Homeostatic Regulation of Copper Uptake in Yeast via Direct Binding of MAC1 Protein to Upstream Regulatory Sequences of FRE1 and CTR1*

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Copper deprivation of Saccharomyces cerevisiae induces transcription of the FRE1 and CTR1 genes. FRE1 encodes a surface reductase capable of reducing and mobilizing copper chelates outside the cell, and CTR1 encodes a protein mediating copper uptake at the plasma membrane. In this paper, the protein encoded by MAC1 is identified as the factor mediating this homeostatic control. A novel dominant allele of MAC1, MAC1<sup>up2</sup>, is mutated in a Cys-rich domain that may function in copper sensing (a G to A change of nucleotide 812 resulting in a Cys-271 to Tyr substitution). This mutant is functionally similar to the MAC1<sup>up1</sup> allele in which His-279 in the same domain has been replaced by Gin. Both mutations confer constitutive copper-independent expression of FRE1 and CTR1. A sequence including the palindrome TTTGCTC...TGAGCAAA, appearing within the 5'-flanking region of the CTR1 promoter, is necessary and sufficient for the copper- and MAC1-dependent CTR1 transcriptional regulation. An identical sequence appears as a direct repeat in the FRE1 promoter. The data indicate that the signal resulting from copper deprivation is transduced via the Cys-rich motif of MAC1 encompassing residues 264–279. MAC1 then binds directly and specifically to the CTR1 and FRE1 promoter elements, inducing transcription of those target genes. This model defines the homeostatic mechanism by which yeast regulates the cell acquisition of copper in response to copper scarcity or excess.

Copper is essential but toxic to cells. Copper is an essential prosthetic group of proteins such as cytosolic superoxide dismutase that is required for detoxification of oxygen free radicals and thus for the fitness of aerobic organisms (1). The copper sites in cytochrome oxidase are essential for the activity of this enzyme and thus for sustained cellular respiration (2). More recently, a role for copper enzymes in iron metabolism has been defined (3). Multi-copper oxidases, ceruloplasmin (mammals) (4, 5) and FET3 (yeast) (6, 7), have been implicated in iron transport. On the other hand, excess copper is toxic. The toxicity of copper may derive from the reaction of Cu(I) with hydrogen peroxide yielding the highly reactive hydroxyl radical that in turn may damage lipids, proteins, or DNA in cells (8). One way that cells solve the problem of acquisition of sufficient copper while avoiding toxic excess is by homeostatic control of copper uptake (2, 9).

In aerated media, copper, like iron, is complexed to media components in its higher valence state. Since data indicate that the metals enter the cell uncomplexed to any extracellular ligand, an initial ligand displacement step must precede the movement of the metal ion across the plasma membrane (9). Ligand coordination to lower valence ions is typically more labile than to the corresponding high valence species (9), and therefore reduction of copper in the environment facilitates cellular uptake. The major surface reductase of Saccharomyces cerevisiae is the FRE1 gene product (10); FRE1 is capable of reducing extracellular copper or iron complexes (11, 12). Consequently, this reductase plays a significant role in cellular copper acquisition (12).

In most laboratory strains of S. cerevisiae, cellular copper acquisition requires CTR1 (13, 14). CTR1 protein exists in the cell as a multimer in the plasma membrane (15), although in some settings it may be internalized into the endocytotic pathway (16). CTR1 contains an unusual amino-terminal domain oriented toward the cell exterior (13). This domain, rich in methionine and serine, is similar to copper-binding domains present in bacterial proteins mediating copper resistance (17, 18). The homology of the methionine-rich domain with copper binding proteins suggests that it will form a pocket with affinity for Cu(I). The subsequent events involved in internalization and translocation of the metal across the cell membrane and into the cytosol have not been characterized.

