Mac1 is a transcriptional activator whose activity is inhibited by copper ions. Mutagenesis studies were carried out to map residues important in the copper inhibition of Mac1 activity. Seven new missense mutations were identified that resulted in copper-independent Mac1 transcriptional activation. All seven mutations were clustered in one of two C-terminal cysteine-rich motifs, designated the C1 motif. All but one of the constitutive Mac1 mutations occurred in one of the conserved six residues in the $^{264}$CXC$(X)_2$CXC$(X)_2$CXC$(X)_2$H$^{279}$ C1 motif. The lone exception was a L260S substitution. Two additional MAC1 mutations exhibiting constitutive activity were in-frame deletions encompassing portions C1. Engineered mutations in the second cysteine-rich motif did not yield a constitutively active Mac1. These results are consistent with the C1 motif being the copper-regulatory switch. Both cysteine-rich motifs exhibited transactivation activity, although the C1 activator was weak relative to the C2 activator. Limited copper metabolism regulation of Mac1 was observed with only the C1 activator fused to the N-terminal DNA binding domain. Thus, the two Cys-rich motifs appear to function independently. The C1 motif appears to be a functional copper-regulatory domain.

Yeast cells respond to nutrient metal ion availability by regulating the expression of cell surface transport proteins. Nutritional deprivation of copper, iron, and zinc ions in Saccharomyces cerevisiae results in up-regulation of specific high affinity transporters arising from the activation of specific transcription factors (1–3). Nutritional deprivation of copper ions in yeast leads initially to the activation of the Mac1 transcription factor and subsequent stimulated transcription of three genes encoding proteins involved in high affinity copper uptake (4, 5). However, Mac1 lacks transcriptional activation activity in copper-replete cells (4). Mac1 is critical for expression of the high affinity copper uptake system; therefore, cells lacking Mac1 exhibit phenotypes consistent with a copper-deficient state. Copper ions are necessary in yeast for formation of active cytochrome oxidase, superoxide dismutase, and the Fet3 ferro-oxidase.

The high affinity uptake system in yeast consists of two copper ion permeases, Ctr1 and Ctr3, and a metalloprotease Fre1 capable of copper ion reduction (6). A fourth gene activated by Mac1 is the Fre1 homolog, Fre7, the function of which remains unknown (7). Reduction of extracellular cupric and ferric ions is an important step in high affinity copper and iron uptake in yeast. An important facet of copper homeostasis in yeast is the ability to restrict entry of copper ions when cells are copper-replete. This is illustrated by the redundant cellular mechanisms to block copper ion uptake and buffer cellular copper levels. The initial event is the specific inhibition of DNA binding and transactivation functions of Mac1 that occurs in cells cultured in medium containing $\mu$M Cu(II) (4, 8, 9). Repression of Mac1 function inhibits transcription of CTR1 and CTR3 (4). In response to elevated cellular copper, the Mac1 protein is degraded, ensuring that expression of the high affinity uptake system is shut down (10). In addition, pre-existing copper transporters are internalized and degraded (11). A further, protective response occurs in cells cultured in medium containing Cu(I) salts. Copper ions activate the nuclear transactivation factor Ace1 which induces transcription of three distinct genes, products of which bind copper ions, protecting the cell from deleterious effects of elevated copper levels (12).

The copper-dependent inhibition of Mac1 function appears to arise from direct interaction of Cu(I) with Mac1 (13). Mac1 contains two C-terminal cysteine-rich sequences with a repeating motif of CXC$(X)_2$CXC$(X)_2$CXC$(X)_2$H (14). These two Cys-rich sequences, designated C1 and C2, bind a total of 12 Cu(I) ions (13). The Mac1 ortholog, Grisrea from Podospora angerina, contains two related C-terminal Cys-rich motifs that are important for metalloregulation (15). Also, the Schizosaccharomyces pombe Mac1 ortholog, Cuf1, has a single Cys-rich motif homologous to the Mac1 motif above (16). The Cys-rich motifs occur in the segment of Mac1 responsible for transactivation (9). The transactivation function of Mac1 is copper-modulated (9). Inhibition of transactivation is seen in chimeric molecules containing Mac1 fused to a heterologous DNA binding domain (8, 9). In addition, the DNA binding function of Mac1 is inhibited in copper-replete cells (4). DNA binding activity of Mac1 maps to the N-terminal 159 residues, but copper repression of DNA binding requires the C-terminal Cys-rich motifs (17). Likewise, full copper repression of transactivation activity is dependent on a portion of the DNA binding domain. Thus, Cu(I) binding in the C-terminal domains inhibits functions of both the N-terminal and C-terminal halves of the protein. The copper repression of Mac1 function appears to arise from a copper-induced, intramolecular interaction of the C-terminal Cys-rich motifs and the N-terminal DNA binding domain (13).

