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Expression of a Yeast Metallothionein Gene Family Is Activated by a Single Metalloregulatory Transcription Factor

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The opportunistic pathogenic yeast *Candida glabrata* elicits at least two major responses in the presence of high environmental metal levels: transcriptional induction of the metallothionein gene family by copper and the appearance of small (γ-Glu-Cys)$_n$Gly peptides in the presence of cadmium. On the basis of a *trans*-activation selection scheme in the baker's yeast *Saccharomyces cerevisiae*, we previously isolated a *C. glabrata* gene which encodes a copper-activated DNA-binding protein designated AMT1. AMT1 forms multiple specific DNA-protein complexes with both *C. glabrata MT-I* and *MT-II$_a$* promoter DNA fragments. In this report, we localize and define the AMT1-binding sites in the *MT-I* and *MT-II$_a$* promoters and characterize the mode of AMT1 binding. Furthermore, we demonstrate that the AMT1 protein *trans* activates both the *MT-I* and *MT-II$_a$* genes in vivo in response to copper and that this activation is essential for high-level copper resistance in *C. glabrata*. Although AMT1-mediated *trans* activation of the *C. glabrata* metallothionein genes is essential for copper resistance, AMT1 is completely dispensable for cadmium tolerance. The distinct function that metallothionein genes have in copper but not cadmium detoxification in *C. glabrata* is in contrast to the role that metallothionein genes play in tolerance to multiple metals in higher organisms.

Environmental metal toxicity is a hazard of increasing significance because of the constant deposition of these pollutants in the biosphere. Fortunately, organisms have evolved a number of diverse mechanisms to cope with high environmental metal levels which allow the appropriate accumulation of essential yet toxic metals such as copper and which prevent the accumulation of harmful metals such as cadmium. In prokaryotic organisms, metal tolerance is accomplished primarily by modulating the efflux or exclusion of metals or by reduction of toxic metals to an innocuous state (39–41). In eukaryotic organisms, the mechanisms implemented for metal balance are not well understood and are currently under intense investigation. Most eukaryotic organisms synthesize one or more forms of a low-molecular-weight, cysteine-rich, metal-binding protein known as metallothionein (MT) (27). Because of their ability to efficiently bind metals, and the fact that all known MT genes are transcriptionally activated by metals, MT proteins are thought to play an important role in metal tolerance and homeostasis and may have other important functions (4, 19, 20, 25, 27, 28, 48).

The organization and expression of MT genes vary in different organisms. In humans, mice, and most other higher eukaryotes, MTs are encoded by gene families (19). Higher eukaryotic MT genes are transcriptionally induced by many metals (such as Cu, Cd, and Zn) and are thought to confer resistance to high levels of these same metals (19). This metal-activated transcriptional response occurs through conserved *cis*-acting metal-regulatory promoter elements (MREs) and putative metal-responsive DNA-binding transcription factors (19, 25, 48). Since little information concerning higher eukaryotic MT gene *trans*-acting metalloregulatory transcription factors is currently available, it is not clear whether each MT gene in a given organism is activated by a distinct or common metalloregulatory transcription factor (48). A further complicating feature is that isoforms of the human MT genes, such as *MT-IB*, *MT-IE*, *MT-IF*, and *MT-IG*, are regulated differentially in response to metals in a cell- or tissue-specific manner (14, 15, 22, 26).

The baker's yeast *Saccharomyces cerevisiae* harbors a single MT gene (*CUP1*), which is essential for resistance to copper toxicity (8–10, 20). *CUP1* transcription is known to be induced in response to two metals, copper and the physiologically irrelevant though chemically similar metal silver (10, 17, 19, 48, 49). *CUP1* transcription induction by either metal is mediated through the action of a Cu- or Ag-activated sequence-specific DNA-binding protein called ACE1/CUP2 (6, 17, 46–49). *S. cerevisiae* cells exposed to Cu or Ag foster the rapid binding of metallated monomeric ACE1 to four distinct regulatory sites within a region of the *CUP1* promoter termed the upstream activation sequence (5, 13, 24, 48). Once bound, ACE1 activates *CUP1* transcription in large part through the carboxy-terminal acidic region (23, 47). ACE1 therefore appears to be a member of the acidic activator class of transcription factors (36, 42). The Cu- or Ag-activated DNA-binding domain of ACE1 resides within the amino-terminal 101 residues, is highly positively charged, and contains 11 cysteine residues which are individually of critical importance for this function (5, 23, 47). Recent reports demonstrate that the ACE1 DNA-binding domain cooperatively binds Cu(I) in a 1:6 stoichiometry as a sulfur-coordinated polynuclear cluster (12, 16, 35).

The opportunistic pathogenic yeast *Candida glabrata* has recently been demonstrated to elicit distinct biological responses in the presence of cadmium or copper salts (31, 33). In an environment containing elevated cadmium concentrations, *C. glabrata* produces (γ-Glu-Cys)$_n$Gly peptides, presumably by a nontranslational mechanism, which are known to bind cadmium and may confer resistance to this metal (31). Furthermore, work by Mehra et al. has demonstrated that, like higher eukaryotes, *C. glabrata* harbors an MT gene family composed of a single *MT-I* gene, multiple tandemly amplified *MT-II$_a$* genes, and a single unlinked *MT-II$_b$* gene

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(29–33). In contrast to higher eukaryotes, however, the *C. glabrata* MT genes are transcriptionally activated only in response to the metal copper or silver (29). Therefore, *C. glabrata* provides an experimental system which possesses an MT gene organization similar to that of higher eukaryotes but a metal specificity for transcriptional induction similar to that of *S. cerevisiae*. To begin to understand how such an organism regulates the metal-dependent transcription of MT genes, we recently cloned a *C. glabrata* gene, called *AMT1*, encoding a copper- or silver-activated DNA-binding protein (51). We demonstrated that AMT1 protein, synthesized in *Escherichia coli*, forms in vitro multiple copper-dependent, sequence-specific DNA-protein complexes with promoter fragments from both the *MT-I* and *MT-II*$_a$ genes (51). Consistent with a role as a transcription factor, AMT1 shares several features at the primary structural level with ACE1 from *S. cerevisiae*. The amino-terminal 110 amino acid residues of AMT1 are 73% similar and 55% identical to the 100 amino-terminal residues encompassing the ACE1 copper-activated DNA-binding domain, and AMT1 also contains an abundance of acidic residues in the carboxy-terminal region, corresponding to a potent ACE1 *trans*-activation domain (23, 51). In this report, we demonstrate that *AMT1* is required for both basal and copper-dependent activation of *MT-I* and *MT-II*$_a$ gene transcription in vivo, indicating that a single metalloregulatory transcription factor activates the family of MT genes in *C. glabrata*. Furthermore, we localize and characterize the AMT1-binding sites in these promoters and assess the subunit composition of DNA-bound AMT1. Moreover, the analysis of cells harboring a chromosomal *AMT1* disruption allele indicates that unlike in higher eukaryotes, cadmium detoxification in *C. glabrata* is independent of metal-activated MT gene transcription.

