Identification and Analysis of a *Saccharomyces cerevisiae* Copper Homeostasis Gene Encoding a Homeodomain Protein

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Yeast metallothionein, encoded by the *CUP1* gene, and its copper-dependent transcriptional activator *ACE1* play a key role in mediating copper resistance in *Saccharomyces cerevisiae*. Using an ethyl methanesulfonate mutant of a yeast strain in which *CUP1* and *ACE1* were deleted, we isolated a gene, designated *CUP9*, which permits yeast cells to grow at high concentrations of environmental copper, most notably when lactate is the sole carbon source. Disruption of *CUP9*, which is located on chromosome XVI, caused a loss of copper resistance in strains which possessed *CUP1* and *ACE1*, as well as in the *cup1 ace1* deletion strain. Measurement of intracellular copper levels of the wild-type and *cup9-1* mutant demonstrated that total intracellular copper concentrations were unaffected by *CUP9*. *CUP9* mRNA levels were, however, down regulated by copper when yeast cells were grown with glucose but not with lactate or glycerol-ethanol as the sole carbon source. This down regulation was independent of the copper metalloregulatory transcription factor *ACE1*. The DNA sequence of *CUP9* predicts an open reading frame of 306 amino acids in which a 55-amino-acid sequence showed 47% identity with the homeobox domain of the human proto-oncogene *PBX1*, suggesting that *CUP9* is a DNA-binding protein which regulates the expression of important copper homeostatic genes.

Copper (Cu) is a trace element that is required for life by all species thus far examined (67). The essentiality of copper is derived from its ability to function as an electron transfer intermediate, and as such it is required as a cofactor for a number of enzymes including copper-zinc superoxide dismutases and cytochrome oxidase (42). It is therefore ironic that the electron transfer capabilities of copper that make it of essential use to the cell are also extremely detrimental to the cell. Copper readily reacts with the superoxide anion and with hydrogen peroxide via a Fenton-like reaction to generate the highly reactive hydroxyl radical, which can attack sugars, amino acids, phospholipids, and nucleic acids with deleterious results (22). Importantly, oxygen free radicals are readily generated as intermediates during the reduction of oxygen to water during normal cellular respiration (20). Consequently for mammalian cells undergoing elevated respiration, or yeast cells growing on nonfermentable carbon sources, from which all energy is derived by respiration, the tight regulation of copper is essential. In addition, Cu can exert toxic effects by nonspecific interaction with cysteinyl thiol groups, rendering proteins that contain exposed cysteine residues inactive (30, 55). Therefore, the regulation of copper, from its entry into the cell to its final functional destination, is a critical but little understood process.

Despite the essentiality of copper, a deficiency of this element in humans is rare, and its clinical significance was debated until Danks and coworkers identified that Menkes' kinky hair disease was an inherited metabolic disorder of copper metabolism (14). Recently, a candidate gene for Menkes' disease has been cloned and sequenced. Comparison analysis of the derived 1,500-amino-acid sequence revealed homology to a family of type P ATPases, which transport

cations across membranes (8, 47, 69). The gene linked to the other known heritable disorder of copper metabolism, Wilson's disease, has also been cloned (5, 53, 63) and also encodes a putative copper-transporting ATPase with homology to the Menkes' gene product (5, 63). Despite the similarity of these two gene products, the genes are expressed in different tissues. The Menkes' gene is expressed predominantly in extrahepatic tissue, while the Wilson's disease gene is predominantly expressed in liver and kidney tissues (5, 63). This tissue-specific expression is reflected in the pathology of the two diseases, an apparent copper deficiency in Menkes' disease, and a hepatic accumulation of copper leading to copper toxicity in Wilson's disease (13), and suggests a requirement for multiple factors, both structural and regulatory, for normal copper homeostasis in different cell types exposed to various physiological conditions.

As the Menkes' and Wilson's disease examples illustrate, in higher eukaryotes the identification of a gene(s) that causes a phenotype is a time-consuming task. The baker's yeast *Saccharomyces cerevisiae* provides an excellent system to rapidly identify and characterize proteins that are involved in cellular copper regulation in eukaryotic cells under a variety of conditions. In this regard, it was demonstrated that the *S. cerevisiae* metallothionein (MT) gene (*CUP1*) expression is transcriptionally induced by a copper-dependent DNA-binding protein, *ACE1* (activator of *CUP1* expression) (65). *S. cerevisiae* MT is functionally similar to mammalian MT, to the extent that mammalian MT can complement the copper detoxification defect in yeast strains in which the *CUP1* gene has been deleted (66). Furthermore, recent studies have demonstrated that both yeast and mammalian MTs can suppress many of the oxygen sensitivity phenotypes of strains bearing a deletion of the yeast Cu-Zn superoxide dismutase gene (*SOD1*) (62). *ACE1*, for which a mammalian analog has not been identified, coordinates six to seven Cu(I) ions per polypeptide as a polynuclear cluster with each Cu(I) bound with trigonal coordination geometry. The current model (65) for the detoxifica-