Expression of FRE1 (11, 12) and CTR1 (15) are homeostatically regulated by copper availability, consistent with the roles of the two gene products in copper acquisition. The regulation of FRE1 (12), like the regulation of CTR1 (15), is mediated at the level of copper-dependent transcription. Copper deprivation induces and copper loading represses transcription of both of these genes. Thus, cellular component(s) must exist through which the signal for available copper levels is transduced into regulated transcription of these genes.

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ACE1 must be considered as a candidate for this transducing function (19). ACE1 encodes a yeast protein that binds copper (20) and activates the transcription of target genes in a copper-dependent fashion (21). However, strains deleted for the ACE1 gene maintain homeostatic regulation of the surface reductase and copper uptake (15). Furthermore, ACE1 protein mediates the induction of genes involved in copper detoxification, including CUP1, the yeast metallothionein (19), and SOD1, the copper-zine superoxide dismutase (22). Because these genes function in cellular protection, the threshold copper concentrations at which these genes are expressed are relatively high (µM concentrations in defined media). In contrast, the copper exposure at which regulation of the copper uptake system occurs is much lower (less than 20 nM in defined media) (7, 15). Also, the copper acquisition system is repressed by copper availability (15), and thus is regulated in a direction opposite from the detoxification system controlled by ACE1 (19). These several factors make ACE1 an unlikely candidate for the copper sensor controlling copper uptake.

A protein with homology to ACE1, called MAC1, must also be considered. The sequence of the MAC1 protein is notable for an amino-terminal domain (residues 1–42) with significant homology to ACE1. The activator for the metallothionein genes in Candida glabrata, AMT1, is also homologous in this region (23). In addition, MAC1 contains a motif conforming to the structure of copper-zinc superoxide dismutase (22). Because these genes function in cellular protection, the threshold copper concentrations at which these genes are expressed are relatively high (µM concentrations in defined media). In contrast, the copper exposure at which regulation of the copper uptake system occurs is much lower (less than 20 nM in defined media) (7, 15). Also, the copper acquisition system is repressed by copper availability (15), and thus is regulated in a direction opposite from the detoxification system controlled by ACE1 (19). These several factors make ACE1 an unlikely candidate for the copper sensor controlling copper uptake.

Here we report evidence that strongly indicates that the MAC1 protein functions as the copper sensor-regulator that controls the expression of surface reductase and copper uptake activity in yeast and thus provides homeostatic control of copper acquisition. We characterize the direct interaction of MAC1 protein with specific sequence elements in the promoters of the CTR1 and FRE1 genes and show that these elements alone support copper- and MAC1-dependent transcriptional activation. The data suggest that MAC1 binding to these elements plays a role in the transduction of environmental copper levels into regulated gene transcription within yeast cells.

**EXPERIMENTAL PROCEDURES**

Yeast Strains—The MAC1mut allele, isolated from strain UCP1 (MATa trpl-1 ade8 his3 gal1 CUP1MAC1), has been previously described (23). Strain MA20 (MATa ino1-13 leu2-3,112 gen4-101 his3-609 2His. This motif occurs twice in the carboxyl-terminal portion of the protein. It is within the first of these repeats, residues 264–279, that the motif conforms to the amino-terminal domain (residues 1–42) with significant homology to ACE1. The activator for the metallothionein genes in Candida glabrata, AMT1, is also homologous in this region (23). In addition, MAC1 contains a motif conforming to the structure of copper-zinc superoxide dismutase (22). Because these genes function in cellular protection, the threshold copper concentrations at which these genes are expressed are relatively high (µM concentrations in defined media). In contrast, the copper exposure at which regulation of the copper uptake system occurs is much lower (less than 20 nM in defined media) (7, 15). Also, the copper acquisition system is repressed by copper availability (15), and thus is regulated in a direction opposite from the detoxification system controlled by ACE1 (19). These several factors make ACE1 an unlikely candidate for the copper sensor controlling copper uptake.