Mac1 was originally identified as a semidominant mutation, designated MAC1<sup>op1</sup>, that abrogated copper inactivation of Mac1 function (14). This mutation results in constitutive expression of CTR1 and FRE1 (4, 5). Copper ion uptake is markedly enhanced in MAC1<sup>op1</sup> cells, and one resulting phenotype is hypersensitivity to elevated copper levels in the growth medium (18). Three additional constitutive mutations in MAC1 have also been identified. All four constitutive mutations map...
to the first cysteine-rich motif, designated C1 (Fig. 1). The constitutive mutations result in substitutions of three of the six conserved residues in the consensus, 264 CXC(X)2CXC(X)2C-(X)6H779. The residues in bold are substituted to yield the four known constitutive mutants designated Mac1up1 (H279Q), Mac1up2 (C271Y), Mac1up3 (C264Y), and Mac1up4 (H279Y) (5, 10).

The copper-induced inhibitory, intramolecular interaction of the N-terminal DNA binding domain and the C-terminal Cys-rich motifs is expected to involve residues in both domains. Since mutations in the C1 motif abolish the inhibitory interaction, we sought to map the interactive region in the N-terminal DNA binding domain of Mac1 by isolating additional constitutive Mac1 mutants. The question was whether any mutations within the DNA binding domain would abolish the repressive intramolecular interaction, without perturbing DNA binding activity. The second motive for the present work was to determine whether the repeated Cys-rich sequence motifs function separately or as a single domain in mediating copper metalloregulation of Mac1. Conceivably, the binding of 8 Cu(I) ions in a polypeptide containing both C1 and C2 sequences may occur due to 4 Cu(I) ions binding in each motif. Alternatively, the two Cys-rich motifs may come together creating a single octa-copper center. The clustering of constitutive mutations in the C1 motif is consistent with C1 being an independent copper regulatory domain. The presence of two Cys-rich motifs in Mac1 and Grisea leaves open the possibility that both motifs function in copper metalloregulation. We show in the current work that the C1 motif is indeed an independent copper regulatory domain.

**MATERIALS AND METHODS**

**Strains and Culture Conditions**—The yeast strains used, CM66J (MATa, trp1-63, gcn4-101, his3-609, ura3-52, FRE1-HIS3::LEU2) and CM66E (MATa, trp1-63, gcn4-101, his3-609, ura3-52, FRE1-HIS3::LEU2) and CM3262 (MATa, ino1-13, gcn4-101, his3-609, ura3-52, leu2-3, 112, ino1-13). Strains 66 and CM3262 were generously provided by Andrew Dancis (19). CM66mac1 was constructed by integrating URA3 at the MAC1 locus in strain CM66E by one-step gene replacement as described (20). The disruption was verified by diagnostic PCR1 and sequencing. CM66mac1 was then crossed with YJ11 (MATa, his3-200, leu2-3, 112, lys2-801, trp1-1, ura3-52, mac1-1) (14), sporulated, and dissected. A resulting spore that formed into strain Y190. The clustering of constitutive mutations in the C1 motif is consistent with C1 being an independent copper regulatory domain. The presence of two Cys-rich motifs in Mac1 and Grisea leaves open the possibility that both motifs function in copper metalloregulation. We show in the current work that the C1 motif is indeed an independent copper regulatory domain.

**Random Mutagenesis**—A 1.7-kilobase pair Sau3A genomic fragment containing MAC1 was subcloned from pYACu1 (generously provided by D. Hamer) into pBluescript (Stratagene) at the BamHI site, generating pBSMAC1. pBSMAC1 was then subjected to XLI-Red mutagenesis according to manufacturer’s instructions (Stratagene). The resulting plasmids were rescued from XLI-Red Escherichia coli, inserts cleaved by XbaI/BamHI digest, cloned into PBS316 (22), and transformed into E. coli DH5α. The resulting library of mutagenized MAC1 was transformed into CM3mac1. Transformants were replica-plated onto LCCM containing 30 μM Cu(II), lacking histidine and uracil and medium containing 1.75 mM CuSO4. Positive growth on the former and negative growth on the latter was indicative of a plasmid-borne MAC1m allele and was verified by loss of histidine prototrophy following shedding of plasmid by growth on media containing 5-fluoro-orotic acid. The ability of each mutagenized plasmid to confer elevated CTR1 transcript levels was determined by S1 nuclease assay. Each plasmid conferring a MAC1m phenotype was rescued to E. coli DH5α and sequenced. DNA sequencing was performed on an ABI Prism 377 Sequencer using Taq FS DNA polymerase.