MATERIALS AND METHODS

Strains and growth conditions. *C. glabrata* strains used in this work are all based on strain 85/038, which was described previously (51). The uracil auxotrophic derivative of 85/038, designated Q, was isolated as a 5-fluoroorotic acid-resistant derivative after ethyl methanesulfonate mutagenesis (data not shown). Yeast strains were grown at 30°C in rich (YPD) or synthetic complete (SC) medium lacking nutrients as specified and as previously described (2, 37). Metal resistance tests and copper treatments were conducted by the addition of metal salts to final concentrations as indicated in the figure legends. *E. coli* XL-1 blue was used for the construction and maintenance of plasmids by standard techniques (2).

RNA analysis. The transcription initiation sites of the *MT-I* and *MT-II*$_a$ genes were determined by primer extension reactions (2, 18). The *MT-I* and *MT-II*$_a$ promoters in the *C. glabrata* 85/038 strain used in these studies have several sequence alterations compared with the published sequences for *C. glabrata* 2001 (29, 30). The *MT-I* gene was isolated four independent times by using polymerase chain reactions (2), and sequencing results indicated that for each isolate, two of the five AACAAAC repeats found in the *MT-I* promoter in strain 2001 (nucleotides 282 to 295) (29) were absent in the *MT-I* gene of strain 85/038. The genomic *MT-II*$_a$ gene was isolated together with the *AMT1* gene in our previous cloning experiments (51). This *MT-II*$_a$ clone was initially identified as a plasmid insert which conferred copper resistance when transformed into a copper-sensitive *S. cerevisiae* strain; however, it failed to *trans* activate an endogenous *MT-I–lacZ* fusion gene (51). Sequence analysis of this insert revealed that it contains the *C. glabrata MT-II*$_a$ gene previously identified (30, 33). Variations and nucleotide insertions in the promoter of the *MT-II*$_a$ gene from *C. glabrata* 85/038 were also observed and are summarized as follows: nucleotide substitutions include G-107→A, G-266→A, T-389→C and T-595→A; nucleotide insertions following the original 5′ nucleotide (underlined) were G-256TT, C-299C, T-315A, C-318A, and A-340T. All nucleotide positions described in this report are based on the *MT-I* and *MT-II*$_a$ sequences of *C. glabrata* 85/038.

Two deoxyoligonucleotides were synthesized for use as primers in extension reactions and labeled at 5′ termini with polynucleotide kinase and [γ-^{32}P]ATP (2). The *MT-I*-specific primer contained 15 nucleotides with the sequence 5′-GCACTCGCACTTGTC-3′, complementary to nucleotide positions +118 to +132 of the *MT-I* gene (29); the *MT-II*-specific primer contained 18 nucleotides with the sequence 5′-CAGTTGACTTGTTCAGGC-3′, which is complementary to nucleotide positions +64 to +81 of the *MT-II*$_a$ gene (30). Wild-type *C. glabrata* 85/038 cells were grown to mid-log phase in SC medium and induced with 100 μM $CuSO_4$ for 45 min. Total RNA from untreated and copper-induced cells was prepared as previously described (2), and 20 μg of RNA was used in each primer extension reaction to detect *MT-I* and *MT-II* mRNAs (18). The transcription start sites of the *MT-I* and *MT-II* genes were determined by comparing the electrophoretic mobilities of the primer extension products with those of sequencing reactions, using plasmid DNA containing the *MT-I* or *MT-II*$_a$ gene, primed with the same oligonucleotides used in the primer extension reactions (2). It should be noted that in both primer extension and RNA blot hybridization assays, the probes used detect both the *C. glabrata MT-II*$_a$ and *MT-II*$_b$ mRNA species (32).

RNA blot assays were carried out to assess steady-state levels of the *MT-I* and *MT-II* mRNA species in the wild-type (85/038) and *amt1-1* disruption strains (2). Total mRNA was prepared from both wild-type and *amt1-1* strains as described above except that 50 μM $CuSO_4$ was used for the copper treatment conditions for 30 min. Under these conditions, both wild-type and *amt1-1* strains continued to grow in the presence of $CuSO_4$, as monitored by cell culture optical density readings. The 0.7-kb *Eco*RI-*Apa*I DNA restriction fragment including the *MT-I* gene and the 0.7-kb *Eco*RI-*Sma*I DNA restriction fragment spanning the *MT-II*$_a$ gene (31, 32) were radiolabeled by nick translation with [α-^{32}P] dCTP (2) and were used as hybridization probes to detect specific *MT-I* and *MT-II* mRNA species. The amount of RNA used in each experiment was judged to be equivalent by comparing the intensity of ethidium bromide-stained rRNA species and by the levels of *C. glabrata URA3* mRNA present when the *URA3* gene probe was used (data not shown).

Subunit composition of DNA-bound AMT1. For the analysis of AMT1 protein-DNA interactions, *E. coli* extracts containing full-length AMT1 protein were prepared by expressing the *AMT1* gene in *E. coli*, using the T7 RNA polymerase system (45). Extracts were subjected to $(NH_4)_2SO_4$ precipitation by the addition of two-thirds volume of saturated $(NH_4)_2SO_4$ to the crude soluble extract. After gentle mixing for 30 min at 4°C on a rotating wheel, the insoluble fraction was pelleted by centrifugation at 16,000 × *g* for 10 min at 4°C and suspended in extract storage buffer (40 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 200 mM KCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol). *E. coli* extracts containing a truncated version

of the AMT1 polypeptide [tAMT1(1-115)], containing the amino-terminal 115 amino acids, were prepared as described above. The plasmid for expression of tAMT1(1-115) was constructed as follows. Site-directed mutagenesis was performed on the wild-type *AMT1* sequence with a mutagenic primer (5'-GCGAATTAAAGCTTTAGTTATC-3'), engineered to replace Leu-116 of the AMT1 protein with a TAA translation termination codon (2, 51). A *Hin*dIII site was also introduced 3' to the TAA to facilitate screening for the presence of the mutation. The DNA sequence of the mutagenized *AMT1* gene was determined to verify that no other coding region changes were introduced, and a 0.9-kb *Nco*I-*Bam*HI DNA restriction fragment encompassing the altered *AMT1* gene was subcloned into the *Nco*I-*Bam*HI sites of plasmid pET-8c (45). Protein concentrations of all extracts were determined by the Bradford assay (2).