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**DELETION ANALYSIS OF THE CTR1 PROMOTER**

Copper concentrations were manipulated in YPD medium (2% yeast extract, 1% peptone, 2% glucose) by the addition of the copper chelator BCS or by the addition of copper sulfate. In some experiments, the strains were grown in defined media containing yeast nitrogen base without amino acids modified to omit iron and copper. 25 mg of ferrous sulfate (97.5% purity) to add glucose (2%, w/v) and MES buffer (25 mM, pH 6.1), and BCS for copper starvation or copper sulfate for copper loading. Iron was added back as ferric ammonium sulfate in a concentration of 10 µM. A 1-mm loopful of yeast grown on a fresh YPD plate was inoculated into 10 ml of liquid medium and grown overnight with aeration and agitation at 30 °C to late log phase (A500 approximatively 2.0). The following day, the culture was diluted into medium of the same composition at an A500 of 0.2 and grown for an additional 5 h prior to harvesting. Methods for crossing, sporulation, spore dissection, and transformation of yeast were as described (25).

Demonstration That MA20 Carried an Allele of MAC1—MA20 (MATa trpl-1 ade8 leu2-3,112) was crossed with WY10 (MAC1up1 trpl-1 ade8 leu2-3,112) and the diploid was sporulated. Spore clones derived from 12 tetrad were evaluated for leucine prototrophy and non-repressing reductase activity. This phenotype has been associated with the MAC1mut mutation (23). Clones with non-repressing reductase were in all cases found to be incapable of growing in the absence of leucine, indicating lack of recombination between the marked MAC1 allele and the non-repressing reductase phenotype of the MA20 mutant.

The MAC1mut allele was also rescued by gap repair (24). A 1.6-kilobase pair SacI fragment containing the entire MAC1 open reading frame was removed from the 3.0-kilobase pair HindIII MAC1 clone in pSEYS (23). The resulting gapped pSEYS that now contained regions 5' and 3' to the MAC1 open reading frame as sticky ends was transformed into MA20 (MAC1up1), and recircularized plasmid was recovered. This plasmid mixture was amplified in Escherichia coli and the recovered MAC1 mutation plasmid in pSEYS was sequenced from +162 to +1585 (numbering relative to MAC1 translation start, with termination at +1251) by the standard chain termination method (Sequenase 2.0, U. S. Biochemical Corp.).

CTR1 mRNA and Protein Analysis—CTR1 (and FRE1) mRNA levels in wild type, MAC1mut, and MAC1up2 containing strains were determined by Northern blot analysis following standard protocols (26). For detection of a CTR1 protein, a myc-tagged CTR1 genomic clone was constructed. To do this, the HindIII fragment was excised from plasmid 352-myc (13) and inserted into the HindIII sites of pRS416, creating plasmid 416-CTR1myc. This construct contains a myc epitope tag inserted at the unique EcoRI site of the genomic CTR1 clone and is carried on a centromere-linked vector.

CTR1 protein visualization was as follows. Cultures of transformants with the 352-myc plasmid (myc epitope labeled CTR1 genomic clone) were grown to log phase. Approximately 2 × 107 cells were harvested and lysed by pelleting and resuspending in 150 µl of 1.85 µM NaOH, 2% 2-mercaptoethanol. Proteins were precipitated by the addition of 150 µl of 50% trichloroacetic acid and incubation on ice for 30 min. The precipitate was then recovered by centrifuging at 16,000 × g for 10 min prior to resuspending in 45 µl of 2 x Laemmli buffer and 5 µl of 1 x Tris base. Ten microliters or 4 × 104 cell equivalents were loaded per lane of an SDS-polyacrylamide gel. The proteins were blotted to nitrocellulose, and the myc-tagged CTR1 protein was visualized using 9E10 ascites anti-myc monoclonal antibody and peroxidase-conjugated rabbit anti-mouse secondary antibody. The enhanced chemiluminescence (Amerham Corp.) was used to develop the signal from the epitope labeled protein.