**Site-directed Mutagenesis**—The 1.7-kilobase pair XbaI/BamHI MAC1 fragment from pBSMAC1 was subcloned into pAlter (Promega). Mutagenesis was carried out according to manufacturer’s instructions. The mutations were verified by sequencing and subsequently cloned into either pRS316 or pRS426. Phenotypes were determined by transforming into CM3mac1.

**Vectors**—MAC1 and MAC1up truncates encoding codons 1–311 were generated by PCR amplification using PstI and SalI cleavage sites for subcloning into vector pTVT102. pTVT102 is a Yep-based vector containing the ADH1 promoter and terminator (23). The HA epitope tag encoding the sequence YPDVPYD was inserted at the 3’ end of codon 311 of MAC1. GAL4/MAC1 fusions, engineered previously (9), were excised as HindIII/SalI fragments and inserted into pRS425, a Yep-based vector containing the MET15 promoter and CYC1 terminator (24). GAL4/MAC1 constructs were transformed into DY2042 that contains the GAL1–10/ lacZ fusion reporter gene. The two hybrid MAC1 and MAC1up fusion vectors were constructed (13). DNA sequences encoding residues 240–417 of Mac1 and containing the double Cys → Ser substitutions (residues 264, 266; 271, 273; 322, 324; and 329, 331) previously engineered (9) were PCR-amplified and subcloned into the vector pVTVP16 at BamHI/ClaI sites, generating vectors fused to the minimal activation domain from the herpes simplex virus VP16 protein (25). Sequences encoding residues 1–159 were subcloned into pBG4D-1 creating vector Mac1–159–pBG4D-1. Two-hybrid vectors were co-transformed into strain Y190.

**RNA Quantitation by S1 Nuclease Analysis**—Total RNA isolated from mid-logarithmic cells by the hot acid phenol method was hybridized with a 32P-labeled single-stranded DNA oligonucleotide and digested with S1 nuclease. The samples were electrophoresed through an 8% polyacrylamide, 5 x urea gel, and data were quantified by PhosphorImager analysis. S1 probes used included 60 nucleotides of the 5’ CTR1 ORF, 40 nucleotides of the calmodulin (CDM1) 5’ ORF, and 60 nucleotides from the lacZ ORF.

**Immunoprecipitation and Western Analysis**—Cellular lysates for immunoprecipitation analysis were prepared by glass bead in 50 mM Tris-Cl, pH 7.5, 150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate and a protease inhibitor mixture. The supernatant was incubated with either a rabbit polyclonal anti-HA antibody or a rabbit polyclonal anti-Gal4 DBD antibody for 1 h at 4 °C. Protein A-agarose was added and incubated overnight at 4 °C. The protein A-agarose was collected and treated with SDS sample buffer. The immunoprecipitated protein was resolved on SDS-polyacrylamide (15%) gel electrophoresis and transferred to nitrocellulose. Membranes were blocked and probed with either mouse monoclonal anti-HA antibody or a rabbit monoclonal anti-Gal4 DBD. Detection was by enhanced chemiluminescence after incubation with a horseradish peroxidase-conjugated secondary antibody.

**Cellular lysates for VP16 Western analysis were prepared by glass bead in 30 mM Tris-Cl, pH 7.5, 500 mM sodium chloride, 5 mM EDTA, 0.1% Triton X-100, 12% (v/v) glycerol, and a protease inhibitor mixture.**
Copper Regulation of Mac1 Function

FIG. 1. Schematic representation of Mac1 showing the N-terminal DNA binding domain and two C-terminal Cys-rich motifs, C1 and C2. The sequence expanded above the C1 motif is the sequence of residues 260-280. The conserved residues are shown in bold. The bold residues on the lines above the C1 sequence are the four known constitutively active Mac1 mutations. The additional residues shown above the C1 sequences are the additional Mac1 mutations identified in the present study.

