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Copper-specific Transcriptional Repression of Yeast Genes Encoding Critical Components in the Copper Transport Pathway*

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Copper is an essential micronutrient that is toxic in excess. To maintain an adequate yet non-toxic concentration of copper, cells possess several modes of control. One involves copper uptake mediated by genes encoding proteins that play key roles in high affinity copper transport. These include the *FRE1*-encoded Cu^{2+}/Fe^{3+} reductase and the *CTR1* and *CTR3*-encoded membrane-associated copper transport proteins. Each of these genes is transcriptionally regulated as a function of copper availability: repressed when cells are grown in the presence of copper and highly activated during copper starvation. Our data demonstrate that repression of *CTR3* transcription is exquisitely copper-sensitive and specific. Although copper represses *CTR3* gene expression at picomolar metal concentrations, cadmium and mercury down-regulate *CTR3* expression only at concentrations 3 orders magnitude greater. Furthermore, copper-starvation rapidly and potently induces *CTR3* gene expression. We demonstrate that the *CTR1*, *CTR3*, and *FRE1* genes involved in high affinity copper uptake share a common promoter element, TTTGCTC, which is necessary for both copper repression and copper-starvation activation of gene expression. Furthermore, the Mac1p is essential for down- or up-regulation of the copper-transport genes. *In vivo* footprinting studies reveal that the *cis*-acting element, termed CuRE (copper-response element), is occupied under copper-starvation and accessible to DNA modifying agents in response to copper repression, and that this regulated occupancy requires a functional *MAC1* gene. Therefore, yeast cells coordinately express genes involved in high affinity copper transport through the action of a common signaling pathway.

Copper is an essential trace element that is required for a number of cellular enzymes including cytochrome *c* oxidase, Cu/Zn-superoxide dismutase, lysyl oxidase, and dopamine-β-monooxygenase (1). Copper also plays a critical role in the assimilation of iron both in microbial and mammalian systems (2, 3). However, when allowed to accumulate in excess, copper is highly toxic due to its proclivity to engage in redox reactions which result in the formation of hydroxyl radical, a reactive species that causes extensive damage to nucleic acids, proteins, and lipids (4, 5). Furthermore, copper may also be toxic through inappropriate incorporation into proteins, such as the estrogen receptor, which normally bind other metal ligands (6). Therefore, all organisms must be able to sense both toxic and nutritional levels of copper to allow sufficient copper to accumulate to drive biochemical reactions, yet prevent the accumulation to toxic levels. Indeed, the failure to appropriately establish and maintain copper homeostasis results in at least two human genetic disorders, Wilson's disease and Menkes disease (1, 7, 8). A number of cellular regulatory responses that result from fluctuations in environmental copper levels have been reported including transcriptional activation or repression, changes in protein stability, and the modulation of protein trafficking (9–13).

Yeast cells have provided an excellent model system for studies of copper transport, distribution, and detoxification (5, 8, 14). In response to high concentrations of copper, yeast cells activate the transcription of the *CUP1* (15–17) and *CRS5* (18) genes, which encode copper-sequestering proteins called metallothioneins, as well as the *SOD1* gene, encoding Cu/Zn-superoxide dismutase (19). This transcriptional activation involves the copper metalloregulatory transcription factors (MRTFs)[1] Ace1p (20) and Amt1p (21) from the bakers' yeast *Saccharomyces cerevisiae* and the opportunistic pathogenic yeast *Candida glabrata*, as well as *cis*-acting promoter regulatory sequences with the consensus sequence HTH*XX*GCTG (H = A, C or T; *X* = any residue). The binding of Cu(I) to Ace1p or Amt1p to form a tetra-copper cluster activates their DNA-binding domains via a conformational change, thereby providing a direct link between the toxic copper sensor and the activation of detoxification genes (22, 23).

S. cerevisiae cells acquire copper as Cu(I) under high affinity conditions through the action of a plasma membrane-associated Cu(II)-Fe(III) reductase activity encoded by the *FRE1* gene (24, 25) and two high affinity copper transport proteins encoded by the *CTR1* (26) and *CTR3* (27) genes. Indeed, cells that are defective in high affinity copper transport exhibit a number of phenotypes which can be corrected by exogenous copper that include respiratory deficiency, sensitivity to superoxide generating agents due to a defect in Cu/Zn-superoxide dismutase activity, and severely diminished iron accumulation due to a defective copper-dependent ferroxidase (Fet3) required for high affinity iron transport (28, 29). As would be expected, the genes encoding proteins involved in high affinity copper or iron transport are repressed, at the level of steady-state mRNA, by low concentrations of their respective metals and induced by copper or iron starvation, respectively (10, 24, 27).

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[1] The abbreviations used are: MRTF, metalloregulatory transcription factor; BCS, bathocuproinedisulfonate; CTR, copper transporter; CuRE, copper-response element; ORF, open reading frame; RI_{50}, repression index 50; bp, base pair(s).

TABLE I
S. cerevisiae strains used in this work

Strain	Genotype	Reference
DTY1	*MAT* **a** *gal1 trp1–1 his3 ade8 CUP1*	33
DTY205	*MAT* **a** *gal1 trp1–1 his3 ade8 CUP1 MAC1^{up1}*	32
SLY1	*MAT* **a** *gal1 trp1–1 his3 ade8 CUP1 ura3::KAN^r*	This study
SLY2	*MAT* **a** *gal1 trp1–1 his3 ade8 CUP1 ura3::KAN^r mac1::URA3*	This study

Furthermore, consistent with the involvement of the *FRE1*-encoded Cu(II)-Fe(III) reductase in both high affinity copper and iron transport, the expression of this gene is repressed by both copper and iron (24, 25). Insight into the mechanism for iron-dependent transcriptional repression in yeast has been gained by the recent identification and analysis of the iron-responsive DNA-binding protein Aft1 (30), which binds to the consensus sequence PyPuCACCCPu found in the promoters of the *FET3*, *FTR1*, *FTH1*, *CCC2*, *FRE1*, and *FRE2* genes (31).

Currently, it is unclear how yeast cells sense very low concentrations of copper and respond by repressing the expression of the *FRE1*, *CTR1*, and *CTR3* genes. One protein implicated in this signaling pathway, Mac1, was discovered based on significant homology to the amino-terminal 40 amino acid residues of the copper-activated DNA-binding domains of the Ace1 and Amt1 proteins (32). Interestingly, *mac1*Δ cells exhibit phenotypes consistent with defective copper transport including respiratory deficiency, defective Cu(II) and Fe(III) reductase activity, impaired regulation of *FRE1* transcription, hypersensitivity to a variety of stresses including metal and oxidative stress, and poor growth on rich medium (25, 32). Furthermore, a dominant mutation in a duplicated region rich in cysteine exhibits high levels of Cu(II) reductase activity and *FRE1* mRNA that are virtually not repressed by copper (25). These characteristics of Mac1p, as well as the nuclear localization of a Mac1-β-galactosidase fusion protein (32), suggests that Mac1 plays a key role in copper homeostasis.