**DELETION ANALYSIS OF THE CTR1 PROMOTER**

Plasmids containing portions of the CTR1 promoter fused to the lacZ gene were constructed as follows. Sequences from the CTR1 promoter, translation start, and codons 1–3 were amplified by PCR between unique SacI and BamHI sites and fused to the β-galactosidase coding region in plasmid pYE-Gal. The sequences of the CTR1 sequences (numbered with respect to the translation start) were made by PCR, creating plasmids pCTRlac1-413, -334, -333, -311, and -226. For pGC-CTR—337/-301, the oligonucleo-
Acid substitution within the same Cys-rich domain, residues a cysteine to a tyrosine. Thus, this mutation causes an amino alter the residue at position 271 in the primary sequence from no. X74551), was identified. This mutation was predicted to accomplished by means of gap repair (24), and the entire DNA Binding Assays—Electrophoretic mobility shift assays (EMSA) were performed with MAC1 protein that was prepared by in vitro transcription and translation. This assay was carried out essentially as described for the AFT1 EMSA (28). MAC1 mRNA was synthesized from the pT7MAC1 plasmid with T7 polymerase and translated in a wheat germ lysate (Promega). The binding reaction was carried out in 20 μl of binding buffer (10 mM HEPES-KOH, pH 7.5, 150 mM KCl, 1 mM dithiothreitol, 0.5 mM EDTA, 0.05% Nonidet P-40, 7.5% glycerol) containing 2 ng of 32P-end-labeled probe, salmon sperm DNA, and in vitro translated MAC1 protein. The binding reaction mixtures were incubated for 10 min at room temperature and then electrophoresed in a 4% native polyacrylamide gel in 6.6 mM Tris-HCl, pH 7.9, 7.5 mM sodium acetate, and 1 mM EDTA at 4 °C.

Enzyme and Copper Uptake Assays—To evaluate reductase activity in yeast on solid media, spore clones were streaked onto YPD plates containing 50 μM copper sulfate and incubated overnight at 30 °C. The reductase activity was then evaluated by filter lift assay as described (29). The MAC1wt or MAC1mut clones gave a strong signal in this assay while the wild-type MAC1 clones were negative. Quantitative Fe(III) and Cu(II) reductase activity was determined in cell suspensions as described using bathophenanthroline disulfonate and bathocuproine disulfonate as indicators of Fe(II) and Cu(II) production, respectively (12). β-Galactosidase activity in yeast transformants was determined by a standard technique (27). For 67Cu uptake measurement, strains were grown in YPD to log phase, washed in assay buffer consisting of 50 mM sodium citrate, pH 6.5, 5% glucose, and incubated at a density of approximately 2 × 107 cells/ml in the presence of 1 μM 65CuSO4 of specific activity 820 dpm/mmol copper. After 1 h incubation at 30 or 4 °C, washed cells were collected on glass fiber filters and counted in a scintillation counter. Copper uptake, reported as pmol/million cells/h, was calculated after subtracting cell-associated counts obtained at 4 °C from the cell-associated counts obtained at 30 °C.

RESULTS

The promoter of the FRE1 gene mediates a wide range of transcriptional changes in response to copper or iron (10, 12). Thus, mutants selected for FRE1 dysregulation included strains with abnormalities of copper or iron metabolism (13). The selection scheme, which has been described previously (13), involved the fusion of the FRE1 promoter element to a HIS3 selectable marker and integration into a his3 deleted strain. The mutants were selected for the inability to repress FRE1 transcription under conditions of abundant copper and iron. A mutant strain identified by this method, MA20, exhibited surface Fe(III) and Cu(II) reductase activity that was poorly repressed by copper (Fig. 1). This phenotype resembled that of the previously described MAC1up1 mutant (12, 23). The MAC20 mutant was crossed with the parental strain 61, and the diploid, like the haploid, was found to possess reductase activity that was not repressible by copper indicating that the mutation behaved as a dominant (not shown). This dominance also resembled the previously described MAC1mut2 mutant (23). The MA20 strain was then mated with WY10, a strain carrying a LEU2-marked MAC1 allele, and the diploid was sporulated. Spore clones analyzed from 12 independent meioses revealed no recombination between the LEU2 marker and the non-repressing reductase phenotype, suggesting that the mutation in MA20 was allelic to MAC1.