FIG. 2. Growth assays of cells harboring various MAC1 mutations. The column on the left lists the various mutants isolated or generated. Substitutions in these mutants are shown. All mutants tested were expressed on a low copy vector. Mutants were cultured on three types of agar. From left to right the culture conditions were growth in complete medium, growth in medium lacking histidine but containing 30 μM CuSO₄, and on the right growth in medium containing 1.75 mM CuSO₄. Ten-fold serial dilutions of cells were plated.

RESULTS
FRE1 is one of the four known Mac1-regulated genes in S. cerevisiae (14). Mac1 activates FRE1 expression in copper-deficient cells. Cells harboring a MAC1 allele show elevated FRE1 expression even in copper-replete conditions (14). In addition, FRE1 expression is also enhanced in iron-deficient cells due to the iron-regulated Aft1 transcriptional activator (19). The dual regulation of FRE1 by the copper and iron status of cells enabled a genetic screen utilizing a FRE1/HIS3 fusion gene to identify a variety of genes whose products were involved in copper and iron uptake (26, 27). This elegant screen also identified Aft1 as the primary iron regulator in S. cerevisiae and uncovered a second Mac1 constitutive mutant designated MAC1 (5, 19).

The successful use of the FRE1/HIS3 fusion gene to identify a Mac1 mutant that was not repressed in copper-replete cells validated its use in mapping other constitutive MAC1 alleles. The starting strain (CM66GJ) exhibited histidine (His) prototrophy only when cultured in low copper medium containing a Cu(I)-specific chelator, bathocuproine disulfonate. The cells were His-auxotrophic when cultured in medium containing 10 μM Cu(II). Following ultraviolet-induced mutagenesis, mutants exhibiting His prototrophy were selected on medium containing 30 μM Cu(II). Subsequently, His⁺ colonies were screened for growth sensitivity to 1.75 mM CuSO₄. Wild-type cells were resistant to elevated copper levels, whereas cells containing a constitutively active mutant Mac1 failed to grow due to unrestricted uptake of copper ions. Colonies conforming to both selection criteria, growth on -His + Cu medium and copper sensitivity (Cu), were analyzed for constitutive expression of chromosomal CTR1 by quantifying CTR1 mRNA levels. Eight mutants exhibited high, constitutive CTR1 expression. The eight mutants were determined to carry a single nuclear, recessive mutation by crossing with the isogenic wild-type strain followed by tetrad analysis. Diploids of the mutants crossed with CM mac1 showed His prototrophy in medium containing 30 μM CuSO₄. These experiments suggested that the mutations were in MAC1. The MAC1 locus was PCR-amplified and sequenced in multiple reactions. All eight clones showed mutations in the C1 region of MAC1 with the following substitutions (number in parentheses indicate number of independent isolates): L260S (2), C264Y, C271Y, C271G, H279Q (3) (Fig. 1). The C264Y, C271Y, and H279Q substitutions represent three of the four known constitutive Mac1 mutants (5, 10). Two new constitutive alleles were L260S and C271G.

The involvement of Leu²⁶⁰ in copper regulation of Mac1 function has not been previously reported. To verify that the L260S substitution abolished copper inhibition of Mac1 function, the mutation was engineered in MAC1, and the mutant gene was subcloned into a low copy YCP vector for transformation into CM mac1 cells containing the FRE1/HIS3 fusion gene. This MAC1 mutant and all subsequent mutants were tested in the context of the MAC1 promoter and terminator on a low copy vector. Cells harboring L260S Mac1 were His prototrophs on medium containing 30 μM CuSO₄ and were Cu⁺, confirming the constitutive nature of the L260S substitution (Fig. 2, line 3).

A second approach, taken simultaneously, to identify constitutive Mac1 mutations was to mutate randomly MAC1 in a mutator E. coli strain (XL1-Red), defective in three primary DNA repair genes. After propagation of episomal MAC1 in XL1-Red cells, plasmid DNA was recovered and the MAC1 locus excised and subcloned into a yeast YCp vector. Eleven transformants of CM mac1 cells were His prototrophs on medium containing 30 μM CuSO₄ and failed to grow on medium containing 1.75 mM CuSO₄. These phenotypes were reversed in cells in which the URA3-based plasmid was shed following selection on medium containing 5-fluoroorotic acid. The 11 transformants also exhibited high, constitutive expression of chromosomal CTR1. Sequencing of the MAC1 locus in rescued plasmids revealed eight distinct MAC1 mutations. Three such mutations were isolated in two independent colonies. All eight mutations occurred within the C1 region of Mac1. Six missense mutations were found with the following codon substitutions: C264F, C266G, C271W, C271F, H279N, and H279Y (Fig. 1). The phenotypes of these mutants are shown in Fig. 2 (lines 5,
7, 9, 10, 15, and 16). Only the H279Y substitution was observed previously, but other substitutions at residues Cys264 and Cys271 have been shown to yield constitutive Mac1 function (5, 10). The remaining two mutations were deletions encompassing portions of C1 (Fig. 2, lines 19 and 20). A six-base deletion of codons 262–264 and a 120-base deletion removing codons 226–267 yielded in-frame Mac1 truncates with constitutive Mac1 activity.