In this report, we demonstrate that the regulation of *FRE1*, *CTR1*, and *CTR3* transcription is exquisitely copper-sensitive and specific. We demonstrate that these three genes share a common promoter element, TTTGCTC, which is necessary for both copper repression and copper-starvation activation of expression. Furthermore, the Mac1p is essential for down- or up-regulation of the copper transport genes. *In vivo* footprinting studies reveal that the *cis*-acting element, termed CuRE (copper-response element), is occupied under copper-starvation and accessible to DNA modifying agents in response to copper repression, and that this copper-responsive occupation of the CuREs requires a functional *MAC1* gene. Taken together these results show that yeast cells coordinately express genes involved in high affinity copper transport through the action of a common signaling pathway to acquire this micronutrient from the environment.

EXPERIMENTAL PROCEDURES

Chemicals—Stock solutions at 10–100 mM $CuSO_4$ (100.2%), $FeCl_2$ (99.0%), $NH_4Fe(SO_4)_2$ (100.4%), $CoCl_2$ (99.0%), $CaCl_2$ (99.0%), $MnCl_2$ (99.0%), $(C_2H_3O_2)Pb$ (99.3%), $AgNO_3$ (99.9%), $CdCl_2$ (99.9%), $ZnCl_2$ (99.9%), $NiSO_4$ (99.9%), $MgSO_4$ (99.9%), $HgCl_2$ (99.9%), $NaO_2CCH_2CH(SAu)CO_2Na$, $AuCl_3$ (99.9%), and BCS (Aldrich) were freshly prepared. Solutions of $AgNO_3$, $HgCl_2$, $CdCl_2$, $ZnCl_2$, $FeCl_2$, and $NH_4Fe(SO_4)_2$ were analyzed by atomic absorption spectroscopy using a Perkin-Elmer PEC 3000 spectrophotometer and found to contain no detectable copper ion contamination.

Yeast Strains and Cell Growth—Yeast strains used in this study are listed in Table I. The *ura3*Δ*::KAN^r* allele was constructed by deleting 391 bp of the *URA3* open reading frame (ORF) and replacing it with the KAN^r marker (34). The *mac1::URA3* allele was constructed using the disruption plasmid pmac1::URA3 (generously provided by Daniel Kosman). This disruption rendered the *MAC1* gene nonfunctional as ascertained by the lack of growth on YPE media. Furthermore, the allele status of each of these loci in the strains generated in this study was verified using diagnostic polymerase chain reaction (35).

Yeast cells were grown in a modified minimal medium (SD), which was depleted for copper as described previously (10). This depleted medium contains 16 nM copper as determined by atomic absorption spectroscopy while standard synthetic complete media contains 150 nM copper. Copper administration or copper starvation of yeast strains were carried out by adding the indicated amount of $CuSO_4$ or bathocuproinedisulfonate (BCS) to cells grown to mid-logarithmic phase (optical density at 600 nm = 1.1 to 1.3) in this modified SD medium. Under nonselective conditions, yeast cells were grown in YPD (1% yeast extract, 2% bactopeptone, and 2% dextrose).

Plasmids and Gene Analyses Methods—Plasmid YEp357R*TRP1* was constructed by insertion of a blunt-ended 1.7-kilobase *TRP1* fragment isolated from p330 (gift of Robert Fuller) into the *Stu*I and Klenow filled-in *Nco*I sites of YEp357R, disrupting the *URA3* genetic marker. The oligonucleotides CTR3-A (5′-CTCGCGGATCCAGTCATAGCATGAACAATTC-3′) and CTR3-B (5′-GTCCGGAATTCGAAGCAGTGCTGCTACTGCCTCC-3′) were used to polymerase chain reaction amplify the *CTR3* promoter (1116 bp of the 5′-noncoding region) and the first 10 codons of *CTR3* gene from genomic DNA of strain DTY1, which expresses both the Ctr1p and Ctr3p copper transporters. The polymerase chain reaction product was sequenced in its entirety and fused in-frame to the *Escherichia coli lacZ* gene using the *Bam*HI and *Eco*RI sites of YEp357R*TRP1* to generate YEp*CTR3-lacZ*. A low-copy number plasmid pRS*CTR3-lacZ* was also constructed. To accomplish this construct, a 4.8-kilobase DNA fragment from plasmid YEp*CTR3-lacZ* containing the *CTR3* sequence from −1116 to +10 fused to the *E. coli lacZ* gene was inserted into the *Bam*HI and *Sma*I sites of pRS314. To perform the RNase protection analyses as described previously (36), two plasmids for making antisense RNA probes were made. pKS*lacZ* was constructed by the insertion of the 233-bp *Eco*RV-*Bcl*I fragment from the *E. coli lacZ* gene into the *Eco*RV and *Bam*HI sites of pBluescript II KS. pKS*ACT1* was made by the insertion of the 132-bp *Hin*dIII-*Eco*RI fragment from the *S. cerevisiae ACT1* gene into the *Hin*dIII-*Eco*RI sites of pBluescript II KS. A series of plasmids containing sequential deletions from the 5′ end of the *CTR3* promoter (Fig. 3*B*) were created from plasmid YEp*CTR3-lacZ* using standard protocols (Exo III/Mung Bean Nuclease Deletion Kit, Stratagene). To assess the capability of the CuREs to mediate copper repression and copper starvation induction, a series of purified oligonucleotides (Figs. 4*C* and 5*C*) were annealed pairwise to form double-stranded DNA and then ligated into the *Bgl*II and *Xho*I sites of a *CYC1-lacZ* fusion plasmid pCM64 (generously provided by Charles Moehle).

RNA was extracted by the hot phenol method as described previously (37). Northern blot analyses were carried out by standard protocols (38). The radioactive bands corresponding to specific transcripts were quantitated using a PhosphorImager SP and ImageQuant 3.3 software (Molecular Dynamics). The data derived from the PhosphorImage quantitation were plotted and analyzed using Kaleidagraph software 3.02 (Synergy Software, Reading, PA). DNA isolation and polymerase chain reaction were performed using standard protocols (38, 35). DNA sequencing was carried out using Sequenase according to the manufacturer's protocol (U. S. Biochemical Corp.).