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tion of copper is that ACE1 serves as a sensor for cellular copper. Excess copper coordinates to ACE1, and the ACE1-Cu(I) complex binds to the UAS_{CUP1} (upstream activation sequence of *CUP1*) and activates *CUP1* expression. The increased levels of MT synthesize chelate copper and thereby protect cells from copper poisoning. This model can, in part, account for how the cell copes with potentially toxic levels of copper. However, the entire pathway by which copper homeostasis is established and maintained under diverse physiological conditions has up to now not been well defined. Recently, factors involved in both iron and copper homeostasis have been identified, and an understanding of their function and regulation is evolving. *FRE1* is an iron and copper reductase; the reduction of iron from ferric to ferrous form is required for transport into the cell; the same is also true for copper (10, 11, 25). A high-affinity transport protein for copper, CTR1, which in environmental conditions of low copper is required, indirectly, for the uptake of iron, has been identified (12). The connection between iron and copper uptake is believed to be via the product of the *FET3* gene, which encodes a multicopper oxidase and is postulated to catalyze the oxidation of Fe(II), permitting its release into the cell (1). This interplay between proteins involved in copper and iron homeostasis is extensive in that the newly identified transcription factor *MAC1*, which contains an amino-terminal domain similar to that of the copper binding region of ACE1, is involved in transcriptional regulation of *FRE1* (33). The regulation and interaction of these metal homeostasis proteins are only poorly understood, and furthermore it is likely that other proteins involved in metal homeostasis under different conditions will play critical roles in Cu and Fe homeostasis.

To identify other proteins involved in yeast cellular copper homeostasis, we investigated our observation that yeast strains grow at very high copper concentrations in the presence of nonfermentable carbon sources, even in the absence of *CUP1* or *ACE1*. This report describes the identification of a gene, *CUP9*, which permits yeast cells to grow at otherwise toxic copper concentrations, on both fermentable and nonfermentable carbon sources. We show that *CUP9* encodes a putative homeodomain protein, the steady-state mRNA level of which is downregulated by copper independently of the ACE1 protein.

MATERIALS AND METHODS

Strains and growth conditions. The genotypes of the yeast strains used in this study are described in Table 1. Strain DTY3 (50.L4) has been previously described (66). Strain DTY86 was constructed from DTY3 by deletion of the *ACE1* gene as described by Butler and Thiele (6) and deletion of the entire *CUP1* gene as described elsewhere (73). The *CUP9* gene in DTY86 and DTY3 was disrupted with *URA3* to generate strains DTY157 and DTY158, respectively, the details of which are described below. The mating type of DTY5 was switched from MAT α to MATa by using HO endonuclease as described by Herskowitz and Jensen (27) to give strain DTY131. Deletion of *CUP1* in strain DTY131 as previously described (73) and integration at the *CUP9* locus of *CUP9* linked to *URA3* generated strain DTY183, which was used for genetic linkage analysis. Yeast strains were grown at 30°C in rich (YPD) or synthetic complete (SC) medium lacking specified nutrients with 2% glucose, 2% lactate, or 3% glycerol plus 3% ethanol as the carbon source (59). To assess the ability of cells to grow at high copper concentrations, CuSO₄ was added to the growth medium at the concentrations specified in the figure legends. To examine the effect of copper on mRNA levels, CuSO₄ was

TABLE 1. Yeast strains used in this study

Strain	Genotype	Source or reference
DTY3	MAT α <i>trp1-1 leu2-3 leu2-112 gal1 ura3-50 his cup1^s</i> (single copy)	66
DTY5	MAT α <i>trp1-1 gal1 met13 can1 ade ura3-50 his cup1^s</i> (single copy)	23
DTY21	MATa <i>trp1-1 gal1 his ura3-50 cup1::URA3 ade</i>	66
DTY86	MAT α <i>trp1-1 leu 2-3 leu2-112 gal1 ura3-50 his cup1-Δ61 ace1-Δ225</i>	73
DTY131	MATa <i>trp1-1 gal1 met13 can1 ade ura3-50 his cup1^s</i> (single copy)	This study
DTY155	MAT α <i>trp1-1 leu 2-3 leu2-112 gal1 ura3-50 his cup1-Δ61 ace1-Δ225 cup9-1</i>	This study
DTY157	MAT α <i>trp1-1 leu2-3 leu2-112 gal1 ura3-50 his cup1-Δ61 ace1-Δ225 cup9::URA3</i>	This study
DTY158	MAT α <i>trp1-1 leu2-3 leu2-112 gal1 ura3-50 his cup1^s</i> (single copy) <i>cup9::URA3</i>	This study
DTY181	MATa <i>trp1-1 gal1 met13 can1 ade ura3-50 his cup1::hisG</i>	This study
DTY183	MATa <i>trp1-1 gal1 met13 can1 ade ura3-50 his cup1::hisG CUP9::CUP9-URA3</i>	This study