Experiments to determine the subunit composition of DNA-bound AMT1 were performed by using extracts from *E. coli* cells expressing the truncated AMT1 polypeptide [tAMT1(1-115)] and the full-length AMT1 protein. Complementary oligonucleotides representing a single AMT1-binding site, d, in the *MT-II*$_a$ promoter, 5'-GCTAGATTTAGC TG-3' and 5'-CAATCAGCTAAATC-3', and containing four-base overhangs at each end were annealed by heating to 100°C for 2 min and slowly cooled to room temperature. The termini of the AMT1-binding site double-stranded oligonucleotide were filled in with [α-^{32}P]dGTP by using Klenow enzyme, and the DNA fragment was purified on a 7.5% native polyacrylamide gel and electroeluted. Truncated or full-length AMT1 extract (8 μg of each) or a mixture of 4 μg of AMT1 and tAMT1(1-115) extract was mixed with 0.5 ng of probe (5,000 cpm) at room temperature for 10 min in separate reactions and in either the absence or presence of 100 μM $CuSO_4$. The reaction mixtures were subsequently loaded onto a 5% native low-ionic-strength polyacrylamide gel (2, 51) and subjected to electrophoresis at 250 V for 4 h at 4°C. In a parallel experiment, AMT1, tAMT1(1-115), or a mixture of both extracts was denatured in 5 M urea at 4°C for 30 min and renatured by dialysis against three changes (2 liters each) of buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 60 mM KCl, 200 μM EDTA, 500 μM dithiothreitol, 500 μM phenylmethysulfonyl fluoride, 20% glycerol) at 4°C overnight. The protein concentration of the resulting extracts was determined, and the extracts were used in electrophoretic mobility shift assays as described above.

DNase I footprinting and methylation interference assays. DNase I footprinting and methylation interference assays were used to identify the AMT1-binding sites on the *MT-I* and *MT-II*$_a$ promoters. Three plasmids were constructed for the isolation of the *MT-I* and *MT-II*$_a$ DNA restriction fragment probes. The 240-bp *Eco*RI-*Bst*YI fragment of the *MT-I* promoter (see Fig. 3A) was inserted into pUC19 to make plasmid pMT-IB. For preparing *MT-II*$_a$ promoter probes, two fragments from the 5' noncoding region of the *MT-II*$_a$ gene were isolated as a 335-bp *Sma*I-*Xba*I fragment containing the upstream AMT1-binding sites c, d, e, and f and a 260-bp *Ssp*I-*Eco*RI fragment harboring the downstream binding sites a, b, and c (see Fig. 3A). These two fragments were inserted into pBluescript SK(+), digested with the appropriate restriction enzymes, and radiolabeled with [α-^{32}P]dCTP and the Klenow fragment of *E. coli* DNA polymerase I to generate uniquely end-labeled DNA fragments. DNase I footprinting and methylation interference assays were carried out as described in detail elsewhere (2). Chemical DNA sequencing reactions with the end-labeled DNA fragments used in DNase I footprinting reactions were carried out (3) and used to identify the regions protected from DNase I by bound AMT1 and the critical purine residues, which, when methylated by dimethylsulfate, interfered with the binding of AMT1 protein. Precise mapping of AMT1-binding sites distal to labeled termini was accomplished by extended electrophoretic fractionations of reaction products (data not shown). All reaction products were fractionated by electrophoresis on 6% polyacrylamide–7 M urea sequencing gels, and the gels were dried and exposed to XAR-5 film at −80°C with intensifying screens.

Disruption of the *AMT1* locus. A disruption allele of the wild-type *AMT1* locus in strain 85/038 (Q) was constructed as follows. The 1.4-kb *Bgl*II-*Pst*I fragment spanning the *AMT1* gene was first inserted into the *Bam*HI-*Pst*I sites of pBluescript SK(+) to construct plasmid pBZ-7. A 3.8-kb *Bam*HI-*Bgl*II DNA restriction fragment cassette, containing the *S. cerevisiae URA3* gene flanked by two direct repeats derived from the *Salmonella hisG* gene, was isolated from plasmid pNKY51 (1), and blunt-ended termini were created with the Klenow fragment of DNA polymerase I enzyme. This 3.8-kb fragment was inserted at the Klenow-repaired *Sty*I site of the 1.4-kb wild-type *AMT1* gene in pBZ-7, between codons 36 and 37 of the *AMT1* open reading frame (51). The resulting 5.2-kb fragment containing the disrupted *AMT1* allele was isolated by agarose gel electrophoresis after digestion with *Xba*I and *Sal*I and used to transform *C. glabrata* 85/038 Q to uracil prototrophy (2). Several *URA*$^+$ transformants were single colony purified and grown on SC agar containing 5-fluoroorotic acid (2) to select for cells having undergone recombination between the two direct *hisG* repeats, resulting in loss of the *URA3* gene. The chromosomal structure of this *amt1-1* allele was verified by Southern blotting using the 1.4-kb *Bgl*II-*Pst*I DNA restriction fragment, which contains the complete *AMT1* open reading frame, as a probe (51) (see Fig. 5B). The genomic DNA of *C. glabrata* wild-type (85/038), Q, and *amt1-1* cells was digested to completion with *Bgl*II and *Pst*I, subjected to electrophoresis on a 1% agarose gel, transferred to a nitrocellulose membrane, and hybridized with the *AMT1* probe (5 $\times 10^6$ cpm/10 ml) according to the standard Southern blotting procedure (2). The three isogenic strains of *C. glabrata*, wild-type 85/038, Q (*ura*), and *amt1-1*, were streaked on SC agar, SC agar containing $CuSO_4$ ranging from 10 to 200 μM, and SC agar containing 25 μM to 1 mM ultrapure $CdCl_2$. The plates were incubated at 30°C for 2 days and then scored for growth of the strains.

RESULTS

Both the *MT-I* and *MT-II* genes have multiple transcription start sites. We initiated our study of copper-inducible transcription of the *C. glabrata* MT genes by mapping the specific transcription initiation sites in both the *MT-I* and *MT-II*$_a$ promoter sequences. Total RNA from untreated or copper-induced *C. glabrata* 85/038 cells was prepared, and *MT-I* and *MT-II* mRNAs were analyzed by primer extension reactions. In the *MT-I* promoter, there are two major starts, designated +1 and +2 (a C and an A), located 44 and 43 bp, respectively, upstream of the ATG translation initiation codon (29; this work), with several minor start sites observable upon longer exposure (Fig. 1, MT-I). A putative TATA box (TATAAA) is located at nucleotide positions −69 to −64 relative to the +1 transcriptional start site. Six major primer extension products were detected in the *MT-II*$_a$ promoter at bases −4 (A), −3 (A), +1 (C), +2 (A), +5 (A),

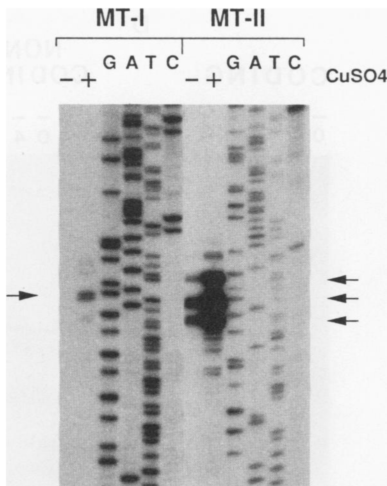

FIG. 1. Primer extension mapping of *MT-I* and *MT-II* mRNAs. Cells of *C. glabrata* 85/038 were grown in SC medium without copper to mid-logarithmic phase. Parallel cultures were untreated (−) or induced (+) with 50 μM $CuSO_4$ for 30 min, and *MT-I* and *MT-II* mRNAs were detected by primer extension reactions. The arrows indicate positions of clusters of the major *MT-I* and *MT-II* extension products. Sequencing reactions (lanes G, A, T, and C) were carried out as described in Materials and Methods to determine the locations of the transcription initiation sites of the *MT-I* and *MT-II* genes.