In Vivo Dimethyl Sulfate Footprinting—Cultures of the isogenic strains DTY1 (*MAC1*), DTY205 (*MAC1^{up1}*), or SLY2 (*mac1*Δ) containing YEp*CTR3-lacZ* were grown to early log-phase in modified SD media. Untreated and copper-treated (10 nM) cultures were incubated for 1 h at 30 °C, 375 rpm. *In vivo* footprinting was carried out as described previously (39). Isolated DNA samples were digested by *Bst*YI prior to G + A-specific DNA cleavage of the dimethyl sulfate-treated DNA. The purified oligonucleotide used as primer in the extension reactions was CTR3E (5′-GCCTCCCATATTCATCTTTGTATAGCCC-3′), which hybridizes to *CTR3* gene positions +15 to −14 with respect to A of the translational start codon.

RESULTS

Metal Ion Specificity and Sensitivity of CTR3 mRNA Down-regulation—Previous investigations have demonstrated that all three yeast genes known to be involved in high affinity copper transport, *CTR1*, *CTR3*, and *FRE1*, are repressed by copper at the level of steady-state mRNA (10, 27). This regulation is independent of the *S. cerevisiae* copper MRTF Ace1p, which activates the *CUP1*, *CRS5*, and *SOD1* genes in response

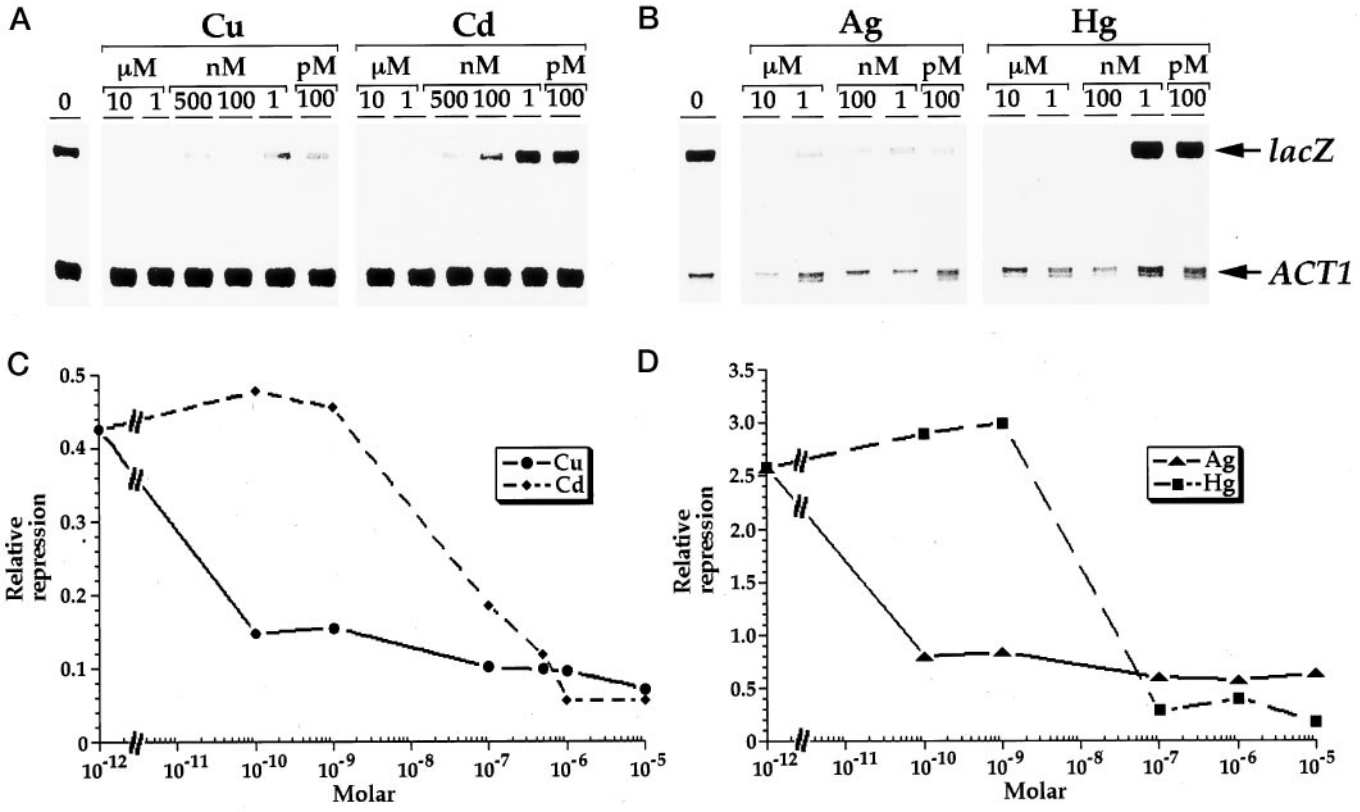

FIG. 1. **Metal selectivity and sensitivity for repression of the *CTR3* promoter.** Strain DTY1, transformed with pRS*CTR3-lacZ*, was grown as described under "Experimental Procedures." Total RNA from control (0), $CuSO_4$ (10, 1 μM; 500, 100, 1 nM; 100 pM) and $CdCl_2$ (10, 1 μM; 500, 100, 1 nM; 100 pM) cultures (*A*) as well as control (0), $AgNO_3$ (10, 1 μM; 100, 1 nM; 100 pM), and $HgCl_2$ (10, 1 μM; 100, 1 nM; 100 pM) cultures (*B*) was isolated. RNase protection analyses (*A* and *B*) show the down-regulation of the *CTR3* promoter-*lacZ* fusion in the presence of these metal ions. The *lacZ* and *ACT1* mRNA steady-state levels are indicated with *arrows*. Results illustrated are representative of three independent experiments. *C* and *D*, graphic representations of the RNase protection analyses by PhosphorImage quantitation. *Solid lines* indicate the response of the *CTR3-lacZ* fusion gene to copper (*C*) and silver (*D*), whereas *dashed lines* show that metal concentrations 3 orders of magnitude greater than for $CuSO_4$ were required for cadmium (*C*) and mercury (*D*) to repress the *CTR3* promoter to 50% maximal repression.