added to logarithmically growing cells for 45 min, after which cells were harvested and washed in preparation for RNA isolation. The *Escherichia coli* strains XL1-Blue and DH5 α F' were used for all DNA manipulations and were grown under standard conditions (44).

Plasmids. The yeast genomic bank used for *CUP9* gene isolation consisted of partial *Sau3A* fragments ligated into the *Bam*HI site of the centromere-based plasmid YCp50 (57). Centromere-based plasmids pRS315 and pRS316 were used for subcloning the genomic 3.6-kb *Hind*III fragment containing *CUP9* (see Fig. 2) (60). A frameshift mutation in the *CUP9* open reading frame (ORF) was created by subcloning an 8-bp *Sal*I linker into the *Sca*I site of the *CUP9* gene. The insertion of this linker was confirmed by digestion with *Sal*I and DNA sequencing using a synthetic oligonucleotide primer (5'-CCCC GCATCCTTTTCC-3'). The resulting plasmid, pcup9::*Sal*I, theoretically encodes a truncated peptide of 157 amino acids which terminates 50 codons downstream of the *Sal*I site. Plasmid YipCUP9-URA3 was created by ligation of the 2.8-kb *Hind*III-*Kpn*I fragment of *CUP9* into the multiple cloning site of the integrating plasmid pRS306 (60).

Yeast cell mutagenesis, genetic analysis, and complementation testing. Strain DTY86 (resistant to >250 μ M CuSO₄ when grown on SC medium with lactate as the sole carbon source) was mutagenized to approximately 82% viability with ethyl methanesulfonate (EMS) as described previously (17). Mutagenized cells were plated on YPD agar to give a density of approximately 150 colonies per 100-mm-diameter plate and incubated at 30°C. After a 6-day incubation, colonies were replica plated to SC agar with lactate as the sole carbon source (SCL), and SCL agar containing 50 μ M CuSO₄. After a 4-day incubation at 30°C, plates were inspected to identify colonies that grew on SCL but not on SCL containing 50 μ M copper. The copper-sensitive isolates were single-colony purified and retested on SCL agar with and without 50 μ M CuSO₄ to confirm sensitivity to copper. To examine whether the copper-sensitive mutation was recessive or dominant in nature, the mutant strain was mated with the wild-type strain DTY21. The resultant diploid strain was purified and tested for the ability to

grow on SCL agar in the presence of CuSO_4 . Sporulation and tetrad analysis were performed as previously described (59).

Subclones of the 3.6-kb *HindIII* DNA restriction fragment (containing the wild-type *CUP9*) were constructed in the centromere-based plasmids pRS315 or pRS316 and introduced by lithium acetate transformation into strain DTY155 (*cup9-1*) or strain DTY157 (*cup9::URA3*) (32). The transformants were single-colony purified, and two independent transformants were tested for complementation of the *cup9* mutation by the ability to grow on SCL plates containing 100 and 250 μM CuSO_4 .

To ascertain whether the cloned DNA which complemented the copper sensitivity phenotype contained the gene in which the EMS-generated mutation had occurred, *CUP1* was deleted from strain DTY131 as described previously (73). This strain, DTY181, was transformed with YIpCUP9-URA3 which had been linearized at the unique *SnaBI* restriction enzyme site of the *CUP9* fragment, to generate strain DTY183. Integration at the *CUP9* locus was confirmed by Southern blotting of *HindIII*-digested genomic DNA with a ^{32}P -labeled 3.6-kb *CUP9 HindIII* fragment as the probe. Strain DTY183 was mated with the original EMS-derived mutant DTY155. Diploids were purified by selection on SC-Met-Leu medium and sporulated, and the tetrads were dissected and tested for the ability to grow on the following solid media: SCL in the presence of 100 and 250 μM CuSO_4 , SCL, SC-Ura, SC-Met, SC-Leu, and SC. The mating types of the spore clones were determined by using the PCR protocol of Huxley and coworkers (31).