The conserved residues in the C1 motif are as follows: CYSX(X)2CXX(X)3CXXH. Substitutions at four of the six conserved residues yielded constitutive Mac1 activity. No mutations were isolated at the remaining codons, Cys273 and Cys276. To determine whether substitutions at these codons yielded a similar constitutive phenotype, Cys → Tyr substitutions were engineered at codons 273 and 276. MAC1 mutants with these changes were evaluated in CMΔmac1 cells containing the FRE1/HIS3 reporter gene on a low copy YCP vector. Unexpectedly, cells harboring either the C273Y or the C276Y mutant Mac1 were His auxotrophic and were copper-resistant (Fig. 2, lines 12 and 14). Expression of either C273Y or the C276Y mutant Mac1 on a high copy YEP vector imparted a weak His prototrophy to cells (Fig. 3, lines 6 and 8). Quantitation of CTR1 expression in cells expressing these mutant Mac1 molecules revealed constitutive mRNA levels (Fig. 4A).

Serine substitutions were engineered at each of the six conserved residues. Surprisingly, cells containing Mac1 with Cys → Ser substitutions at any of the conserved Cys residues in C1 failed to show constitutive Mac1 activity when mutant genes were expressed on low copy vectors. In each case, FRE1/HIS3 expression was reduced in copper-treated cells resulting in a His auxotrophy, and the cells were also wild-type in copper resistance (Fig. 2, lines 4, 6, 8, 11, and 13). CTR1 mRNA levels were assessed under copper-deficient and copper-replete growth conditions. Although aromatic residue substitutions at residues 264 and 271 and a Cys → Gly substitution at position 266 resulted in high, un-regulated CTR1 expression, Mac1 with Cys → Ser substitutions at the same positions expressed from low copy vectors show limited copper metallogulation of CTR1 expression (Fig. 4B). Although the Cys → Ser mutants (substitutions at codons 264, 266 and 271) did not yield high constitutive Mac1 activity, residual CTR1 expression was clearly elevated in copper-treated mutant cells compared with cells with wild-type Mac1 (Fig. 4B). When the Cys → Ser mutants were expressed from high copy YEP vectors, the residual expression seen in copper-treated cells was sufficient to yield constitutive FRE1/HIS3 expression and a Cu⁺ phenotype.

Fig. 3. Growth assays of cells harboring various MAC1 mutations expressed from a high copy vector. Plating conditions are as described in the legend to Fig. 2.

Fig. 4. A, expression of CTR1 in cells harboring MAC1 mutants expressed on high copy YEp vectors. Cells were cultured in low copper medium containing 100 μM bathocuproine sulfonate (–Cu) or 100 μM CuSO₄. RNA was extracted from cells at A₅₅₀ = 0.5, incubated with the CTR1 and calmodulin (CMD1) DNA probes, and digested with S1 nuclease. B, expression of CTR1 in cells harboring MAC1 mutants on low copy vectors as detected by S1 analysis of mRNA. Cells harboring MAC1 or mac1 variants on low copy vectors were cultured in low copper medium containing 100 μM bathocuproine sulfonate or 100 μM CuSO₄. WT, wild type.

(Data from Fig. 3, lines 2–4 and 7). Overexpression of wild-type Mac1 did not result in these phenotypes (Fig. 3, line 1). Although the His prototrophy was weak in cells with Cys → Ser mutants at codons 266, 273, and 276, CTR1 expression in the mutant cells cultured in the presence of Cu(II) was clearly elevated relative to cells with wild-type Mac1 (Fig. 4A). Thus, substitutions at any of the conserved cysteinyl residues in the C1 motif yield incomplete copper inhibition of Mac1 function.