to micromolar copper concentrations (10).[2] To characterize in detail the regulation of *CTR3* by metals, the low-copy number plasmid pRS*CTR3-lacZ* was transformed into DTY1, a strain that expresses both high affinity copper transporters Ctr1p and Ctr3p, and the level of *lacZ* steady-state mRNA was assayed by RNase protection experiments. Among the metal ions tested at many concentrations, Cu(II), Fe(II), Fe(III), Ag(I), Cd(II), Zn(II), Hg(II), Pb(II), Co(II), Mn(II), Ni(II), Mg(II), Ca(II), Au(I), and Au(III), only four were capable of repressing expression of *lacZ* mRNA under the control of the *CTR3* promoter: copper, silver, cadmium, and mercury (Fig. 1). Moreover by using a range of concentrations for each of these four metal ions, $CuSO_4$ and $AgNO_3$, were most effective for the repression of *CTR3-lacZ* transcription. Only 2.0×10^{-11} M and 1.4×10^{-11} M $CuSO_4$ and $AgNO_3$, respectively, was required for half-maximal repression (repression index 50% (RI_{50})) of expression from the *CTR3* promoter. In contrast, $CdCl_2$ and $HgCl_2$ repress transcription from the *CTR3* promoter, but at concentrations 3 orders of magnitude greater than for $CuSO_4$ and $AgNO_3$. The RI_{50} for $CdCl_2$ and $HgCl_2$ were 4.0×10^{-8} M and 1.7×10^{-8} M, respectively. Although we cannot eliminate the possibility that the $CdCl_2$ and $HgCl_2$ solutions contain trace levels of copper, atomic absorption spectroscopy failed to detect any copper in the $CdCl_2$ and $HgCl_2$ stock solutions. Therefore, the repression of *CTR3* gene expression exhibits a high degree of selectivity for copper ions. Moreover, the observation that Ag(I), a metal which is electronically similar to the reduced form of cupric Cu(II), represses *CTR3* gene expression with an efficacy similar to $CuSO_4$, suggests that cuprous Cu(I) might be the active form of copper in the signaling process resulting in repressing *CTR3* gene expression.

Time Course of Repression and Activation of CTR3 Gene Expression—Because yeast cells alter the expression of the copper transport machinery as a function of changing environmental copper levels, we analyzed *CTR3-lacZ* steady-state mRNA levels over time in response to either copper repletion or starvation. Using the low-copy number plasmid pRS*CTR3-lacZ* in strain DTY1, we followed the time course of down- and up-regulation of *CTR3* gene expression in the presence of copper (1 μM) or BCS (100 μM), respectively. The derepression of *CTR3-lacZ* gene expression is rapid with 91% of the maximal level of transcript detected 10 min after treatment with BCS (Fig. 2). On the other hand, 79% of the *CTR3-lacZ* transcript levels remained detectable after a 10-min exposure to copper. The time course data observed using the low-copy number plasmid pRS*CTR3-lacZ* were virtually identical to those observed for the endogenous *CTR3* gene.[3] These data demonstrate that yeast cells respond to changes in environmental copper levels by rapidly altering steady-state levels of the *CTR3* copper transport mRNA. The temporal differences observed between derepression and repression of the *CTR3-lacZ* reporter gene expression may be due to changes in the stability of the mRNA or may reflect unidentified differences in the repression *versus* the derepression signaling pathways.

[2] S. Labbé, and D. J. Thiele, unpublished data.

[3] M. Peña and D. J. Thiele, unpublished data.

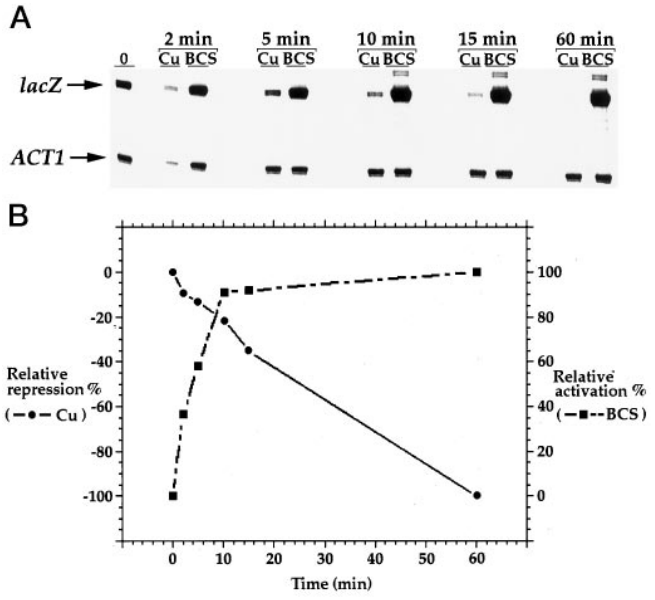

FIG. 2. **Time course of copper repression and copper-starvation activation of the *CTR3* promoter.** Strain DTY1, transformed with pRS*CTR3-lacZ*, was grown as described under "Experimental Procedures." *A, lacZ* and *ACT1* mRNA levels were analyzed in a time course for both repression with 1 μM $CuSO_4$, and copper deprivation with 100 μM BCS using RNase protection assays. For simplicity one control lane (0) is shown since the mRNA signals detected from untreated cultures for each subsequent time point were virtually identical. Results shown are representative of three independent experiments. *B*, graphic representation of quantitation of the RNase protection assay by PhosphorImaging. *Solid* and *dashed lines* show, respectively, the repression by copper and the activation by BCS of the *CTR3* promoter-*lacZ* fusion with respect to time.

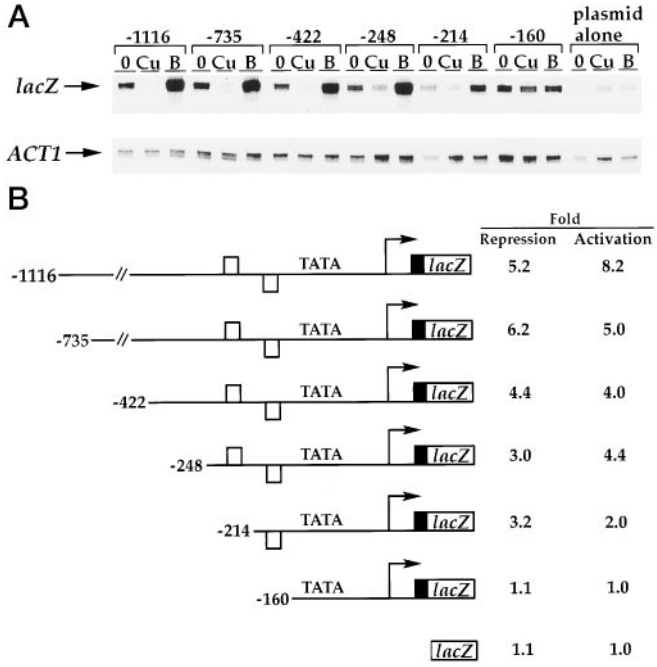

FIG. 3. **Identification of *CTR3* promoter elements critical for copper-repression and copper-starvation-mediated activation.** *A*, RNase protection assay from strain DTY1 harboring the *CTR3* promoter-*lacZ* fusion. Total RNA was isolated from each DTY1 transformant harboring the indicated *CTR3-lacZ* promoter derivatives. The steady-state mRNA of *lacZ* and *ACT1* are indicated with *arrows. B*, schematic representation of *CTR3-lacZ* promoter derivatives assayed in RNase protection experiments. For each of these derivatives the corresponding values for fold repression by copper (1 μM) and copper-starvation activation by BCS (100 μM) are shown. The *boxes* indicate the location of the repeat element TTTGCTC within the *CTR3* promoter and TATA indicates the location of the TATA box. The nucleotide *numbers* refer to the position relative to the A of the ATG codon of the *CTR3* ORF. Plasmid alone represents YEp357R*TRP1*.

