, E. 1989. Autoregulation—A common property of eukaryotic transcription factors? *Trends Genet.* **5**: 131–133.
- Sikorski, R.S. and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**: 19–27.
- Sinnott, J.T., J.P. Cullison, and M.P. Sweeney. 1987. *Candida* (*Torulopsis*) *glabrata*. *Infect. Control* **8**: 334–336.
- Studier, F.W., A.H. Rosenberg, J.J. Dunn, and J.W. Dubendorff. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* **185**: 60–89.
- Szczypka, M.S. and D.J. Thiele. 1989. A cysteine-rich nuclear protein activates yeast metallothionein gene transcription. *Mol. Cell. Biol.* **9**: 421–429.
- Tamai, K.T., E.B. Gralla, L. Ellerby, J.S. Valentine and D.J. Thiele. 1993. Yeast and mammalian metallothioneins functionally substitute for yeast copper-zinc superoxide dismutase. *Proc. Natl. Acad. Sci.* (in press).
- Thanos, D. and T. Maniatis. 1992. The high mobility group protein HMG I(Y) is required for NF- κ B-dependent virus induction of the human IFN- β gene. *Cell* **71**: 777–789.
- Thiele, D.J. 1988. ACE1 regulates expression of the *Saccharomyces cerevisiae* metallothionein gene. *Mol. Cell. Biol.* **8**: 2745–2752.
- . 1992. Metal-regulated transcription in eukaryotes. *Nucleic Acids Res.* **20**: 1183–1191.
- Zhou, P. and D.J. Thiele. 1991. Isolation of a metal-activated transcription factor gene in *Candida glabrata* by complementation in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci.* **88**: 6112–6116.
- Zhou, P., M.S. Szczypka, T. Sosinowski, and D.J. Thiele. 1992. Expression of a yeast metallothionein gene family is activated by a single metalloregulatory transcription factor. *Mol. Cell. Biol.* **12**: 3766–3775.

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References

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