and +6 (A), located 66, 65, 62, 61, 58, and 57 bases 5′ to the translation start (30; this work). Several minor primer extension products were also observed (Fig. 1, MT-II). A putative TATA box (TATAAA) within the $MT\text{-}II_a$ promoter is located from −80 to −85 relative to the +1 nucleotide. We demonstrate here by primer extension analysis that both the *MT-I* and *MT-II* mRNA levels are induced by copper and that the choice of the transcription initiation sites for at least the *MT-II* gene is not altered in control versus copper-treated cultures. In addition, the *MT-II* gene is expressed at higher basal and copper-induced levels than is *MT-I*, consistent with previous results obtained by RNA blot analysis (29). Since the $MT\text{-}II_a$ and $MT\text{-}II_b$ genes are almost identical in their promoter and protein-coding sequences (32), the $MT\text{-}II_a$ primer that we used in the primer extension reaction also hybridizes to the same sequence found in the $MT\text{-}II_b$ open reading frame and presumably also primes the synthesis of $MT\text{-}II_b$ mRNA-templated extension products. Therefore, we could not determine which, if any, of the six major transcription initiation sites are specific for $MT\text{-}II_a$ mRNA. However, it has been previously demonstrated that the $MT\text{-}II_a$ gene is present in multiple tandem copies and that $MT\text{-}II_b$ exists as a single-copy gene (30, 33). We propose that at least the strongest primer extension products (the C at +1 and A at +2) represent the major transcription initiation sites for the $MT\text{-}II_a$ gene.

AMT1 protein binds to multiple sites in both the *MT-I* and $MT\text{-}II_a$ promoters. Previously, we demonstrated that copper- or silver-activated AMT1 protein binds to multiple sites in *MT-I* and $MT\text{-}II_a$ promoter DNA fragments in vitro (51). To precisely map the number and location of AMT1-binding sites, we performed DNase I footprinting assays. Copper-activated AMT1 protein protected two regions in the *MT-I* promoter, from −153 to −142 and −116 to −101 on the coding strand and from −119 to −107 and −158 to −145 on the noncoding strand, against DNase I cleavage. A 4-bp sequence (−139 to −136) located 6 nucleotides downstream of the upstream-most AMT1-binding site was also protected on the noncoding but not the coding strand (Fig. 2A). The two AMT1-binding sites within the *MT-I* promoter, designated a and b (Fig. 3), represent those sites found from −254 through +1; however, it is unknown whether other AMT1-binding sites exist further upstream in the *MT-I* gene promoter. Copper-activated AMT1 protein also protected five regions within the $MT\text{-}II_a$ promoter from DNase I digestion. Using the upstream $MT\text{-}II_a$ promoter fragment as a probe (Fig. 2B, U), we detected copper-activated AMT1 protein-mediated protection from DNase I cleavage at positions −545 to −528, −381 to −359, and −342 to −325 on the coding strand and at positions −538 to −519, −378 to −359, and −342 to −327 on the noncoding strand. AMT1 also protected regions within the downstream promoter fragment (Fig. 2B, D) from positions −230 to −216 and −199 to −187 on the coding strand and from positions −233 to −219 and −199 to −183 on the noncoding strand. One nucleotide residue (−209) on the coding strand and three nucleotides (−211, −210, and −209) on the noncoding strand, located between binding sites a and b, were also protected by copper-activated AMT1 against DNase I cleavage (Fig. 2B). Using electrophoretic mobility shift assays with the end-labeled upstream or downstream $MT\text{-}II_a$ probe and partially purified AMT1 protein, we detected an additional copper-dependent protein-DNA complex which could not be accounted for by DNase I footprinting (data not shown). The increased sensitivity of electrophoretic mobility shift assays relative to DNase I footprinting (2) suggests that this may represent a low-affinity AMT1-binding site. Consistent with this possibility is the presence of a sequence resembling the other AMT1-binding sites located between −270 to −256 in the $MT\text{-}II_a$ promoter (Fig. 3B, site c). Furthermore, methylation interference assays with the upstream $MT\text{-}II_a$ probe indicated that the G residue on the coding strand (−263), when methylated by dimethylsulfate, partially inhibited AMT1 binding, suggesting a weak interaction between copper-activated AMT1 protein and this sequence (data not shown). DNase I footprinting using the downstream $MT\text{-}II_a$ probe also suggested that AMT1 exhibited higher affinity to binding site b than to binding site a, since higher doses of AMT1 extract were required to observe occupancy of site a than site b (Fig. 2B). Figure 3B summarizes the positions and nucleotide sequences of the AMT1-binding sites on both strands of the *MT-I* and $MT\text{-}II_a$ promoters. The differences in number and organization of AMT1-binding sites in the *MT-I* and $MT\text{-}II_a$ promoters might suggest a difference in the promoter strength of the *MT-I* and $MT\text{-}II_a$ genes in response to copper and AMT1; however, this possibility must be tested in vivo.

AMT1 binds to its target site as an apparent monomer. The DNA-binding and copper-binding domains of the *S. cerevisiae* ACE1 protein lie within the amino-terminal 101 amino acids, wherein lie 11 cysteine residues critical for copper-activated DNA binding (5, 23). To begin to investigate the location of the copper-activated DNA-binding domain of AMT1, we expressed the amino-terminal 115 amino acids of AMT1 in *E. coli*, using the T7 RNA polymerase-driven expression system (45). The truncated AMT1 protein, designated tAMT1(1-115), was made by introducing a translation termination signal at codon 116 of the *AMT1* open reading frame (51). This region was chosen as a truncation point on the basis of the primary sequence homology between AMT1 and ACE1 (51) and the known preliminary