Direct rescue of MAC1 mutant allele was then accomplished by means of gap repair (24), and the entire MAC1 coding region in MA20 was sequenced. A single mutation, a G to A missense mutation at nucleotide 812 (SCMAC1 accession no. X74551), was identified. This mutation was predicted to alter the residue at position 131 in the primary sequence from a cysteine to a tyrosine. Thus, this mutation causes an amino acid substitution within the same Cys-rich domain, residues

![Fig. 1. Copper-regulated surface reductase activity in MAC1 wild-type and mutant strains.](image-url)
FIG. 2. MAC1 protein sequence motifs and homologies. Cysteine and histidine residues of the MAC1 primary amino acid sequence (accession no. X74551) are plotted using an algorithm from DNAStrider. The position of the two UP mutant substitutions are indicated by the vertical arrows. Motifs A, B, and C, each containing six clustered cysteine and histidine residues, are indicated on this map and in the key below. The key also indicates the location of each motif in the MAC1 sequence (MAC1 up) and the single letter amino acid code for the sequence of each motif (SEQUENCE). Cysteines and histidines are shown in bold in this key. Motif A shares 53 and 41% amino acid identity with ACE1 and AMT1, respectively, and is part of a larger domain (residues 1–42) implicated in DNA binding of those proteins (see text). Motifs B and C resemble each other in conforming to the pattern CX-CS-CX-CS-CX-CS-X.H where X is any residue. The mutations in the MAC1up (C271Y) and MAC1upl (H279Q) alleles are indicated in the key for motif B.

FIG. 3. Copper uptake in MAC1 wild-type and mutant strains. Strains CM3262 (MAC1), MA20 (MAC1up2), 3262mac (mac1Δ), and FTRUNB1 (ΔcTR1) were grown in YPD, washed, and resuspended in uptake buffer. Uptake was initiated by addition of 67Cu and was determined as described under “Experimental Procedures.” Error bars represent 1 S.D. for n = 3.

MAC1 in Copper Homeostasis in Yeast

FIG. 4. Copper-regulated CTR1 expression in wild-type and MAC1 mutant strains. A, congeneric strains BR10 (MAC1), BR10mac (mac1Δ), and UPC31 (MAC1up2) and congeneric strains CM3262 (MAC1) and MA20p (MAC1up2) were grown in YPD in the presence of either 100 μM CuCl2 (+Cu) or 100 μM BCS (+BCS) to early log phase. RNA was then extracted, electrophoretically size-fractionated on a formaldehyde-agarose gel, capillary transferred to nitrocellulose, and probed with random-primed DNA fragments for FRE1 and CTR1 mRNA species. The blot shown was developed by autoradiography. B, congeneric strains CM3262 (MAC1), MA20 (MAC1up2), and 3262mac (mac1Δ) were transformed with the plasmid 352-myc carrying a myc-tagged CTR1 clone. Cultures were grown in synthetic media in the presence of the concentrations of CuSO4 as indicated; δ indicates growth in the presence of 10 μM BCS. Protein lysates derived from approximately 4 × 107 cells were loaded per lane and were electrophoretically fractionated in a polyacrylamide gel. CTR1 protein expression was evaluated by blotting to nitrocellulose and development of the signal using anti-myc monoclonal primary, anti-mouse secondary antibodies, and enhanced chemiluminescence reagents (Amersham Corp.). Molecular mass markers are shown in lane 1.