Identification of cis-Acting Elements Necessary for Copper Repression and Copper-starvation Activation of CTR3, CTR1, and FRE1 Gene Expression—To identify *cis*-acting elements necessary for the copper repression and copper-starvation-mediated induction of *CTR3* expression, we fused 1116 bp of the 5′-noncoding region and the first 10 codons of *CTR3* in-frame with the *E. coli lacZ* gene on both low- and high-copy number plasmids pRS*CTR3-lacZ* and YEp*CTR3-lacZ*, respectively. *CTR3-lacZ* expression from both reporter plasmids was down-regulated in the presence of copper (approximately 5-fold), and up-regulated in the presence of BCS (approximately 8-fold) (Fig. 3 and data not shown). To identify the copper-responsive *CTR3* promoter elements, a series of nested 5′ deletions of promoter sequences beginning at position −1116 were created in the high-copy number plasmid YEp*CTR3-lacZ* (Fig. 3). Removal of the *CTR3* upstream region between −1116 and −248 had little effect on the copper and BCS-regulated expression of the *CTR3-lacZ* fusion, although the magnitude of the response decreased progressively. When the *CTR3* promoter was further deleted to position −214, the overall magnitude of the response decreased by approximately 75% as compared with the parental plasmid, but the reporter gene was still regulated in response to copper levels. Further deletion to position −160 was found to completely abolish both the down- and up-regulation of the reporter gene (Fig. 3). Due to the observation that the integrity of the region between positions −248 and −160 was essential for driving copper repression and copper-starvation activation of the *CTR3-lacZ* fusion, we examined whether this sequence could regulate a heterologous reporter gene in a copper-dependent fashion. A double-stranded DNA oligomer derived from the *CTR3* promoter (positions −237 to −173) was inserted in its natural orientation into the minimal promoter of the *iso-1-cytochrome c* (*CYC1*) gene fused to *lacZ* (40). This fusion promoter was able to down-regulate (approximately 5-fold) and up-regulate (approximately 9-fold) *lacZ* mRNA expression in the presence of copper or BCS, respectively (Fig. 4). Within this 64-bp *CTR3* promoter DNA fragment, we noted the presence of two copies of a repeated sequence, TTTGCTC, that are similar to the binding sites for the Ace1 and Amt1 copper MRTFs. To ascertain if this element plays a role in *CTR3* regulation by copper, all seven of these residues were mutationally altered in either or both repeats and the cells carrying *CTR3-CYC1-lacZ* fusion plasmids were assayed for copper-regulated expression of *lacZ* mRNA (Fig. 4*C*). Multiple point mutations in either or both elements abolished copper responsiveness of the *CTR3-CYC1-lacZ* fusions (Fig. 4). Moreover, a *CTR3-CYC1-lacZ* promoter fusion plasmid in which the last C in each TTTGCTC element was changed to G, failed to respond to the presence of a wide range of copper concentrations or copper-starvation to repress or activate gene expression (Fig. 4 and data not shown). Our data do not allow us to establish the reason why a single CuRE cannot regulate the *CTR3-CYC1-lacZ* fusion derivative, but does regulate with a low magnitude a 5′-truncated *CTR3* promoter-*lacZ* derivative which has one element (Fig. 3).

Interestingly, the TTTGCTC element was also observed to be perfectly conserved and repeated in the *CTR1* and *FRE1* promoters, both of which drive the expression of proteins involved in high affinity copper transport. As shown in Fig. 5, fusion of the regions from the *CTR1* or *FRE1* promoters encompassing these TTTGCTC repeats mediated copper repression and copper-starvation activation of *lacZ* mRNA expression. For both the *CTR1-CYC1-lacZ* and *FRE1-CYC1-lacZ* promoter fusions, the integrity of the TTTGCTC repeats was essential for copper-