Sequence analysis of the *CUP9* gene. The DNA sequence of the *CUP9* locus from the *AluI*-to-*KpnI* restriction enzyme sites was obtained by subcloning the 2.8-kb *HindIII-KpnI* DNA restriction fragment, a 1.3-kb *HindIII-ScaI* DNA restriction fragment, and a 1.5-kb *ScaI-KpnI* DNA restriction fragment (see Fig. 2) into the multiple cloning site of pBluescript plasmids (Stratagene, La Jolla, Calif.). A series of nested deletions of these fragments was constructed by using exonuclease III (Erase-a-Base; Promega, Madison, Wis.) according to the manufacturer's directions. The templates were sequenced on both strands with the Sequenase dideoxy-chain termination kit (U.S. Biochemical Corp., Cleveland, Ohio). The sequencing reactions were resolved on buffer gradient polyacrylamide-urea gels, dried, and visualized by exposure to Kodak XAR-5 X-ray film (4). Additional sequence information was obtained by the use of custom synthetic oligonucleotide primers in conjunction with the Sequenase kit. The sequence analysis and database searches were carried out by using the University of Wisconsin Genetics Computer Group package.

Disruption of the chromosomal *CUP9* allele. The *CUP9* gene was disrupted with the *S. cerevisiae URA3* gene as outlined in Fig. 5A. A 1.1-kb *SmaI URA3* DNA fragment isolated from plasmid pURA310 was subcloned into *ScaI* of the *CUP9* gene in plasmid pCUP9HK. The resulting plasmid, pcup9::URA3, was digested with *NotI* (in the multiple cloning site) and *KpnI* to release a 4-kb DNA fragment which was agarose gel purified and used to transform yeast strains DTY3 and DTY86. Uracil prototrophs were isolated and tested for their abilities to grow on SCL agar with 0, 100, and 250 μM CuSO_4 . Southern blot analysis of copper-sensitive isolates confirmed the presence and structure of the chromosomal disruptions (44).

Mapping of the *CUP9* locus. To map the position of the *CUP9* locus in the yeast genome, membranes prepared from a set of overlapping recombinant clones containing inserts from the genome of *S. cerevisiae AB972* were probed with a 2.8-kb *HindIII-KpnI* DNA fragment which contains the *CUP9* gene (43). The membranes and instructions for use were obtained

from the American Type Culture Collection. In brief, the membranes were prehybridized in a solution of $5\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50% (vol/vol) formamide, 5% (wt/vol) blocking reagent (Genius 1 kit; Boehringer Mannheim, Indianapolis, Ind.), 0.1% (wt/vol) *N*-lauroylsarcosine Na salt, and 0.02% (wt/vol) sodium dodecyl sulfate (SDS) at 42°C for 6 h. The membranes were then hybridized at 42°C for 17 h, with the 2.8-kb *HindIII-KpnI* DNA fragment and control DNA fragments, supplied by the American Type Culture Collection, which had been random prime labeled with digoxigenin-labeled deoxyuridine triphosphate (Genius Kit 1). After hybridization, the membranes were washed twice with $2\times$ SSC-0.1% SDS for 5 min at room temperature and then twice with $0.1\times$ SSC-0.1% SDS for 15 min at 68°C . Positive hybridization was detected with a Genius 1 kit, which employs an anti-digoxigenin antibody conjugated to alkaline phosphatase and a colorimetric substrate.

Quantitation of cellular copper concentrations. Yeast strains DTY86 and DTY155 were grown in SC or SCL medium to an optical density at 650 nm of between 0.9 to 1.5. To the growing cells, CuSO_4 was added to give concentrations of 0, 100, and 250 μM Cu. The cells were incubated at 30°C with shaking for 45 min and harvested by centrifugation at 4°C , and the cell pellets washed twice in 0.1 M Tris-succinate (pH 6.0) containing 10 mM EDTA and twice in glass-distilled water. At the final wash, a sample of cell suspension was taken for protein determination, using the modified Lowry method of Herbert and coworkers (26). The remaining cells were pelleted by centrifugation, and the pellets were digested by a gentle boiling with concentrated nitric acid. The acid-digested cells were resuspended in 1 ml of 0.5% nitric acid and analyzed for copper content by atomic absorption spectroscopy (aa/ae Spectrophotometer 451; Instrumentation Laboratory, Wilmington, Mass.). These experiments were repeated a total of five times.