MAC1up2, and mac1Δ-containing strains. This region attracted attention because it included a perfect palindrome of the sequence TTTGCTCA, a likely candidate for a regulatory sequence (cf. Fig. 6A). Furthermore, this same sequence was found in the FRE1 promoter as a direct repeat in a region shown to confer metal-dependent expression of that locus (10). These three transformants were grown in the presence of BCS (–Cu) or copper sulfate (+Cu), and β-galactosidase activity was determined as a measure of CTR1 promoter activity. As the data in Fig. 5 show, in MAC1 wild-type strain CM3262 this CTR1 promoter fragment did confer copper- and MAC1-dependent expression of the reporter gene (pCTR lacZ-413, first entry). In contrast, in the MAC1up2 strain, MA20, this expression was copper-independent, whereas it was essentially absent in the mac1Δ strain.

To more closely define this copper-responsive element, progressive 5′ deletions of this fragment were constructed and analyzed. Upon deletion to –334 (leaving the 5′ TTTGCTCA intact, pCTR lacZ-334, second entry) the expression of the reporter gene was qualitatively unaltered in that both copper and MAC1 dependent were proportionately unaffected. However, deletion of even the 5′-terminal T of this sequence (pCTR lacZ-333, third entry) significantly reduced the copper dependence of this expression, as did deletion of the entire 5′-half of the palindrome (pCTR lacZ-311, fourth entry). In these two constructs, expression was less MAC1-dependent also as indicated by the increased β-galactosidase activity in the mac1Δ-containing strain. Removal of the palindrome entirely (pCTR lacZ-226) abolished lacZ expression, suggesting that this re-
 FIG. 5. Localization of a copper- and MAC1-responsive element in the CTR1 promoter. Progressive 5'-deletions of the CTR1 promoter constructed by PCR and cloned in front of a β-galactosidase reporter gene are depicted by horizontal bars indicating the CTR1 sequences included in each construct. The plasmids are named according to the number of the initial nucleotide of the CTR1 region included in the construct (i.e., pCTR lacZ-413 contains CTR1 sequences from −413 to −3; numbering with respect to the translation start). The last entry, pGCT-CTR (−357/−301), is a hybrid construct created by cloning an oligonucleotide with the indicated CTR1 sequences into a CYC1-lacZ fusion plasmid lacking its own upstream activating sequences. The CTR1 promoter-lacZ fusion constructs were introduced into strains CM3262 (MAC1), MA20 (MAC1<sup>Δm</sup>), or 3262Δmac1 (Δmac1). Cells were grown in defined medium in the presence of 10 μM BCS (−Cu) or 50 μM CuSO<sub>4</sub>, β-Galactosidase activities represent the average of three independent assays (± 1 S.D.).

In this paper, the MAC1 protein was identified as a regulatory DNA-binding protein through which cellular copper levels are transduced into the regulated transcription of genes involved in copper acquisition. Specifically, copper-regulated expression of FRE1 and CTR1 was found to be altered in MAC1 mutant strains, with copper-independent expression in the MAC1<sup>Δm</sup> and MAC1<sup>Δup</sup> strains and negligible expression in the mac1Δ strains. A homeostatic feedback loop for the control of cellular copper levels can thus be defined. Copper uptake into the cell, requiring reduction of copper chelates (FRE1-mediated) and translocation across the plasma membrane (CTR1-mediated), subsequently inhibits transcription of FRE1 and CTR1 (MAC1-mediated).

To define further this feedback loop, we identified a regulatory sequence from within the CTR1 promoter that was sufficient for conferring copper-dependent and MAC1-dependent expression to a reporter gene. MAC1 was shown in vitro to bind specifically to a motif within this sequence. This element (A/T)TTGCTCA appears as a palindromic in the native CTR1 promoter region and is capable of direct interaction with MAC1 protein. A direct repeat of an identical sequence (Fig. 6B, lane 4) was able to compete (again at 100-fold excess) with the CTR1 palindromic for binding to MAC1 protein (lane 8). Subsequently, a directly labeled oligonucleotide with the same sequence was tested, and a specific MAC1-dependent shift was observed in the EMSA (lane 10) which was reduced upon addition of the FLAG antibody (lane 11).