As mentioned, all MAC1 mutations mapped to the C1 region. None were found in the DNA binding domain or in the conserved Cys-rich C2 motif. The clear prediction was that substitutions within the C2 motif would not alter copper metallogulation of Mac1. To test this prediction, a H337Q substitution was engineered in the C2 sequence motif. His337 is analogous in sequence position to the His279 residue in C1, in which various substitutions yield constitutive Mac1 activity. Cells (CMΔmac1) harboring YCPMAC1 with a H337Q substitution showed wild-type FRE1/HIS3 expression (Fig. 2, line 18) and wild-type copper modulation of CTR1 expression (Fig. 5). Expression of the H337Q mutant on a high copy YEP vector also showed wild-type phenotypes (Fig. 3, line 10).

The conservation of His in the two Cys-rich motifs is suggestive of histidinyl coordination of one or more Cu(I) ions. We demonstrated previously that the two Cys-rich motifs bind 8 Cu(I) ions, presumably within polycopper clusters (13). The copper binding stoichiometry is reduced to 4 Cu(I) ions in the Mac1 mutant with a H279Q substitution. Since Cu(I) coordination in proteins with known polycopper clusters is dominated by cysteinyl ligands, we tested the copper metallogulation of a H279C mutant Mac1. Cells (CMΔmac1) harboring YCPMAC1 with a H279C codon substitution were wild-type in FRE1/HIS3 expression (Fig. 2, line 17) and exhibited limited copper regulation of CTR1 expression (Fig. 5). However, overexpression of this mutant from a high copy YEP vector resulted in constitutive FRE1/HIS3 expression and copper hypersensitivity (Fig. 3, line 9). In contrast, overexpression of wild-type Mac1 did not elicit constitutive FRE1/HIS3 expression or copper hypersensitivity (Fig. 3, line 1). Thus, Gln, Asn, or Tyr substitutions at position 279 are more disruptive of copper metallogulation than a cysteine substitution. However, because a
His → Cys substitution did yield considerable metalloregulation of CTR1 expression, a prediction to be tested is that a cysteinyl residue at position 279 may provide a thiolate ligand to the putative polycopper cluster.

Previously, we used a modified two-hybrid assay to show a copper-induced interaction between the N-terminal DNA binding domain of Mac1 and the C-terminal segment containing the C1,C2 motifs (13). One vector contained Mac1 (residues 1–159) fused in-frame to the minimal Gal4 DNA binding domain. This chimera lacked any transactivation activity. The second vector consisted of a fusion of Mac1-(240–417) or H279Q-Mac1-(240–417) to the VP16 activation domain of the herpes simplex virus (25). The two vectors were transformed into strain Y190, and expression of the GAL1/lacZ reporter gene was assessed. β-Galactosidase activity was observed only in copper-treated cells containing both Mac1-(1–159)/Gal4 and Mac1-(240–417)/VP16. The copper-induced interaction was abrogated if the C1 motif contained a H279Q substitution. In the present work, to assess whether mutations in the conserved Cys residues in the C1 or C2 motif altered the two-hybrid interaction, a series of double Cys → Ser substitutions was engineered in the C1 motif and corresponding positions in the conserved C2 motif. Yeast transformed with the two vectors encoding either wild-type sequences or mutated MAC1 in the Mac1/VP16 fusion were asayed for lacZ expression in medium either containing or lacking 100 µM CuSO4 (Fig. 6A). Double Ser substitutions at the Cys264-Cys266 pair or the Cys271-Cys273 pair in the C1 motif inhibited copper-induced lacZ expression, whereas double Ser substitutions at the corresponding Cys pairs in the C2 motif (Cys322-Cys324 or Cys329-Cys331) yielded Cu-induced β-galactosidase activity (Fig. 6A). Western analysis of the extracts revealed that all mutant Mac1/VP16 fusion proteins were present except the C271C273 mutant Mac1/VP16 hybrid. The double Cys271-Cys273 substitution clearly destabilized the Mac1 protein.

The clustering of constitutive mutations within the C1 motif and the failure of C2 mutations to alter significantly copper metalloregulation suggest that the C1 motif is the key switch in copper regulation of Mac1 function. To address whether the C1 motif is functionally independent of the C2 motif, a Mac1 truncate was engineered consisting of residues 1–311 with and without a C-terminal HA epitope tag. The truncates on YEp vectors enabled CΔmac1 cells to grow on medium lacking methionine or medium with glycerol as the carbon source (Fig. 7). These cells showed limited copper metalloregulation of CTR1 expression (Fig. 8A). Immunoprecipitation followed by Western analysis on cells with the HA epitope-tagged Mac1 truncate revealed that the reduced CTR1 expression observed in copper-replete cells was not due to lower Mac1 protein levels. In fact, truncated Mac1 was stabilized in copper-replete cells (Fig. 8B), as was observed previously with intact Mac1 (13).