RNA analysis. Total RNA was isolated from 5 to 10 ml of early- to mid-log-phase (optical density at 650 nm, 1 to 1.5) yeast cultures grown in SC or SCL medium with CuSO_4 as indicated in the figure legends (24). The yield of total RNA from the SCL cultures was typically 50 to 75% of that from the SC cultures when cells were grown to similar optical density. For RNA blot analysis, 20 μg of total RNA was subjected to electrophoresis in a 1% formaldehyde-agarose gel and capillary blotted to Nytran membranes (Schleicher & Schuell, Keene, N.H.). Hybridization was carried out by the Schleicher & Schuell protocol, with a nick-translated ^{32}P -labeled 0.7-kb *ScaI-NsiI* probe encompassing the majority of the *CUP9* translational ORF. After exposure to Kodak XAR-5 X-ray film, the membranes were washed in 50% formamide- $6\times$ SSPE ($1\times$ SSPE is 0.18 M NaCl, 10 mM NaPO_4 , and 1 mM EDTA [pH 7.7]) at 65°C for 30 min, rinsed in $2\times$ SSPE, and then prehybridized and reprobbed with a nick-translated ^{32}P -labeled *ACT1* probe, derived from the *S. cerevisiae* actin gene, using the aforementioned Schleicher & Schuell protocol. The size of the *CUP9*-specific mRNA was estimated by using RNA molecular weight markers (Boehringer Mannheim) and rRNA.

The start site of transcription of the *CUP9* gene was determined by using 20 μg of total RNA in a primer extension reaction with a synthetic oligonucleotide primer (5'-GGGT GGGAAGATCAAC-3' complementary to nucleotides -101 to -116 of the *CUP9* gene) 5' end labeled with [γ - ^{32}P]ATP. The labeled reaction products were subjected to electrophoresis alongside DNA sequencing reactions conducted with the same primer, on a 10% polyacrylamide-urea sequencing gel, and exposed to Kodak X-AR film at -70°C with intensifying screens.

FIG. 1. Yeast copper resistance as a function of carbon source. Strains DTY3 (*CUP1*), DTY4 (*cup1Δ*), DTY67 (*ace1Δ*), and DTY86 (*ace1Δ cup1Δ*) were streaked onto SC agar with either glucose or lactate as the sole carbon source. CuSO_4 was added to the final concentrations indicated. The SC-glucose plates were incubated at 30°C for 4 days. The SC-lactate plates were incubated at the same temperature for 6 days. The key indicates the relevant genotype of the strains streaked onto each quadrant of the plate.

Nucleotide sequence accession number. The sequence of the *CUP9* gene has been assigned GenBank accession number L36815.

RESULTS

Isolation of a copper-sensitive mutant unlinked to *CUP1* or *ACE1*. These studies were initiated by our observation that yeast strain DTY86, in which the *CUP1* and *ACE1* genes encoding MT and the Cu metalloregulatory transcription factor ACE1 had been deleted, was able to grow at greater than 20-fold-higher copper concentrations on SC agar plates containing lactate than on SC agar plates containing glucose as the sole carbon source (Fig. 1). Examination of small incremental changes in copper concentration indicated that DTY86 grows on SC agar containing 12.5 μM Cu but grew on SC agar plates containing lactate in the presence of 250 μM CuSO_4 (data not shown). These results suggested that an additional gene(s) is involved in the protection of *S. cerevisiae* cells from high environmental copper concentrations under respiratory growth conditions and that perhaps the function or expression of these gene products is altered by the physiological state of the cell. To identify the gene(s) that allowed growth at high copper concentrations when yeast cells were grown on nonfermentable carbon sources, we mutagenized strain DTY86 with EMS and isolated copper-sensitive mutants. Of approximately 3,400 colonies screened for sensitivity to 50 μM copper sulfate when grown on a lactate carbon source, a single copper-sensitive candidate was identified. This mutant grew normally on both SC and SCL media in the absence of exogenous copper, and growth was not discernibly different from that of the wild-type strain when examined for sensitivity to zinc, cadmium, or silver at any concentration tested (data not shown).

To determine the nature of the EMS-generated mutation,

the mutant strain (DTY155) and the parent strain (DTY86) were crossed with a wild-type tester strain (DTY21). The diploids from the mutant cross were all resistant to at least 200 μM copper sulfate (data not shown), demonstrating that the copper sensitivity mutation is recessive. Sporulation of diploids from a cross of DTY155 with DTY21 and analysis of the meiotic progeny indicated Mendelian segregation of the mutation leading to copper sensitivity on SCL medium, demonstrating that this recessive mutation, designated *cup9-1*, lies in a single nuclear gene.