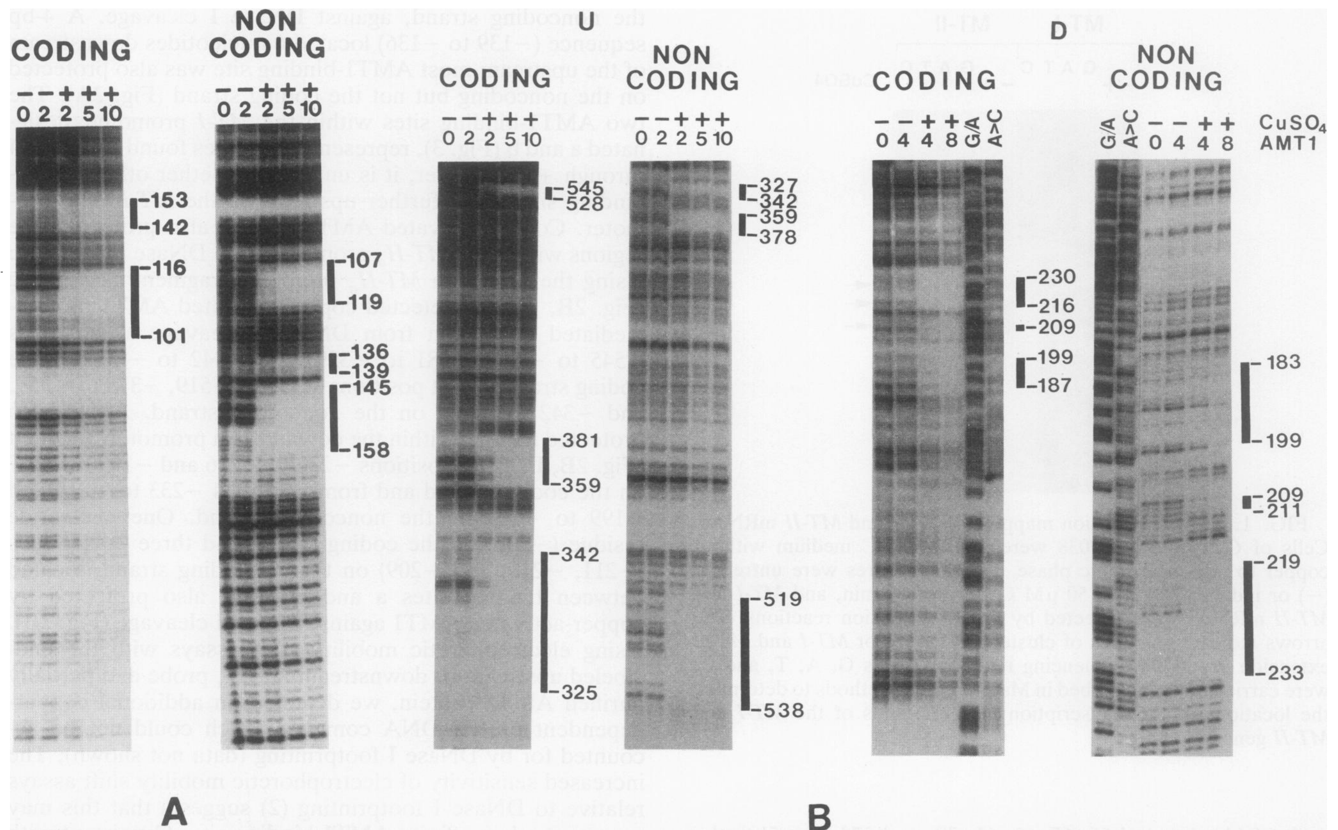

FIG. 2. DNase I footprinting analysis of the *MT-I* and *MT-II*$_a$ promoters. (A) The *MT-I* promoter fragment (−256 to −15) and (B) two *MT-II*$_a$ promoter fragments from −570 to −230 (U) and −304 to −42 (D) were uniquely end labeled on the coding and noncoding strands and used in DNase I footprinting reactions with AMT1 extract. The levels of AMT1 extract used (in micrograms) and the omission (−) or inclusion (+) of 50 μM $CuSO_4$ in the binding reactions are indicated above the lanes. The bars to the right indicate the regions protected from DNase I cleavage on each strand of the probe by copper-activated AMT1. The nucleotide positions bordering each site are numbered with respect to the major 5′ transcription start site of the *MT-I* or *MT-II*$_a$ gene. G/A and A>C indicate chemical DNA sequencing reactions identifying the positions of G and A residues and of A residues preferentially over C residues, respectively.

localization of the ACE1 copper-activated DNA-binding domain (23). The tAMT1(1-115) polypeptide, synthesized in *E. coli*, was used in electrophoretic mobility shift assays with the upstream *MT-II*$_a$ probe (U) used in the DNase I footprinting experiments shown in Fig. 2. The same number of copper-dependent DNA-protein complexes was observed with the truncated AMT1 protein as was observed with the full-length AMT1 protein, although they exhibited a correspondingly faster electrophoretic mobility (data not shown). These results indicate that the amino-terminal 115 amino acids of AMT1 contain the copper-activated DNA-binding domain, consistent with our previous observation of the conserved primary structure of the amino-terminal domains of AMT1 and ACE1 (51).

To determine whether AMT1 binds to its target sequences as a monomeric or multimeric protein, we performed electrophoretic mobility shift assays using independent extracts containing full-length and tAMT1(1-115) proteins. Figure 4A shows a diagrammatic representation of the two forms of AMT1 produced in *E. coli*. The electrophoretic mobility shift assay shown in Fig. 4B indicates that both tAMT1(1-115) and full-length AMT1 extracts gave rise to a single copper-inducible complex with the double-stranded oligodeoxynucleotide probe, representing a single AMT1-binding site, d, from the *MT-II*$_a$ promoter. Although tAMT1(1-115) appears to bind to the probe less efficiently in this experiment, other experiments indicate that tAMT1(1-115) binds with an affinity indistinguishable from that of full-length AMT1. When equal amounts of full-length AMT1 and tAMT1(1-115) extracts were mixed together in the presence of the ^{32}P-labeled DNA probe, we detected two DNA-protein complexes, one with the same migration properties as the full-length and truncated AMT1 proteins, respectively. The absence of an intermediate complex between the two proteins indicates that no heterodimers were formed between AMT1 and tAMT1(1-115) complexes. Moreover, no slower-migrating DNA-protein complexes were observed with use of an AMT1 or tAMT1(1-115) extract level which saturated the probe (data not shown). To test the possibility that the independent folding of the partially purified AMT1 and tAMT1(1-115) may inhibit multimerization, we performed denaturation-renaturation experiments with AMT1 and tAMT1(1-115) prior to addition to the binding reaction. Also, the probe and the AMT1 and tAMT(1-115) polypeptides were added in different orders in the binding reactions and then subjected to electrophoresis. No multimeric complexes were observed under any of the binding reaction conditions used (data not shown). The experiments described above do not exclude the possibility that the dimerization domain was deleted in the truncated form of AMT1 protein [tAMT1(1-