The limited transcriptional activation by Mac1-(1–311) is consistent with this fragment containing weak transactivator activity. The truncated Mac1 contains the intact DNA binding domain (residues 1–159). When this minimal DNA binding domain is fused to the strong VP16 activator, potent transcriptional activation is observed (17). Confirmation that the activator in the Mac1-(1–311) truncate is weak was obtained by three truncated Mac1 activation domains fused to the Gal4 DNA binding domain. Gal4/Mac1 truncates with Mac1 residues 240–311 (C1 alone); residues 240–350 (C1 and C2) and residues 290–350 (C2 alone) were engineered in a YCp under the MET25 promoter. Cells containing these fusions and a GAL1/lacZ reporter gene were tested for lacZ expression. Cells cultured in high methionine to limit expression levels of the Gal4/Mac1 fusions showed potent transactivation activity for Gal4/Mac1 constructs containing C2 motifs (constructs 240–350 and 290–350) (Fig. 9A). In contrast, cells containing the C1 motif alone had very weak transactivation activity, although the Gal4/Mac1 C1 fusion protein was well expressed (Fig. 9B).

**Fig. 5. Expression of CTR1 in cells containing MAC1 mutants with substitutions at codon 279.** S1 analysis was as described in Fig. 4. BCS, bathocuproine sulfonate.

**Fig. 6. Copper-induced two-hybrid interaction in Mac1.** Cells contained two vectors. One expressed a fusion of the Mac1 DNA binding domain fused to the Gal4 DNA binding domain. The second vector was a fusion of the C-terminal segment of Mac1 fused to the VP16 activation domain. The dots show the fusion of cysteinyl residues in Mac1. Double Cys → Ser substitutions in either the C1 or C2 motifs are shown by the X in place of dots. Cells were cultured in low copper medium and medium containing 100 µM CuSO4. A, shows the expression of the GAL1/lacZ fusion gene quantified by β-galactosidase assays. Each sample was assayed in triplicate, and the standard deviation is shown on each bar. B, shows Western analysis of the Mac1/VP16 fusion protein and mutant variants. Equal protein was loaded on each lane, and protein blots were probed with anti-VP16 monoclonal antibodies.
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Fig. 7. Growth of CMA\Delta mac1 cells transformed with intact MAC1 or a fragment of MAC1 encoding codons 1–311 on YEp vectors. In A transformants were cultured on low copper medium lacking methionine without or with 100 μM CuSO4. The medium used in B was low copper medium with glycerol as the carbon source without or with 100 μM CuSO4.

Fig. 8. A, expression of CTR1 by S1 analysis in cells harboring either full-length Mac1 or a C-terminal truncate (residues 1–311). The Mac1 truncate was tested with or without a C-terminal HA epitope tag. Cells were cultured in the presence or absence of 100 μM CuSO4. B, Western blot of the HA-tagged Mac1 truncate in cells containing an untagged Mac1 truncate (311) or HA-tagged Mac1 truncate (311-HA) cultured in either low copper medium or medium containing 100 μM CuSO4. The band labeled CRM is a cross-reacting yeast protein with the HA monoclonal antibody.

DISCUSSION

Mutagenesis studies were conducted on MAC1 to map residues important in the copper inhibition of Mac1 activity. Seven new missense mutations were identified that exhibited loss of copper metalloregulation of Mac1 function. All seven mutations were clustered in the first Cys-rich C1 sequence motif. The absence of constitutive mutations in the second Cys-rich C2 motif is consistent with the C1 motif being the copper-regulatory switch. One major conclusion of the present study is that mutation of any residue may perturb cluster formation. Although mutations at each conserved residue attenuated copper inhibition of Mac1 function, not all substitutions were equally disruptive of copper metalloregulation. Substitutions at positions 264, 271, and 276 dramatically attenuate copper inhibition of Mac1 function, not all substitutions were equivalently a constitutively active Mac1. In contrast, a H279C substitution in Mac1 enabled limited but not wild-type copper modulation. Thus, His279 is a critical residue in the copper-regulatory domain. This information implies, but does not prove, that the imidazole is a Cu(I) ligand.