Isolation of the *CUP9* gene by complementation cloning. The recessive nature of the copper sensitivity phenotype associated with the *cup9-1* allele permitted the isolation of the wild-type *CUP9* gene by in vivo complementation. Strain DTY155, harboring the *cup9-1* allele, was transformed with a YCp50-based yeast genomic library, and approximately 22,000 transformants were selected on SC agar lacking uracil. Replica plating of these transformants to SCL agar containing 100 μM CuSO_4 identified nine transformants that were resistant to copper. The nine transformants were patched onto SC agar containing 5-fluoroorotic acid to select for cells which had eliminated the plasmid. Replating of these cells to SCL agar containing 100 μM CuSO_4 demonstrated that for three of the nine transformants, copper resistance was plasmid dependent. The other copper-resistant isolates may have been due to reversion of the *cup9-1* allele; however, this has not been experimentally verified. These plasmids were rescued from two of the three copper-resistant isolates and retransformed into strain DTY155 (*cup9-1*), restoring resistance to copper on SCL agar. Restriction endonuclease digestion of these two plasmids revealed that the two independently isolated clones contained nonidentical genomic DNA fragments but did share a common 3.6-kb *HindIII* DNA restriction fragment. This fragment was subcloned into pRS316 to generate plasmid pCUP9H3 (Fig.

FIG. 2. Subcloning and complementation analysis of the cloned *CUP9* locus. Plasmid pCUP9H3 and subclone derivatives were transformed at a single copy into *cup9-1* mutant strain DTY155 and tested for complementation of the copper sensitivity defect on SCL agar containing 250 μ M CuSO_4 . The *cup9-1* mutant harboring control vectors pRS315 and pRS316 failed to grow on SCL medium containing 25 μ M CuSO_4 . +, plasmids containing DNA fragments that permitted growth at 250 μ M CuSO_4 are indicated; -, no growth above a copper concentration of 25 μ M. Plasmid pcup9::Sal contains an eight-nucleotide *SalI* linker inserted at the *ScaI* site within the *CUP9* ORF. The solid arrow shows the position of the *CUP9* ORF and the direction of transcription. The line that extends from the *AluI* to *KpnI* restriction enzyme sites indicates the region of *CUP9* DNA sequenced. The *cup9::URA3* construct depicts the chromosomal *CUP9* allele disruption at the *ScaI* site by insertion of the *URA3* gene (see Fig. 5A).

2), retransformed into the *cup9-1* mutant strain DTY155, and shown to restore copper resistance to wild-type levels.

Subcloning of the *CUP9* gene. In order to map more precisely the portion of DNA responsible for conferring copper resistance to the *cup9-1* mutant, fragments of the complementing 3.6-kb *HindIII* fragment were subcloned into pRS316 by using restriction endonuclease sites as shown in Fig. 2. The plasmids generated were transformed into DTY155 and tested for the ability to confer growth on SCL agar containing 250 μ M copper sulfate. A 2.8-kb *HindIII*-*KpnI* DNA restriction fragment (plasmid pCUP9HK) which completely restored the copper resistance of the *cup9-1* mutant to wild-type levels was identified. Bisection of the 2.8-kb *HindIII*-*KpnI* DNA with *ScaI* generated two further subclones, pcup9HS and pcup9SK, neither of which complemented the copper sensitivity phenotype of the *cup9-1* mutant (Fig. 2). These results suggested that the *ScaI* site is located within the ORF of the gene or within a region essential for correct expression of the *CUP9* gene. The smallest DNA fragment tested which complemented the *cup9-1* mutant was a 1.4-kb *AluI*-*DraI* fragment, which conferred the same degree of copper resistance as the 3.6-kb *HindIII* fragment. This suggests that the 1.4-kb *AluI*-*DraI* fragment contains the complete ORF and the necessary elements for *CUP9* expression.

To determine if this complementing DNA fragment is identical to the gene which contained the original mutation or is an extragenic suppressor of the *cup9-1* mutation, the EMS mutant (DTY155) was mated with strain DTY183 in which the selectable marker *URA3* had been integrated at the wild-type genomic *CUP9* locus by homologous recombination. The resulting diploid sporulated poorly and produced few complete tetrads, though three-spore asci were abundant. Thirty tetrads

were dissected, and of these only one gave rise to four viable spores; this was not unexpected, as low spore viability is usually associated with poor sporulation (19). A total of 33 spore clones were analyzed for linkage of uracil prototrophy to copper resistance on a lactate carbon source. Of these, 32 spore clones exhibited linkage of either uracil prototrophy with copper resistance (16 spore clones) or uracil auxotrophy with copper sensitivity (16 spore clones). Leucine and methionine markers and mating type all segregated independently and in Mendelian fashion. The one complete tetrad exhibited 2:2 segregation of all markers and cosegregation of copper resistance with uracil prototrophy. This demonstrates that the cloned complementing DNA fragment is tightly linked to the genomic *CUP9* locus. Mapping of *CUP9*, using membranes prepared from overlapping recombinant clones containing inserts from genomic *S. cerevisiae*, indicated that the *CUP9* allele was located on chromosome 16 between *TPK2* and *PEP4* (data not shown).