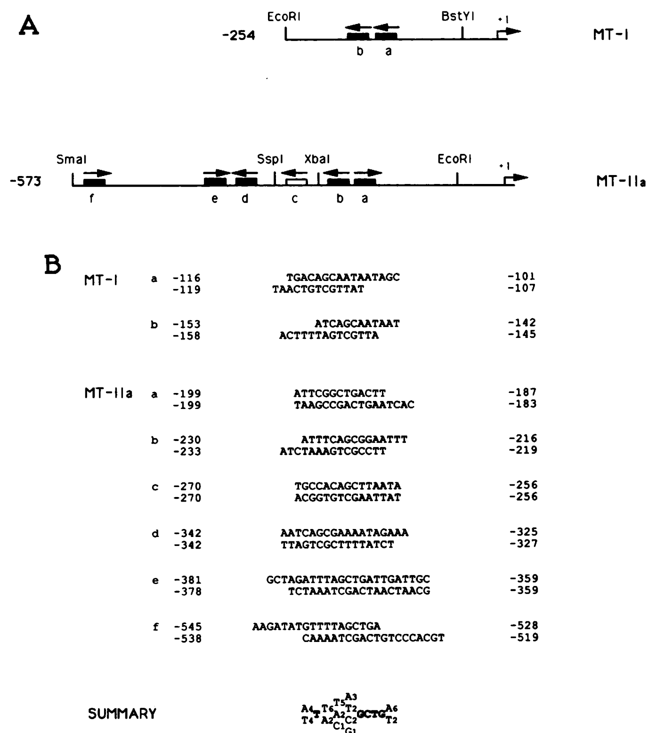

FIG. 3. Summary of the AMT1-binding sites on the *C. glabrata MT-I* and *MT-II$_a$* promoters. (A) Schematic diagram of the *MT-I* and *MT-II$_a$* promoters depicting AMT1-binding sites. Filled boxes represent AMT1-binding sites at locations determined by in vitro DNase I footprinting, and the white box represents a putative low-affinity AMT1-binding site on the *MT-II$_a$* promoter. The arrows indicate the orientation of each site corresponding to the orientation of the 5′-GCTG-3′ core sequence. Restriction endonuclease sites are shown with respect to their positions in both promoters. (B) Positions and nucleotide sequences of the AMT1-binding sites on the *MT-I* and *MT-II$_a$* promoters on the coding and noncoding strands as determined by DNase I footprinting. The nucleotide sequence of AMT1-binding site c is derived from the sequence similarity with other AMT1-binding sites and the importance of the G at −263 for the binding of copper-activated AMT1 protein as determined by methylation interference assays (data not shown). The summary of the AMT1-binding sites shows the tetranucleotide core (GCTG) and conserved T residue in bold. Other residues flanking the tetranucleotide core are shown with their frequencies of occurrence in the AMT1-binding sites on the *MT-I* and *MT-II$_a$* promoter fragments.

FIG. 4. Subunit composition of DNA-bound AMT1 protein. (A) Schematic diagram of AMT1 polypeptides synthesized in *E. coli* and used in electrophoretic mobility shift assays. T represents a truncated form of the wild-type AMT1 protein containing the 115 amino-terminal residues [tAMT1(1-115)]; F corresponds to the full-length (265-amino-acid) wild-type AMT1 protein. The filled rectangle represents the amino-terminal AMT1 copper-activated DNA-binding domain with 11 cysteine residues (indicated by C). The open rectangle indicates the carboxy-terminal 150 amino acids of AMT1. (B) Electrophoretic mobility shift assays with full-length and truncated polypeptides. A 14-mer oligonucleotide (5′-GCTAGATTT AGCTG-3′) was hybridized to a complementary oligonucleotide with the sequence 5′-CAATCAGCTAAATC-3′. The resulting duplex probe (P) represents a single AMT1-binding site which corresponds to site d on the *MT-II$_a$* promoter. Binding reaction mixtures contained truncated (lanes T), full-length (lanes F), or full-length plus truncated (lanes FT) extracts and were incubated with the probe for 10 min in the absence (−) or presence (+) of 100 μM $CuSO_4$ and subjected to electrophoresis on a 5% native polyacrylamide gel. T and F indicate the protein-DNA complex obtained with the probe and copper-treated T and F extracts, respectively.

115)]; however, two lines of evidence are against this possibility. (i) The maximum number of complexes that we detected with use of either *MT-I* or *MT-II$_a$* promoter fragments as probes were equal to the number of AMT1-binding sites in the fragments as determined by DNase I footprinting and methylation interference assays. Supershifts due to potential protein-protein interactions between one AMT1 molecule bound to the DNA and another free AMT1 molecule have not been observed, even under conditions in which the probe was saturated with copper-activated AMT1 protein. (ii) We observed similar patterns of binding between AMT1 and tAMT1(1-115) to the *MT-I* or *MT-II$_a$* promoters containing multiple sites (data not shown). The absence of heterodimer or multimer formation suggests that AMT1 binds to its target sequences in vitro preferentially as a monomer.

AMT1 activates *MT-I* and *MT-II$_a$* gene transcription in vivo. Because copper-activated AMT1 binds to both the *MT-I* and *MT-II$_a$* promoters in vitro, we tested whether this copper-dependent binding correlates with metal-activated MT gene transcription in vivo. An *AMT1* disruption allele (*amt1-1*) was constructed by insertional inactivation of the wild-type *AMT1* gene within the region encoding the copper-activated DNA-binding domain. The parental strain used for the construction of the *amt1-1* allele was the *ura* strain Q, which contains the wild-type *AMT1* locus as verified by Southern blotting (Fig. 5B) and shows the same level of copper resistance (up to 1 mM) as does the wild-type strain 85/038 (Fig. 6). Hybridization with the *AMT1* probe demonstrated that wild-type and Q cells gave rise to a 1.4-kb fragment representing the wild-type *AMT1* gene. Two DNA fragments, 3.0 and 2.1 kb, were observed upon digestion of *AMT1*::*URA3* genomic DNA due to the insertion of the *URA3-hisG* cassette at the *Sty*I restriction site of the chro-

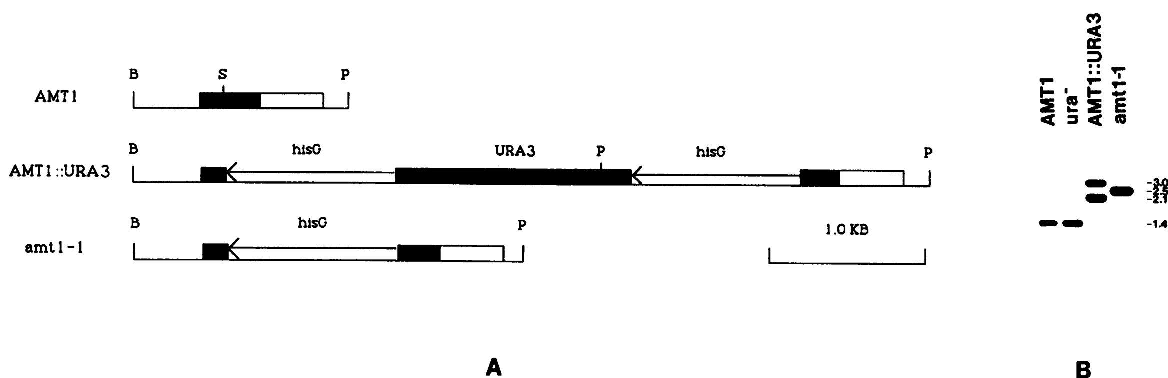

FIG. 5. Construction of a chromosomal disruption allele of the *AMT1* gene. (A) The *AMT1* gene was disrupted as shown diagrammatically and described in Materials and Methods. The *URA3* cassette, flanked by direct repeats derived from the *Salmonella hisG* gene, was inserted at the *Sty*I site located after AMT1 codon 36 within the AMT1 DNA-binding domain. Recombination between the repeats resulted in the loss of the *URA3* marker and a single *hisG* element, generating the disruption allele *amt1-1*. Abbreviations for restriction endonuclease recognition sites: B, *Bgl*II; S, *Sty*I; P, *Pst*I. (B) Genomic DNA from *C. glabrata* wild-type 85/038 (lane AMT1), the *ura* (Q) strain (lane ura⁻), a disruption intermediate (lane AMT1::URA3), and the *amt1-1* strain (lane amt1-1) was prepared and digested to completion with *Bgl*II and *Pst*I and analyzed by Southern blotting as described in Materials and Methods. Numbers on the right indicate the sizes (in kilobase pairs) of DNA fragments that hybridized to the *AMT1* probe.