All but one of the constitutive Mac1 mutations occurred in one of the conserved six residues in the 264(CX)6(CX)6C-(X)2H279 motif. The lone exception was the L260S substitution. Curiously, Leu260 is not conserved in the C2 motif in Mac1 nor in either of the Cys-rich motifs in Grisea or Cuf1. We reported previously that Mac1 binds 8 Cu(I) ions, and the number is reduced to about 4 Eq in Mac1up1 which harbors the H279Q substitution in the C1 motif (13). One prediction is that each motif binds 4 Cu(I) ions in polycopper clusters. Coordination of 4 Cu(I) ions within a single polycopper cluster can occur by coordination with six protein ligands. The C1 motif has five conserved Cys residues and a single His residue. If the copper center is formed by the six conserved residues, a prediction is that mutation of any residue may perturb cluster formation. Although mutations at each conserved residue attenuated copper inhibition of Mac1 function, not all substitutions were equally disruptive of copper metalloregulation. Substitutions at positions 264, 271, and 276 dramatically attenuate copper metalloregulation regardless of whether the substitution was an aromatic or serine. In contrast, a glycine substitution at Cys266 abolished copper regulation more severely than a serine substitution. Thus, the five cysteinyl residues in C1 do not appear to be equivalent in copper inhibition of Mac1 function. If the C1 motif forms a single polycopper cluster, the curious observation is that certain Cys → Ser substitutions do not abrogate copper metalloregulation. Coordination of Cu(I) ions is preferentially through soft ligands. The Ser hydroxyl group may contribute a weak coordinate bond to Cu(I), or alternatively a similar cluster forms in the absence of one ligand.

Mutations at codon 279 that convert the conserved His to Asn, Gln, or Tyr resulted in a constitutively active Mac1. In contrast, a H279C substitution in Mac1 enabled limited but not wild-type copper modulation. Thus, His279 is a critical residue in the copper-regulatory domain. This information implies, but does not prove, that the imidazole is a Cu(I) ligand.

We demonstrated previously that copper repression of Mac1 arises from a copper-induced intramolecular interaction between the C-terminal Cys-rich motifs and the N-terminal DNA binding domain (13). The prediction is that the copper-regulatory switch is the C1 domain that may form a tetracopper...
thiolate, imidazole cluster. Consistent with this postulate was the observation that double Ser substitutions in C1 abolished the copper-induced two-hybrid interaction between the N-terminal DNA binding domain and the C-terminal activator domains, whereas double substitutions in C2 had minimal effect. The observed non-equivalence of cysteiny1 residues in the C1 motif may arise from certain cysteine residues having a dual function of providing a thiolate ligand to the polycopper cluster as well as being functionally important in the inhibitory intramolecular interaction. Thus, Cys^{264} and Cys^{271} may be key residues in the interaction with the N-terminal DNA binding domain. No constitutive Mac1 mutants were identified in the N-terminal segment of Mac1. Since the repressed conformer of Mac1 lacks DNA binding activity, it is possible that the intramolecular interaction involves residues important for DNA binding. This would preclude identification of copper-independent, constitutive mutations in the DNA binding domain.

Although the boundaries of the transcriptional activator domain in Mac1 are unclear, it appears that the molecule contains two transactivators associated with the two Cys-rich motifs. The minimal transcriptional activity of the Mac1 truncate (residues 1–311) was consistent with the C1 activator being weak. In contrast, the minimal C2 activator was potent, but copper ions did not modulate this activity. The C2 activator is restrained in the full-length protein, consistent with some segment of Mac1 restraining the C2 activator. Masking of strong activators in intact transcription factors is also observed in Grisea and Leu^{15} (15, 28). The function of the C2 copper domain remains unclear and will constitute the focus of future experiments. It is likely that multiple intramolecular interactions are stabilized in the copper conformer of Mac1. Copper-induced intermolecular interactions are also possible as Mac1 was recently shown to exist as a dimer with a dimerization motif existing between residues 388 and 406 (29).

Genetic screens used in three laboratories have identified various MAC1^{109} mutants, but to date no accessory genes have been identified that influence copper metalloregulation of Mac1 function. One likely accessory factor would be a metallochaperone that shuttles Cu(I) ions to the nucleus for inhibitory interactions with Mac1. If additional genes exist, and if such mutants are suppressed by high exogenous Cu(II), the current strategy may have eliminated their identification. For example, high exogenous copper is known to suppress mutations in the mitochondrial copper chaperone Cox17 (30).

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