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 Cu²⁺/Fe³⁺ 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 coordinate expression 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 (2, 3). However, when allowed to accumulate in excess, copper is highly toxic due to its proclivity to engage in redox reactions (2, 3).

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). 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 baker’s yeast Saccharomyces cerevisiae and the opportunistic pathogenic yeast Candida glabrata, as well as cis-acting promoter regulatory sequences with the consensus sequence HTHLXGCTG (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).

¹ The abbreviations used are: MRTF, metalloregulatory transcription factor; BCS, bathocuproinedisulfonate; CTR, copper transporter; CuRE, copper-response element; ORF, open reading frame; R150, repression index 50; bp, base pair(s).

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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 cis-acting element, termed CuRE (copper-response element), is occupied under copper-starvation conditions (26). Studies reveal that the CuRE, as identified by a 233-bp DNA fragment from plasmid YEp357TRTP1 isolated from p330 (gift of Robert Fuller) into the StuI and Klenow filled-in Xol4 sites of YEp357TRTP1, disrupting the URA3 genetic marker. The oligonucleotides CTR3-A (5′-CTCCGCGATCTAGCATGCT-GAACAATTTC-3′) and CTR3-B (5′-GTCCGGAATTCGAGTCATGCT-GCTACTGCTCC-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 expressed both the Ctr1p and Cyp51p copper transmembrane transport product was sequenced in its entirety and fused in-frame to the Escherichia coli lacZ gene using the BamHI and EcoRI sites of YEp357TRTP1 to generate YeCTR3lacZ. A low-copy number plasmid prsCTR3-lacZ was also constructed. To accomplish this construct, a 4.8-kilobase DNA fragment from plasmid YeCTR3-lacZ containing the CTR3 sequence from −1116 to +10 fused to the E. coli lacZ gene was inserted into the BamHI and Smal1 sites of pBluescript II KS. To perform the RNase protection analyses as described previously (36), two plasmids for making antisense RNA probes were made. pSklacZ was constructed by the insertion of the 233-bp EcoRV-BclI fragment from the E. coli lacZ gene into the EcoRV and BamHI sites of pBluescript II KS. prsACT1 was made by the insertion of the 132-bp HindIII-EcoRI fragment from then Act1 gene into the HindIII-EcoRI sites of pBluescript II KS. A series of plasmids containing sequential deletions from the 5′ end of the CTR3 promoter (Fig. 3B) were created from plasmid YeCTR3-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. 4C and 5C) were annealed pairwise to form double-stranded DNA and then ligated into the BglII and XhoI sites of a CTC1-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 quantified 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 (MAC110), and SLY2 (mac1) containing YeCTR3-lacZ were grown to early log-phase in modified SD medium. Untreated and copper-treated (10 μM) 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 BstXI 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′-GCTCCCCATATTTTATTTTATGCC-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 transporter, 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 COP1, CRS5, and SOD1 genes in response to 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, 36).

Yeast strains were grown to early log-phase on modified minimal medium (SD), which was depleted for copper as described previously (10). This depleted medium contains 16 μM copper as determined by atomic absorption spectroscopy while standard synthetic complete media contains 150 μM copper. Copper administration or copper starvation of yeast strains were carried out by adding the indicated amount of CuSO4 or bathocuproinedisulfonate (BCS) to optimal medium 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).

### TABLE I

| Strain | Genotype | Reference |
|-------|----------|-----------|
| DTY1  | MAT a gal1 trp1–1 his3 ade8 CUP1 | 33        |
| DTY205| MAT a gal1 trp1–1 his3 ade8 CUP1 MAC1up1 | 32        |
| SLY1  | MAT a gal1 trp1–1 his3 ade8 CUP1 ura3::KAN | This study |
| SLY2  | MAT a gal1 trp1–1 his3 ade8 CUP1 ura3::KAN mac1::URA3 | This study |
to micromolar copper concentrations (10). To characterize in detail the regulation of CTR3 by metals, the low-copy number plasmid pRSCTR3-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), 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, CuSO4 and AgNO3 were most effective for the repression of CTR3-lacZ transcription. Only $2.0 \times 10^{-11}$ and $1.4 \times 10^{-11}$ M CuSO4 and AgNO3, respectively, was required for half-maximal repression (repression index 50% (RI50)) of expression from the CTR3 promoter. In contrast, CdCl2 and HgCl2 repress transcription from the CTR3 promoter, but at concentrations 3 orders of magnitude greater than for CuSO4 and AgNO3. The RI50 for CdCl2 and HgCl2 were $4.0 \times 10^{-8}$ and $1.7 \times 10^{-8}$ M, respectively. Although we cannot eliminate the possibility that the CdCl2 and HgCl2 solutions contain trace levels of copper, atomic absorption spectroscopy failed to detect any copper in the CdCl2 and HgCl2 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 CuSO4, 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 pRSCTR3-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 pRSCTR3-lacZ were virtually identical to those observed for the endogenous CTR3 gene. 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.

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

3 M. Peña and D. J. Thiele, unpublished data.
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 the iso-1-cytochrome c (CYC1) gene fused to lacZ. 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 mutagenically 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. 4C). 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-
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-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 ATG codon of the CTR1 (26) and FRE1 ORFs (24). C, sequences of the synthetic oligomers used to create the fusions.

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 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 mac1Δ 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 (mac1Δ), and a dominant gain-of-function allele (MAC1up1), 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 with respect to the MAC1up1 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 MAC1Δ 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
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 immediately 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 CuSO₄, we observed that all of 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 revealed by dimethyl sulfate footprinting. Conversely, the CTR3 promoter region in the isogenic mac1Δ 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 CuSO₄ or 1.4 × 10⁻¹¹ M AgNO₃ (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

| Strain | MAC1 configurations |
|--------|---------------------|
| DTY1   | MAC1Δ               |
| SLY2   | MAC1Δ              |
| DTY205 | MAC1up1            |

**Fig. 6.** CTR1, CTR3, and FRE1 gene expression is regulated by cellular copper status through Mac1p. The isogenic strains DTY1 (MAC1), SLY2 (mac1Δ), and DTY205 (MAC1up1) were grown to early log-phase in YPD media. CuSO₄ (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 (MAC1up1), and SLY2 (mac1Δ), transformed with YEplac139-CTR3-lacZ, were grown as described in the legend to Fig. 1. Cultures were incubated in the absence (−) or presence of 10 nM CuSO₄ (+) 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.
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 transporter 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 (HTHXXGCTG, 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 repression and 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 of 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 carboxy-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 Mac1p g-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 transporter 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 CuSO4 (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 MAC1op1 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 CuSO4). In contrast, mac1Δ 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, MAC1op1 mutants, which constitutively express the copper transporter 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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