mosomal *AMT1* locus. Deletion of the *URA3* gene and one copy of the *hisG* gene, as a result of the recombination between the two *hisG* genes, produced the expected 2.5-kb DNA restriction fragment representing the disruption of the *AMT1* gene by one copy of the residual 1.1-kb *hisG* gene (Fig. 5). Figure 6 indicates that the *amt1-1* strain grows indistinguishably from the parental wild-type strain on SC agar but fails to grow on SC agar containing 50 μM $CuSO_4$. When the *AMT1* gene was transformed back into the *amt1-1* cells on an episomal plasmid, the *amt1-1* strain was resistant to 1 mM $CuSO_4$ (data not shown). These experiments demonstrate that *AMT1* is not an essential gene for growth in the absence of high levels of exogenous copper but is indispensable for copper detoxification in *C. glabrata*.

The demonstration that copper-activated AMT1 protein binds to both the *MT-I* and *MT-II_a* promoters strongly suggested that AMT1 activates the expression of these genes in vivo. To test this possibility, we carried out RNA blotting experiments to determine whether the copper-sensitive phenotype of the *amt1-1* strain correlates with the inability of the mutant AMT1 allele to activate MT transcription in vivo. Total RNA from both wild-type and *amt1-1* strains was prepared from cultures grown under control or copper-induced conditions, and *MT-I* and *MT-II* mRNAs were analyzed by RNA blotting and hybridization with *MT-I* and *MT-II_a* gene probes. The results shown in Fig. 7 demonstrate that disruption of the *AMT1* locus completely abolished the ability of this strain to induce *MT-I* and *MT-II* transcription in response to exogenous copper. These experiments reveal the physiological function of AMT1 protein as the major if not sole mediator of copper-inducible *MT-I* and *MT-II* gene transcription in *C. glabrata*. We also observed that the mRNA levels of the *MT-II* gene in the *amt1-1* strain had decreased to lower than wild-type basal levels in comparison with levels in cells harboring the *AMT1* allele. Since the *MT-II_a* probe also hybridizes to *MT-II_b* mRNA, the mRNA detected by the *MT-II_a* probe actually reflects the accumulation of transcripts of both the *MT-II_a* and *MT-II_b* genes in either the wild-type (85/038) or *amt1-1* strain under control or copper-treated conditions. The *MT-I* mRNA in wild-type *C. glabrata* cells is expressed at very low but detectable levels under normal growth conditions; however, disruption of the *AMT1* locus also resulted in a decrease in basal-level

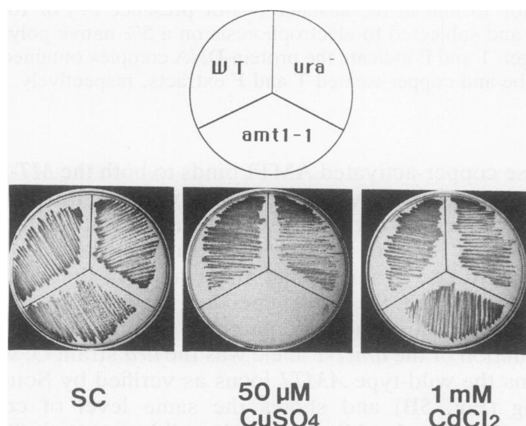

FIG. 6. Evidence that *AMT1-1* is critical for copper but not cadmium detoxification. The wild-type 85/038 strain (WT), *ura* strain (ura⁻), and *AMT1* disruption strain (amt1-1) were streaked onto SC agar and SC agar containing 50 μM $CuSO_4$ or 1 mM $CdCl_2$. Plates were incubated at 30°C for 2 days. The grid indicates the relevant genotypes of the strains streaked onto each sector of the plates.

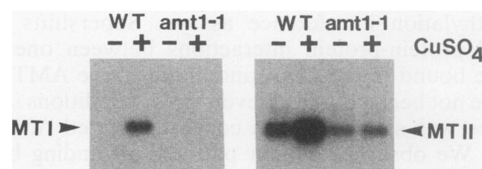

FIG. 7. *MT-I* and *MT-II* mRNA levels in wild-type and *amt1-1* disruption strains. Total RNA was isolated from control (−) or copper-induced (+) cultures of wild-type (85/038) and *amt1-1* strains and analyzed by RNA blotting and hybridization with *MT-I* and *MT-II*-specific probes. The arrowheads indicate the locations of the *MT-I* and *MT-II* mRNA species.

expression of the *MT-I* gene, which can be detected with a longer exposure of the RNA blot (data not shown). These observations suggest that AMT1 contributes to basal-level as well as copper-induced transcription of both the *MT-I* and *MT-II$_a$* genes.

Cadmium resistance in *C. glabrata* is independent of AMT1-mediated induction of MT gene expression. Previous work by Mehra et al. suggested that the nontranslationally synthesized peptides (γ-Glu-Cys)$_n$Gly are the major cadmium-binding molecules which confer cadmium resistance to *C. glabrata* (31). To study the relationship of the cadmium and copper detoxification pathways in *C. glabrata*, we tested the growth of the three isogenic strains, *AMT1* wild type, Q, and *amt1-1*, on SC agar supplemented with $CdCl_2$. In contrast to the observed sensitivity of the *amt1-1* strain to copper, strains containing either the wild-type or *amt1-1* allele grew equally well on SC agar containing up to 1 mM $CdCl_2$ (Fig. 6). This observation indicates that *AMT1* is not directly involved in the pathways which confer cadmium resistance to *C. glabrata*, consistent with the finding that cadmium is incapable of inducing *MT-I* and *MT-II$_a$* transcription and slightly represses the basal levels of *C. glabrata* MT mRNA levels in vivo (29). Furthermore, we conducted electrophoretic mobility shift assays and found that cadmium does not induce the binding of AMT1 protein to the *MT-I* or *MT-II$_a$* promoter (data not shown). Taken together, these observations indicate that *C. glabrata* utilizes two independent pathways to mediate resistance to the metals copper and cadmium.