**DISCUSSION**

The role of the direct repeat sequence in the 5'-flanking region of FRE1 (Fig. 6A) was also evaluated (Fig. 6B, lanes 8–11). An oligonucleotide including this direct repeat from FRE1 (Fig. 6A) was able to compete (again at 100-fold excess) with the CTR1 palindromic for binding to MAC1 protein (lane 8). Subsequently, a directly labeled oligonucleotide with the same sequence was tested, and a specific MAC1-dependent shift was observed in the EMSA (lane 10) which was reduced upon addition of the FLAG antibody (lane 11).
siae and C. glabrata, respectively. The GRP motif (residues 37–39 in MAC1), conserved among MAC1, AMT1, and ACE1, is also shared with a larger family of transcription factors (PAXI, HMGI, and Drosophila prd and Hin recombinase) (30). This motif, and in particular the R residue, has been implicated in the AMT1 interaction with the AT-rich minor groove of its binding site (30). The Tyr<br>Cys<br>Cys<br>2Cys<br>3His<br>4Cys motif (residues 9–23 in MAC1) is likewise shared with AMT1 and ACE1. In AMT1, this motif appears to be a zinc-binding element (31, 32), and there is evidence for its involvement in the DNA-binding activity of both ACE1 and AMT1. Thus, a potential DNA-binding domain can be identified in MAC1 that resembles the apparent DNA-binding domains in ACE1 and AMT1.

The DNA sequences recognized by ACE1/AMT1 or MAC1 proteins are also strikingly similar to each other (Fig. 7). The AMT1 metal response element possesses a critical T thought to lie in the minor groove of the DNA helix and to interact with the GRP motif in the AMT1 protein. This T residue of the metal response element site in AMT1 (nucleotide 2195) is conserved in three of four MAC1 interaction sites (Fig. 7). Only the 5'-CTR1 site has an A in this position instead. The other residues of the core region identified by methylation interference as contacts for AMT1 are all conserved with the MAC1 binding site with the exception of the G at position 2189 (Fig. 7). The MRE element from the CUP1 (copper thionein) promoter that interacts with ACE1 regulatory protein includes the sequence TTTTCCG*CTG*A (the asterisk-marked bases indicate methylation protection in the ACE1-bound state) (33). The TTTTCCG constitute sites were also shown to be critical by base substitution analysis (34). This pattern of base selectivity is similar to that shown for the MAC1 binding site in the CTR1 promoter, in which the GCT of TTTTGCTCA was identified as critical for DNA binding.

ACE1 and MAC1 share primary sequence homology within an amino-terminal domain thought to be important for DNA interaction, and they share features between their DNA recognition sequences. Together these observations could suggest that the protein-DNA complexes may be similar. However, ACE1 and AMT1 activate copper detoxification activity (thionein genes) and are active as trans-factors in a copper-bound state. In contrast, MAC1 activates copper acquisition and the
data indicate is most likely active in DNA binding and transactivation when in a copper-depleted or copper-free form. With this difference in mind, the evolutionary relationship between these copper regulatory proteins is interesting to consider. Coordinated but opposite regulation of iron uptake (transferrin receptor) and iron detoxification (ferritin) by a common regulatory molecule (IRP1) has been described in mammals (35). Perhaps the regulatory molecules MAC1 and ACE1 in *S. cerevisiae*, controlling copper uptake and copper detoxification, respectively, diverged from a common ancestral regulator.

The copper-regulated activity of MAC1 could occur at least in part as a consequence of copper-regulated DNA binding. However, we were unable to detect a copper dependence of this binding in the EMSA. This result was similar to what has been observed with *in vitro* AFT1-DNA binding in which this interaction was independent of iron, although *in vivo* AFT1 trans activity is iron-dependent (28). Despite this negative result for MAC1, a suggestion about how copper might regulate MAC1 is provided by the amino acid sequence. That is, cysteine and histidine residues are known to coordinate copper in proteins, and the Cys-His element in the carboxy-terminal domain of MAC1 (Fig. 2) might be able to reversibly interact with copper, generating a biological signal. In this model, a copper-depleted MAC1 protein form would bind to DNA followed by transcriptional activation. The gain-of-function alleles of MAC1 fit this model in that they contain mutations in potential copper-coordinating residues in this domain (H279Q, C271Y). Thus, by interfering with the copper binding ability of the Cys-His repeat domain of MAC1, these mutations result in a constitutively active protein.