Sequence of the *CUP9* gene. The DNA sequence on both strands of the 1.4-kb *AluI*-*DraI* restriction fragment, which complements the *cup9-1* mutation, was determined by dideoxy nucleotide termination sequencing. Computer analysis of this DNA sequence indicated the presence of a single extended ORF for translation (Fig. 3A). The ORF has the potential to encode a polypeptide of 306 amino acids with a predicted molecular mass of 34,632 Da. The first potential translation initiation ATG triplet is sited within a Kozak consensus (purines at positions -3 and +4) (39). A second potential translation initiation site is present within the proposed ORF at nucleotide position 107; however, which of these functions initiates translation *in vivo* is currently unknown. The evidence that supports the proposed ORF as the functional *CUP9* is as

sensitivity of the *cup9-1* mutant. This insertion shifts the ORF by two bases, which would result in an altered peptide product that terminates 53 amino acids downstream of the *ScaI* site. The ORF is located within the smallest 1.4-kb *AluI*-*DraI*

complementing DNA fragment, and the approximately 1-kb size of the *CUP9* mRNA corresponds to a message size that could encode the predicted size of the CUP9 protein. A computer search of the GenBank and EMBL databases, using the TFASTA Pearson and Lipman algorithm for proteins that resemble the predicted CUP9 polypeptide, was conducted (51). This search identified similarity to the homeobox regions of *S. cerevisiae* α and α mating type loci (2), to the *Schizosaccharomyces pombe* *mat1-P* locus (37), and to the human PBX1 gene product, a proto-oncogene associated with pre-B-cell lymphocytic leukemia (36, 50). Little to no similarity was detected outside the homeobox region with these or other proteins. Comparison of the 55 amino acids that compose the homeoboxes of these proteins using the Gap analysis program of the Genetics Computer Group package (49) at the default parameters, revealed that within this sequence CUP9 was most similar to PBX1 (47% identity) and MAT α 2 (33% identity) (Fig. 3B). Comparison of the putative CUP9 homeobox sequence to a compiled list of homeobox sequences (40) indicated that 7 of the 10 nearly invariant amino acids are conserved in the region of CUP9 encoding a homeobox-like domain. Overall, the sequence comparison data suggest that CUP9 encodes a homeodomain protein which binds DNA.

Outside the homeobox region, no similarity with other known proteins in the database was found. The primary sequence contains three cysteine and six histidine residues. Although the arrangement of these residues does not conform to the Cys-Xaa-Cys or Cys-Xaa₂-Cys metal binding motifs of the copper binding proteins CUP1 and ACE1 (21, 29, 34, 71), the possibility that CUP9 protein can bind metal has not yet been excluded. There are three positively charged clusters at positions 156 to 160, 217 to 220, and 286 to 289; similar clusters have been identified to be important in the localization of nuclear proteins (56, 58). An array of four isoleucines and one leucine starting at Ile-200 and spaced seven amino acids apart might lead to the formation of a coiled-coil structure, the site of dimerization for leucine zipper proteins (3). Overall, the protein encoded by the *CUP9* gene is made up of 67% polar amino acids and has a net positive charge of 16. The acidic and basic amino acids are distributed throughout the primary structure of CUP9 and are not partitioned into the carboxy- and amino-terminal regions as observed for ACE1 (61). Primer extension analysis of the *CUP9*-specific mRNA identified the major start site for transcription to be 191 bases upstream from the first ATG of the *CUP9* ORF (Fig. 4). The position of this start site is consistent with the size of the *CUP9*-specific mRNA detected by RNA blotting (see below). Two minor transcriptional start sites were also detected, one and nine bases upstream from the major start site (Fig. 4).

Construction of isogenic *CUP9* disruption strains. The *cup9-1* mutant, generated by EMS mutagenesis, may harbor other EMS-induced mutations which may complicate analysis of *CUP9* gene function. Therefore, in order to examine the effect of a single mutation at the *CUP9* locus, isogenic strains harboring either a wild-type *CUP9* gene or a *cup9* allele which had been insertionally inactivated were constructed. To generate isogenic strains of DTY86 (*ace1 Δ cup1 Δ CUP9*) and DTY3 (*ACE1 CUP1 CUP9*) in which the single endogenous *CUP9* gene is nonfunctional, these strains were transformed with a linear *CUP9* fragment containing the *URA3* gene inserted at the *ScaI* site (plasmid pcup9::URA3 [Fig. 2 and 5]). Transformants were selected on SC-Ura agar, purified, and tested for copper sensitivity, and total genomic DNA was isolated and digested with *HindIII* for analysis by Southern blotting. Figure 5B shows the results of a Southern blot which was probed with a radionucleotide-labeled 3.6-kb *HindIII*