DISCUSSION

Metal-inducible gene transcription has been observed in a wide variety of eukaryotic organisms (48). Currently, only two genes encoding metal-activated transcription factors, *ACE1* from the baker's yeast *S. cerevisiae* (47, 50) and *AMT1* from the opportunistic pathogenic yeast *C. glabrata*, have been isolated (51). ACE1 and AMT1 represent a class of transcription factors which sense elevated environmental copper levels and directly transmit this information to the transcription machinery. Therefore we designate ACE1 and AMT1 as metalloregulatory transcription factors (48).

The transcription factors ACE1 from *S. cerevisiae* and AMT1 from *C. glabrata* share several distinguishing features: (i) the metal ions Cu(I) and Ag(I) markedly stimulate sequence-specific DNA-binding activity to multiple sites within the *CUP1* promoter and *MT-I* and the *MT-II$_a$* promoters, respectively; (ii) the amino-terminal domains of both ACE1 and AMT1 contain both the copper-activated and DNA-binding functions; (iii) the DNA-binding domains of both proteins do not resemble, at the primary structural level, any of the known DNA-binding motifs such as the zinc finger, helix-turn-helix, or others found in many eukaryotic or prokaryotic sequence-specific DNA-binding transcription factors (21); (iv) both ACE1 (16) and AMT1 proteins preferentially bind to their promoters in the monomeric form; and (v) DNA sequences recognized by ACE1 in the *CUP1* and *SOD1* promoters of *S. cerevisiae* (5, 13, 18, 24) and by AMT1 in the *MT-I* and *MT-II$_a$* promoters of *C. glabrata* are similar in that they contain a four-nucleotide core sequence, 5′-GCTG-3′, with a T- or A-rich region immediately 5′ to the core sequence (Fig. 3B) (48). Previously, both G residues in this 5′-GCTG-3′ core sequence were shown by methylation interference assays to be important for the interaction of ACE1 with binding sites in the *CUP1* and *SOD1* promoters (5, 16, 18). Methylation interference studies with AMT1 and the AMT1-binding sites in both the *MT-I* and *MT-II$_a$* promoters also indicate the critical nature of both G residues in the AMT1 core (data not shown). This assay also supports the presence of a low-affinity binding site, c, in the *MT-II$_a$* promoter, which we could not detect by DNase I footprinting. Consistent with these observations in *C. glabrata* MT gene promoters, it has been shown that in the mouse *MT-I* promoter region, there is a low-affinity MRE. This MRE has little activation function independently in vivo but has been shown to be bound in vivo by a metal-dependent factor (34). Furthermore, the MREs in the mouse *MT-I* promoter exhibit a range of metal-activated transcription potencies in vivo (11, 43, 44).

The complexity of MT gene organization in *C. glabrata* allowed us to examine whether the copper-activated AMT1 protein activates transcription of multiple members of an MT gene family. It has been previously demonstrated that both the mRNA and protein levels of *MT-II* are higher than those of *MT-I* (31, 33). Our footprinting results indicate that AMT1 recognizes two binding sites in the *MT-I* promoter fragment used and five major binding sites and an additional low-affinity site in the *MT-II$_a$* promoter, although we cannot exclude the possibility that sequences further upstream of the *MT-I* and *MT-II$_a$* promoters contain additional AMT1-binding sites. The differences in the number and organization of AMT1-binding sites in these two promoters may, in part, be responsible for the lower magnitude of copper-inducible *MT-I* transcription than of *MT-II* transcription. Although tandem amplification of the *MT-II$_a$* locus must also contribute to the difference in the accumulation of the *MT-I* and *MT-II* gene products (30), the observation that a *C. glabrata* strain carrying a single *MT-II$_a$* locus produces significantly more MT-II than MT-I mRNA and protein supports the observation that *MT-II* is expressed at higher levels than is *MT-I* (33). We interpret the differential expression of the *MT-I* and *MT-II$_a$* genes previously observed as the combination of the number or organization of *cis*-acting elements, the *MT-II* gene dosage effect, and perhaps differential stabilities of the mRNAs or proteins.

The in vivo function of the *C. glabrata AMT1* gene was established by the disruption of the wild-type *AMT1* locus by using a genetic transformation system that we have recently developed for *C. glabrata*. The copper-sensitive phenotype of the *amt1-1* strain and the inability of this strain to activate *MT-I* and *MT-II$_a$* transcription demonstrated that AMT1 is the major copper-activated transcription factor for the *MT-I* and *MT-II$_a$* genes. In the *amt1-1* strain, both the *MT-I* and *MT-II* mRNAs decreased to below basal levels of the wild-type *C. glabrata* cells, suggesting that AMT1 protein also contributes to the basal-level transcription of the MT genes. Similarly, the *ACE1* gene product has been shown to contribute to basal-level transcription of the *CUP1* gene in *S. cerevisiae* (7), presumably by utilizing endogenous copper for DNA-binding function activation. Although the strain bearing the *amt-1* allele is incapable of fostering copper-inducible MT gene transcription, we observed a significant level of *MT-II* mRNA compared with that of *MT-I* (Fig. 7). This finding might be attributed to the amplification of the endogenous *MT-II$_a$* locus or to the action of unknown transcription factors specific to the *MT-II* promoter. In our initial electrophoretic mobility shift assays, we detected a copper-independent DNA-protein complex with the *MT-II$_a$* but not the *MT-I* promoter with use of whole cell extracts from *C. glabrata* (51). The nature of this complex is currently unknown; however, like *S. cerevisiae* and higher eukaryotic MT genes, the *MT-II$_a$* gene might be subjected to

multiple regulatory controls in response to different environmental stimuli (19, 25). At present, two other transcription factors, ACE2 (7) and heat shock transcription factor (38), have been demonstrated to activate *CUP1* transcription.

In higher eukaryotes, MTs confer resistance to a wide range of metals, including cadmium, copper, and others (19). In *C. glabrata*, however, a strain bearing an *amt1-1* disruption allele exhibited no discernible difference in cadmium resistance compared with wild-type cells. The facts that cadmium actually reduces *MT-I* and *MT-II* transcription (29) and that Cd–MT-I/II complexes have not been isolated from *C. glabrata* cells grown in the presence of cadmium (30) suggest that MTs are not a primary mechanism for cadmium resistance in this organism. It has been found that (γ-Glu-Cys)$_n$Gly peptides were synthesized in response to cadmium and were isolated as Cd-(γ-Glu-Cys)$_n$Gly complexes, suggesting that (γ-Glu-Cys)$_n$Gly peptides are important components in the *C. glabrata* cadmium detoxification process (30, 33). Our analysis of *C. glabrata* strains harboring a wild-type or *amt1-1* disruption allele indicates that AMT1 is completely dispensable for cadmium resistance and provides additional evidence that different sensory molecules are responsible for the manifestation of distinct pathways for copper and cadmium detoxification in *C. glabrata*. Therefore, *C. glabrata* provides an interesting model system with which to study the metal detoxification and homeostatic processes in eukaryotic cells.

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