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 amtl 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]

Received April 20, 1993; revised version accepted June 22, 1993.

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 Schaeffer 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 [UASCuP1] [Fürst et al. 1988; Huibregste et al. 1989; Szczypta 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*] or multiple tandem copies [cup1*] 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 transcripational 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 BglII–SspI 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 32P-labeled 169-bp SspI–RsaI 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-
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 interaction, we carried out methylation interference analysis 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) 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 BglII–StyI 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...
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 (Kagi 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 AMTI 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 µM CuSO4, 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 µM CuSO4 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 pAMTI-lacZ recipient cells was also rapidly induced with kinetics identical to that of the authentic endogenous AMT1 gene expression.

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 pAMTI-lacZ, pAMT1(m)-lacZ, and pRS316 plasmids either untreated (-) or treated (+) with 100 µM CuSO4. 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 AMTI transcription. Dideoxy sequencing reactions [lanes G,A,T,C] were carried out using pAMTI-lacZ plasmid DNA and the lacZ-specific primer, and were used to map the primer extension products.

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 µM CuSO4 for the time (in min) indicated at the top of each lane. Total RNA was prepared, and 30 µg 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 amtl-1 [amtl 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 CuSO₄. 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 CuSO₄, a three- to fourfold decrease compared with the wild-type or AMT1::URA3 strains. As demonstrated previously, the amtl-1 strain is hypersensitive to copper and fails to grow even in the presence of 25 μM CuSO₄ [Zhou et al. 1992]. In these experiments, copper-sensitive cells arrested at the single-cell stage when challenged with toxic concentrations of CuSO₄ [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 containing ~10 times the concentration of exogenous copper as the amtl-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 amtl-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)::URA3] 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-II 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-
of the gel in this experiment. Figure 7 (right) shows a quantitation of the steady-state mRNA levels of AMT1, AMTI::hisG, MT-I, and MT-II in AMT1::URA3 and AMT1(m)::URA3 cells exposed to the range of copper concentrations. The wild-type AMTI 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 μM CuSO₄. The endogenous amti-1 locus, which carries an insertion of the Salmonella hisG gene within the AMT1 open reading frame, synthesized a hybrid AMTI::hisG mRNA whose identity was confirmed by its hybridization to a 32P-labeled hisG probe by RNA blotting (data not shown). The expression of this hybrid gene was also induced by copper-activated AMTI with a similar dose response and a 4.2-fold induction, presumably mediated through the intact AMTI-binding site within the promoter of the hisG insertional inactivated amti-1 locus. In contrast, no copper-inducible mRNA accumulation was observed for the AMTI(m) gene in the AMT1(m)::URA3 strain. However, mRNA driven from the AMTI::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 AMTI::hisG mRNA were also detected in the amti-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 AMTI promoter is dependent on both the presence of a functional AMTI-binding site and a functional AMTI protein. The AMTI or AMTI::hisG mRNA levels in the AMT1::URA3 cells were highest when cells were treated with 100 μM 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 μM). The overall level of the MT-I mRNA in the AMT1(m)::URA3 strain is ~1.4-fold lower than that in the AMTI::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 μM of CuSO₄. 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 μM and 1 mM]. It should be noted that 10-min exposure of AMTI(m)::URA3 with 500 μM or 1 mM copper did not immediately impair cell growth, as judged by similar increases of OD₆₀₀ as well as similar levels of actin mRNA observed between AMT1::URA3 and AMTI(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-labeling proteins in AMT1::URA3 and AMT1(m)::URA3 cells with [35S]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 AMTI(m)::URA3 cells. The MT-I protein reached similar maximum levels upon treatment with low copper (100 μM) 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, and 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. [35S]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 μg) was subjected to electrophoresis on a 25% non-denaturing polyacrylamide gel. The arrowheads at left indicate the MT-I and MT-II protein species.
fication 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 (Lin and Kosman 1990). The rapid expression of several copper homeostatic genes regulated by AMT1, for example, is constitutively expressed (Szczypka and Thiele 1989), 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-IIa 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, AMTI, 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 ure3- [Q] strain and the AMTI disruption strain amtl-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 amtl-1 strain is the parental strain for the integration of wild-type (AMTI) or mutant (AMTl(m)) genes at the ure3 locus to construct isogenic AMTI::URA3 and AMTl(m)::URA3 strains by transformation and homologous recombination (Ausubel 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 (Ausubel 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 Theile 1989], which contains the wild-type ACEI and three copies of the tandemly amplified CUP1 genes, was used to examine the mRNA levels from the ACEI 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 (Ausubel et al. 1987).