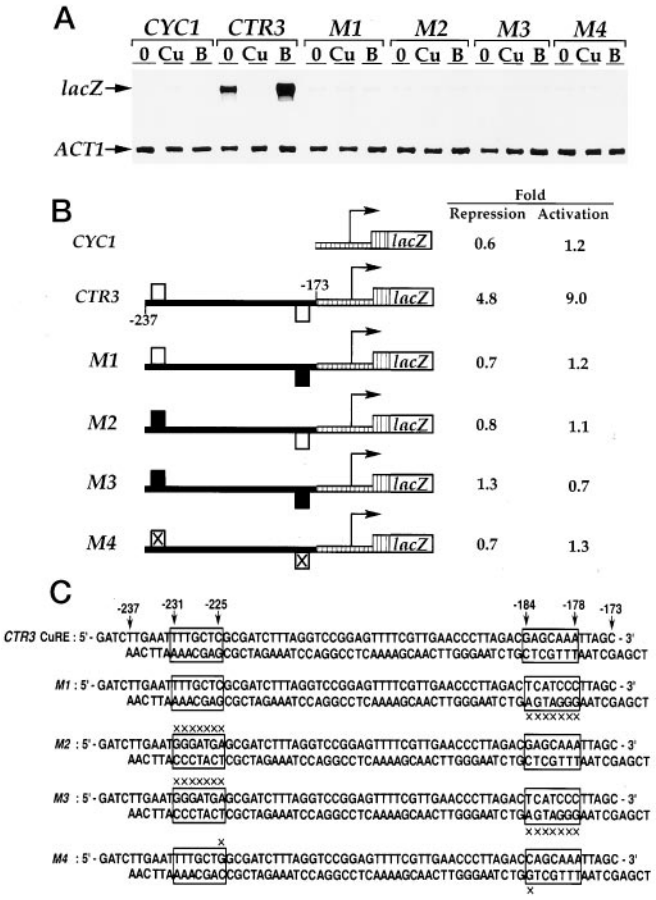

FIG. 4. **The *CTR3* promoter CuREs confer copper responsiveness to the minimal promoter *CYC1-lacZ*.** *A,* RNase protection analysis of repression by copper (1 μM), and derepression by BCS (100 μM) of the wild type repeated element from the *CTR3* promoter and mutant (*M1*, *M2*, *M3*, and *M4*) *CTR3-CYC1* minimal promoter-*lacZ* fusion derivatives. *lacZ* and *ACT1* mRNA levels are indicated with *arrows*. *B*, schematic representation of the plasmid derivatives assayed in the RNase protection assay, and quantitation of fold copper repression and copper-starvation activation. The *open boxes* indicate the wild type repeated element TTTGCTC (CuRE), the *filled boxes* represent the mutated versions harboring 7 substitutions, and the boxes marked with a "*X*" indicate that the element contains a G to C substitution. The nucleotide *numbers* refer to the position relative to the A of the start codon of the *CTR3* ORF, and the *hatched boxes* represent sequences derived from the *CYC1* minimal promoter. *C*, sequences of the synthetic oligomers used to create the fusions.

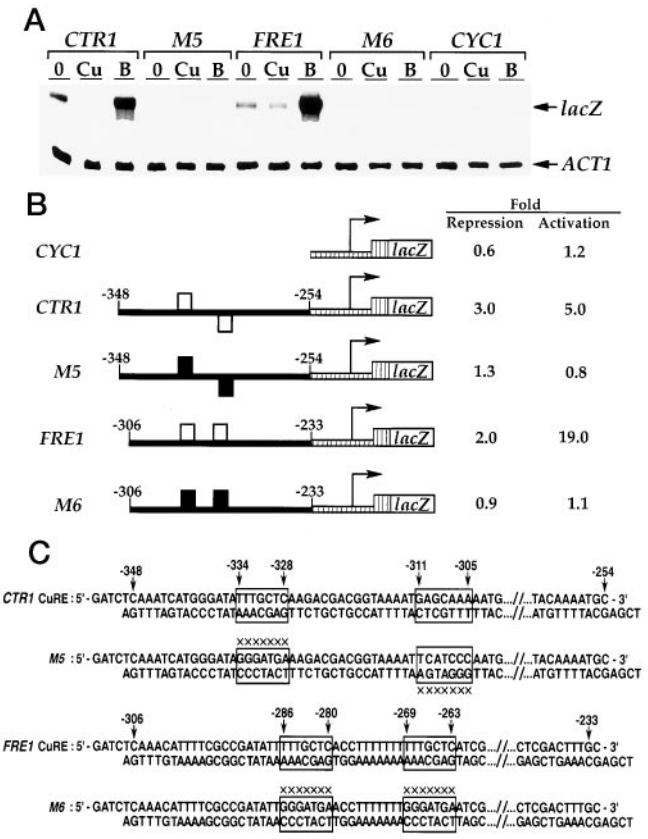

FIG. 5. **The copper-response elements are conserved in the *CTR1* and *FRE1* promoters.** *A*, *CYC1-lacZ* fusion genes containing wild type DNA fragments and CuRE mutants (*M5* and *M6*) derived from *CTR1* and *FRE1* promoters were analyzed by RNase protection as described in Fig. 4*A*. *B*, quantitation of the RNase protection experiment as in Fig. 4*B* except that the nucleotide numbers refer to the position relative to the A of the ATG codon of the *CTR1* (26) and *FRE1* ORFs (24). *C*, sequences of the promoter elements from the *CTR1* and *FRE1* genes and mutant derivatives.

responsive gene expression (Fig. 5). Therefore, based upon these studies the TTTGCTC element, denoted CuRE, plays a critical role in copper-regulated gene expression for all three yeast genes encoding components of the high affinity copper transport machinery. Furthermore, the CuREs in *CTR1*, *CTR3*, and *FRE1* function in both copper-repression and copper-starvation-mediated gene expression.

Yeast High Affinity Copper Transport Genes Require MAC1 for Expression and Regulation by Copper—The repression of *CTR3* gene expression is highly specific for copper and occurs at exquisitely low extracellular copper concentrations. Since *CTR1* and *CTR3* gene repression by copper is independent of the Ace1p copper MRTF (Ref. 10, and data not shown), we sought to identify components of the copper-signaling pathway that play a key role in the copper-regulated expression of the high affinity copper transport genes. The *S. cerevisiae MAC1* gene encodes a nuclear protein with a high level of homology to the first 40 amino acids of the Ace1 and Amt1 copper MRTFs. A hallmark of *mac1*Δ mutants is that virtually all of the pleiotropic phenotypes associated with these cells can be overcome by exogenous copper, thereby clearly implicating Mac1p in copper metabolism (32). Using isogenic strains harboring a wild type *MAC1* gene, an insertionally inactivated *mac1* allele (*mac1*Δ), and a dominant gain-of-function allele ($MAC1^{up1}$), we ascertained whether *MAC1* plays an essential role in *CTR1*, *CTR3*, and *FRE1* gene regulation as a function of cellular copper status by Northern blot analysis (Fig. 6). In the wild type strain (DTY1), basal levels of *CTR1*, *CTR3*, and *FRE1* mRNA are clearly visible. However, in the presence of 1 or 10 μM copper the steady-state levels of *CTR1*, *CTR3*, and *FRE1* mRNA were strongly repressed. Conversely, in the presence of 100 μM BCS, *CTR1, CTR3,* and *FRE1* mRNA levels were induced 3-, 14-, and 6-fold over basal levels, respectively. No mRNA was detected for *CTR1* and *CTR3* in the *mac1*Δ mutant strain (SLY2) even under conditions of copper starvation, although a low level of *FRE1* mRNA was still observed, perhaps due to the action of Aft1p (30). In the $MAC1^{up1}$ strain (DTY205), all three genes were highly expressed (8-fold for *CTR1*, 37-fold for *CTR3,* and 8-fold for *FRE1* with respect to the basal level detected in DTY1), and were virtually unregulated by copper or copper-starvation. Taken together, these data demonstrate that Mac1p is an essential *trans*-acting component of the copper signaling pathway for appropriate expression and regulation of genes involved in high affinity copper transport.