How MAC1 may function to activate transcription is not known. However, the distribution of charged amino acid residues in MAC1 is asymmetric, with the amino-terminal domain (residues 1–201) strongly basic in nature (31 Arg and Lys, 21 Asp and Glu) and the carboxy-terminal domain (residues 202–417) predominantly acidic (27 Asp and Glu, 16 Arg and Lys). Also, the putative nuclear targeting domain is found in the amino-terminal region (24). These features are consistent with a model in which the amino-terminal domain is associated with nuclear targeting and DNA binding, whereas the carboxy-terminal domain is associated with copper sensing and transactivation. This model remains to be tested. For example, we have not rigorously excluded the possibility that the copper dependence of MAC1 function requires another protein(s), with this other protein being the copper sensor. While this model is not excluded by our data, we believe the model that most simply explains the phenotype of the UP alleles is based on their having a diminished affinity for copper.

The interrelationships between iron and copper homeostasis in yeast are defined by a complex network of genes. Cellular iron uptake requires the activity of the plasma membrane iron transport complex composed of the FTR1 and FET3 gene products (36). FTR1 most likely encodes an iron permease function (36), whereas the FET3 gene encodes a multi-copper oxidase that is required for iron uptake (6). Thus, the cellular copper uptake and utilization pathway mediating delivery of copper to FET3 must be intact for high affinity iron uptake to occur. This copper delivery pathway includes CTR1, required for cellular copper uptake (13). We may now add MAC1 to this scheme, because MAC1 is required for CTR1 expression and consequently is required for iron uptake.

At the level of gene regulation, however, the pathways for responding to changes in iron or copper levels are distinct. Iron deprivation induces increased activity of the iron uptake system through a homeostatic feedback loop involving the AFT1 regulatory protein (28). In iron-deprived cells, AFT1 binds to a specific recognition sequence in the enhancer regions of genes involved in iron uptake, including FET3 and FTR1 (28). AFT1 induces transcription of the target genes under conditions of iron deprivation. A separate but analogous pathway mediates the cellular response to copper deprivation; MAC1 is induced to bind to the specific recognition sequences identified in this paper, leading to expression of the genes involved in copper acquisition (*FRE1, CTR1*). The two systems are distinct in that the iron regulatory protein, AFT1, is distinct from the copper regulatory protein, MAC1, and each recognizes different DNA sequence elements. Only the *FRE1* gene possesses binding sites for both AFT1 and MAC1 (10) and thus constitutes a special case. The dual role of surface reductases in facilitating iron and copper acquisition by reduction of extracellular metal chelates may explain this dual regulation of the *FRE1* gene although the MAC1- and copper-dependent regulation of *FRE1* seems to predominate as demonstrated by the Northern analysis shown in Fig. 4A.

Humans, much like yeast, have Cu/Zn superoxide dismutase and copper-based cytochrome oxidase (2), and defects in these proteins have been implicated in human disease (37, 38). Cytoplasmic thioneins exist in humans as in yeast and are involved in protection against copper toxicity (39). Recently, a human homologue of *CTR1* has been identified that is capable of complementing the phenotype of the yeast *ctr1A* mutant (40). This protein may be involved in copper uptake in human cells. Human *FRE1* homologues are also likely to exist (41). Since the problems of acquiring copper and avoiding its toxicity must be confronted by virtually all organisms, copper-dependent, homeostatic gene regulation is likely to occur in humans, too. Whether a MAC1 homologue functions to mediate copper homeostasis in humans must await further study.

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