To study the role that the single AMTI-binding site plays in AMTI autoregulation, two guanine residues (G-192 and G-189) worthy that the AMTI promoter nucleotide positions –201 to –168 relative to the +1 transcription start site (this paper; Ausubel et al. 1987; P. Zhou, M. Szczypka, R. Young, and D. Theile, in prep.) 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 AMTI 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 (AMTI) or mutant (AMTl(m)) AMTI genes were then subcloned into U1(b) to make integrative plasmids YIpAMTI1::URA3 and YIpAMTI(m)::URA3. These two plasmids were digested to completion with Stul 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 amtl-1 strain, in which the authentic AMTI locus was disrupted by an insertion of the Salmonella hisG gene [Zhou and Theile 1991].

Because our original AMTI genomic clone lacks the 3' untranslated sequences of this gene, which contains the AMTI 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 AMTI mRNA transcript [Ochman et al. 1990, GenBank accession number M90146]. This experiment also allowed us to identify a short extragenic sequence that was ligated 3' to the AMTI-coding sequence during our initial cloning; however, this did not alter the AMTI promoter or coding sequence.

RNA analysis

The steady-state levels of AMTI, 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 [Ausubel et al. 1987]. C. glabrata wild-type 85/038, Q [ura3-], or amtl-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 AMTI, 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 Theile 1991], 0.7-kb EcoRI–ApaI [Mehra et al. 1989]; 0.7-kb EcoRI–Smal [Mehra et al. 1990], 1.5-kb Xhol–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. Deoxoyguonucleotides were synthesized for use as primers in extension reactions and were labeled at 5' termini with polynucleotide kinase and [γ-32P]ATP, the AMTI-specific primer contained 26 nucleotides with the sequence 5'-GATTACTACCATGTCGAAATG-3', complementary to nucleotide positions +65 to +90 of the AMTI gene [Zhou and Theile 1991] and was used to determine the AMTI transcription initiation sites. The AMTI-specific primer was also employed in the primer extension reactions using the AMTI–lacZ mRNA as a template. This experiment demonstrated that the transcription of the AMTI–lacZ fusion gene initiated at sites identical to those of the authentic AMTI gene [P. Zhou, unpubl.].

Analysis of DNA–protein interactions

AMTI DNA-binding studies were carried out with partially purified AMTI protein expressed in E. coli using the T7 RNA polymerase system [Studier et al. 1990; Zhou and Theile 1991]; Zhou et al. 1992]. Electrophoretic mobility shift assays were used to detect binding of the copper-activated AMTI protein to the [32P]-labeled 326-bp BglII–SpeI DNA restriction fragments containing either the wild-type or mutant AMTI-binding site. DNase I footprinting and methylation interference assays...
we were 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 BamHI–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-32P]dCTP and the Klenow fragment of DNA polymerase I [Aubusel et al. 1987]. DNase I footprinting sites of pBluescript SK(+) to make plasmid pAMTI(U). The

To analyze the MT-I and MT-II protein levels in C. glabrata cells induced with copper, a 5-ml culture of the isogenic strains 2001-L5, RM2, and RM4; L. Gedamu for advice on MT protein analysis; and R. Young for technical assistance. We are grateful to Geraldine Butler for providing information on the AMT1 nucleotide sequence. P.Z. was supported in part by a Rackham pre-doctoral fellowship from the Horace H. Rackham School of Graduate Studies at the University of Michigan and a Loeb pre-doctoral fellowship from the University of Michigan Cancer Center. This work was funded by grants GM41840 from the National Institutes of Health, and by grant M01-RR00042 to the General Clinical Research Center, University of Michigan Medical Center.

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References


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

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*Genes Dev.* 1993, 7: Access the most recent version at doi:10.1101/gad.7.9.1824