The CuRE Is Differentially Occupied under Copper Deprivation and Repletion—Although Mac1p exhibits homology to the Ace1p and Amt1p copper-activated DNA-binding proteins, we

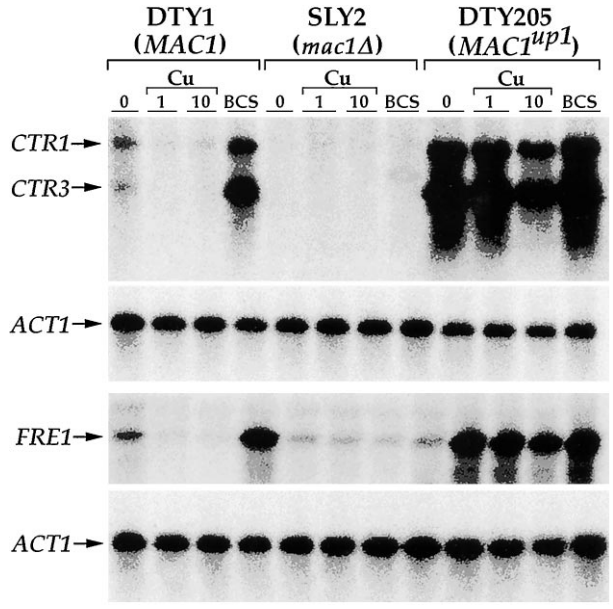

FIG. 6. ***CTR1, CTR3,* and *FRE1* gene expression is regulated by cellular copper status through Mac1p.** The isogenic strains DTY1 (*MAC1*), SLY2 (*mac1*Δ), and DTY205 ($MAC1^{up1}$) were grown to early log-phase in YPD media. $CuSO_4$ (0, 1, and 10 μM) or BCS (100 μM) was added and after a 1-h incubation at 30 °C total RNA was isolated. Shown is an RNA blot of *CTR1*, *CTR3*, *FRE1*, and *ACT1* mRNA steady-state levels indicated by *arrows*, respectively.

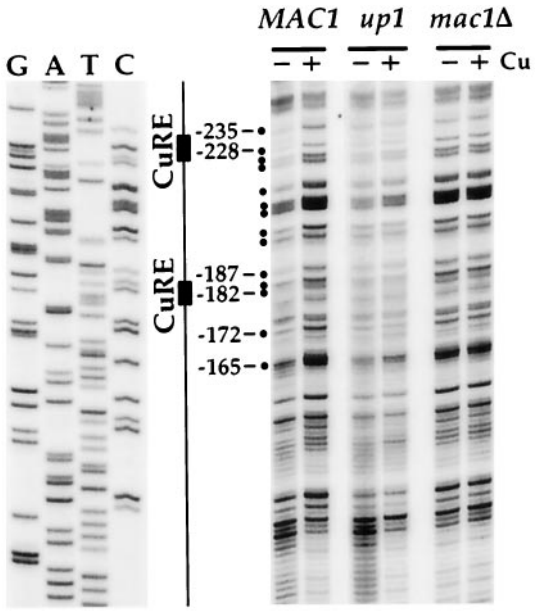

FIG. 7. **The *CTR3* CuREs are differentially occupied in a copper- and Mac1p-dependent manner.** Strains DTY1 (*MAC1*), DTY205 ($MAC1^{up1}$), and SLY2 (*mac1*Δ), transformed with YEp*CTR3-lacZ*, were grown as described in the legend to Fig. 1. Cultures were incubated in the absence (−) or presence of 10 nM $CuSO_4$ (+) for 1 h followed by a 5-min incubation with dimethyl sulfate, and DNA was isolated and processed for primer-extension footprinting as described under "Experimental Procedures." Results illustrated are representative of three independent experiments. *Dots* indicate the position of the protected G residues. *Boxes* represent the location of two CuREs within the *CTR3* promoter. The *numbers* refer to the position relative to the A of the start codon of *CTR3* ORF. Shown to the *left* is the reference DNA sequencing reactions performed using the oligonucleotide primer CTR3E.

have been unable to demonstrate CuRE DNA binding activity for Mac1p expressed in *E. coli* or by *in vitro* transcription translation. To begin to understand the molecular responses to copper mediated by the CuREs, and the function of Mac1p in this pathway, we have examined the *CTR3* promoter in cells starved for copper or grown in the presence of copper by *in vivo* dimethyl sulfate footprinting. In the wild type strain (DTY1) under copper deprivation conditions that parallel induced *CTR3* gene expression, we observed that a region encompassing and flanking the two CuREs located in the *CTR3* promoter is strongly protected from methylation by dimethyl sulfate relative to copper-treated cells in which *CTR3* gene expression is repressed (Fig. 7). Upon copper treatment of the wild type strain with 10 nM $CuSO_4$, we observed that all of the G residues encompassing and immediately flanking the CuREs were strongly modified by dimethyl sulfate. Specifically, the G residues corresponding to positions −182 and −184 inside of the downstream TTTGCTC element (oriented in the opposite direction relative to the direction of the transcription), and the G located at position −228 inside of the upstream TTTGCTC element were strongly protected from dimethyl sulfate modification. The protection detected for the G residue at position −184 correlates with the critical requirement for this G for copper-responsive gene regulation in *CTR3-CYC1-lacZ* fusion gene (see construct *M4*, Fig. 4). Furthermore, each G residue between the two CuREs, plus two additional G residues (at positions −172 and −165) located downstream of the repeat were found to be protected from dimethyl sulfate under conditions in which *CTR3* is expressed, but not when *CTR3* expression is repressed, suggesting the presence of one or more proteins occupying this region under condition of expression. Furthermore, a similar pattern as the one described for the wild type strain under copper deprivation conditions was observed when this strain was grown in the presence of BCS (data not shown). Consistent with the constitutive high level *CTR3* gene expression observed in the $MAC1^{up1}$ strain, a constitutive protected pattern in the *CTR3* promoter, comparable to that found for the wild type strain (*MAC1*) under copper deprivation conditions, was revealed by dimethyl sulfate footprinting. Conversely, the *CTR3* promoter region in the isogenic *mac1*Δ strain revealed no copper-dependent changes in dimethyl sulfate reactivity and strongly resembled the copper-repressed promoter configuration in the wild type strain (Fig. 7). Therefore, a functional *MAC1* gene is required for copper-dependent changes in the *CTR3* promoter region encompassing and flanking the CuREs.

DISCUSSION

Because of its essential yet toxic nature, all cells must maintain tight homeostatic regulation of the levels of bioavailable copper. Regulatory responses to copper include the activation or repression of gene transcription, copper-modulated turnover of proteins that transport or utilize copper, and alterations in the intracellular trafficking of copper transporting ATPases in response to fluctuations in copper concentrations (9–13, 27). In this report, we have characterized the molecular mechanisms responsible for the copper-responsive regulation of *CTR3*, *CTR1*, and *FRE1* genes that play a critical role in high affinity copper transport in yeast. Detailed studies of *CTR3* gene regulation have revealed that half-maximal repression of *CTR3* mRNA levels occurs in the presence of as little as 2.0×10^{-11} M $CuSO_4$ or 1.4×10^{-11} M $AgNO_3$ (Fig. 1). This represents an exquisitely sensitive and selective metal responsive system since at least 1000-fold higher concentrations of cadmium or mercury were required to achieve the same level of repression and no other metal tested was able to repress *CTR3* mRNA levels. Since repression is observed with cadmium and mercury, however, we cannot at present eliminate the possibilities that either trace levels of copper, undetectable by atomic absorption, were present in our stock solutions or that there are

significant differences in the efficiency with which copper and silver, *versus* cadmium and mercury, are transported or distributed in yeast cells. The similarity in the potency of silver in fostering the repression of *CTR3* mRNA levels resembles previous results on the Ace1 and Amt1 copper MRTFs, which are known to form tetragonal coordinates with Cu(I) via cysteine thiolates (23). The electronic similarity of Ag(I) to Cu(I), but not Cu(II), suggests that the copper sensing machinery involved in the repression of yeast copper transport genes may sense Cu(I), rather than Cu(II).

Our studies have identified strictly conserved repeated CuREs present in two copies in each of the *CTR1*, *CTR3*, and *FRE1* promoters. Inspection of the *CTR1* promoter suggests the presence of a third CuRE, located between positions −529 and −523, however, the contribution of this element to copper-responsive regulation of *CTR1* has not yet been determined. Since single or multiple point mutations within the CuREs abolish both copper-dependent repression and copper-starvation-induced expression of *CTR1*, *CTR3*, and *FRE1*, this suggests that yeast cells utilize a common mechanism to coordinately regulate the expression of the copper transport machinery. Examination of the CuRE sequences in *CTR1*, *CTR3*, and *FRE1*, suggest a consensus sequence WWWTTT-GCTCR (W = A or T; R = purine). The CuRE sequence bears an interesting sequence similarity to the binding sites for the Ace1 and Amt1 copper MRTFs (HTH*XX*GCTG, H = A, C or T; *X* = any residue), however, the inability to convert a CuRE to an Ace1p activation site by substituting the terminal C residue for a G (Fig. 4, plasmid derivative *M4*) suggests that other nucleotides within or flanking the CuRE confer specificity for copper repression and copper-starvation induction of gene expression which is independent of Ace1p (10).[2] Although the center-to-center distances between the CuREs, for each of the *CTR1*, *CTR3*, and *FRE1* promoters predict that they lie on opposite faces of the DNA, it is currently unknown whether this geometry plays a role in the regulation of copper transporter gene expression via the CuREs. The *FRE1* promoter fragment, which has the shortest distance between the two CuREs, gave rise to the poorest repression by copper and the strongest activation by copper-starvation. Since this promoter fragment lacks binding sites for the Aft1 iron-responsive regulatory protein (31), this supports the notion that de-repression of the *FRE1-CYC1-lacZ* fusion gene represents a response to copper starvation rather than an indirect response to iron starvation. However, whether other promoter elements contribute to the magnitude of these regulatory responses must await a comprehensive dissection of each of the promoters of these genes encoding proteins that function in high affinity copper transport.

It is currently unknown how such exquisitely low extracellular copper concentrations are capable of signaling the repression of *CTR1*, *CTR3*, and *FRE1* gene expression. Based on previous observations that *S. cerevisiae* cells harboring a deletion of the *MAC1* gene display pleiotropic defects that are corrected by the addition of copper, and are defective in the regulation of *FRE1* transcription (25, 32), we tested the possibility that *MAC1* plays a role in the regulation of all the genes known to be involved in high affinity copper transport in yeast. Indeed, we have demonstrated that cells bearing a disruption of the *MAC1* gene are severely defective in the expression and regulation of mRNA levels from each of these genes (Fig. 6). Furthermore, isogenic cells harboring a dominant allele of *MAC1*, in which a His residue in the first Cys-rich carboxyl-terminal cluster repeat has been altered to Gln, fail to respond to copper administration to repress *CTR1*, *CTR3*, or *FRE1* mRNA levels. Although Mac1p exhibits significant sequence similarity to the amino-terminal 40 amino acids of Ace1p and Amt1p, and a Mac1-β-galactosidase fusion protein has been localized to the yeast cell nucleus (32), we have been unable to demonstrate specific CuRE binding activity for Mac1p either expressed in *E. coli* cells or produced by *in vitro* transcription and translation (data not shown). Therefore, Mac1p may function in the copper-signaling pathway to regulate copper transport gene expression through a number of potential mechanisms. First, Mac1p may require specific post-translational modifications or partner proteins for sequence-specific DNA binding that would not be present through expression in heterologous systems. Alternatively, Mac1p could be required for the synthesis or activity of another protein(s) which directly interacts with CuREs, or Mac1p may play a critical nuclear signaling role for copper that is upstream of a direct DNA binding activity. Consistent with all of these models, electrophoretic mobility shift experiments using extracts from yeast cells expressing a functional epitope-tagged *MAC1* allele have revealed the presence of a specific CuRE-protein complex from control or BCS-treated cells which is absent in extracts from cells treated with 10 nM $CuSO_4$ (data not shown). Furthermore, the formation of this CuRE-protein specific complex was dependent on the presence of a functional *MAC1* gene, and the complex was not abolished by the addition of copper to a strain bearing a $MAC1^{up1}$ allele. On the other hand, the inability of the anti-epitope monoclonal antibody to supershift this complex leaves open the possibility that Mac1p may not directly contact DNA sequences within the CuRE. Our *in vivo* footprinting studies clearly demonstrate that in the wild type *MAC1* strains the *CTR3* CuREs are occupied under conditions of active *CTR3* gene expression (*i.e.* copper-starvation), and are highly accessible to dimethyl sulfate modification under conditions in which *CTR3* gene expression is repressed (*i.e.* addition of 10 nM $CuSO_4$). In contrast, *mac1*Δ mutants give rise to constitutive dimethyl sulfate modifications in the *CTR3* promoter region encompassing the CuREs that resemble the repressed state in the wild type strain. Furthermore, $MAC1^{up1}$ mutants, which constitutively express the copper transport genes and are unresponsive to copper for repression, give rise to constitutive protection from dimethyl sulfate modification within and flanking the *CTR3* CuREs. Taken together these results demonstrate that Mac1p is an essential component of the copper-signaling pathway that directly or indirectly modulates coordinated copper-responsive gene expression of yeast high affinity copper transport genes through the CuREs.

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