FIG. 4. Mapping of *CUP9*-specific mRNA transcription initiation sites. Primer extension reactions were carried out with an oligonucleotide primer corresponding to *CUP9* antisense strand residues -101 to -116 and 15 μ g of total RNA isolated from DTY86 (lane P.E.). The same oligonucleotide was used in dideoxynucleotide chain termination sequencing reactions with a *CUP9* DNA template cloned into pBlue-script (lanes G, A, T, and C). The major and minor primer extension products are indicated by the large and small arrows, respectively.

fragment containing the *CUP9* gene. This probe hybridized to a single 3.6-kb DNA fragment in DNA isolated from strains DTY86 (lane 1), DTY155 (lane 2), and DTY3 (lane 4). The size of this fragment agrees with the predicted size determined from the restriction map of the isolated *CUP9* locus. The presence of a 3.6-kb *HindIII* fragment in strain DTY155 (*cup9-1*) is consistent with the absence of large rearrangements of the *cup9* locus as a result of EMS mutagenesis. The hybridization of the 3.6-kb *HindIII* fragment to two DNA fragments in DNA isolated from the two *cup9::URA3* disrupted strains also concurs with the predicted result; the 1.1-kb *URA3* gene inserted within the *CUP9 ScaI* site contains a *HindIII* site which is cleaved to generate two fragments of 3.4 and 1.3 kb derived from the *cup9::URA3* locus (Fig. 5B, lanes 3 and 5).

Strains DTY86 and DTY3 in which *CUP9* was disrupted were denoted DTY157 (*cup9::URA3*) and DTY158 (*cup9::URA3*), respectively. These strains and the wild-type parental strains were examined for the ability to grow on solid media of SC, SCL, and SC with 3% glycerol-ethanol at a range of copper concentrations (Fig. 6). All strains grew more slowly in the absence of exogenous copper when glycerol-ethanol or lactate was used as the sole carbon source. This is a common observation for yeast cells grown under conditions in which energy is derived solely from respiration (70). In our initial observations (Fig. 1), we did not observe a difference in copper resistance on a glucose carbon source between DTY86 (*CUP9*) and DTY155 (*cup9-1*). However, when small incremental changes in copper sulfate concentrations were examined, DTY86 (*CUP9*) but not DTY157 (*cup9::URA3*) was able to grow on SC glucose agar containing 10 μ M copper sulfate. We did not detect a phenotypic difference between strains DTY3 and DTY158, which contain wild-type *CUP1* and *ACE1* genes, on SC medium at the copper concentrations examined. When the strains were grown on SCL agar, there were clear phenotypic differences (Fig.

FIG. 5. Insertional inactivation of the chromosomal *CUP9* locus. (A) A 1.2-kb *SmaI* *URA3* DNA fragment was inserted into the *ScaI* site of a plasmid-borne *CUP9* gene as described in Materials and Methods and is shown schematically in Fig. 2. Strains DTY86 and DTY3 were transformed with the linear *cup9::URA3* fragment, and successful transformants were selected on SC-Ura agar. The stippled line indicates the ORF of *CUP9*. The following abbreviations indicate restriction sites: S, *SmaI*; Sc, *ScaI*; K, *KpnI*; and H, *HindIII*. (B) Southern blot analysis. Genomic DNA was isolated from the following strains: DTY86 (*cup1Δ ace1Δ CUP9*) (lane 1), DTY155 (*cup1Δ ace1Δ cup9-1*) (lane 2), DTY157 (*cup1Δ ace1Δ cup9::URA3*) (lane 3), DTY3 (*CUP1 ACE1 CUP9*) (lane 4), and DTY158 (*CUP1 ACE1 cup9::URA3*) (lane 5). The genomic DNA was digested with *HindIII*, and the sizes of DNA fragments that hybridized to the probe are indicated on the right of the autoradiogram.

6A). DTY86 grew on SCL containing up to 250 μ M copper sulfate, whereas DTY157 would grow only on SCL containing up to 15 μ M copper. Strain DTY3 (*CUP1 ACE1 CUP9*) grew on SCL agar containing up to 1 mM CuSO_4 ; however, DTY158 (*CUP1 ACE1 cup9::URA3*) grew on SCL agar containing only 700 μ M CuSO_4 . Thus, on SCL medium, *CUP9* confers a growth advantage at high copper concentrations even in the

presence of functional *CUP1* and *ACE1* genes, whereas there was no discernible contribution of *CUP9* to copper resistance for cells which contained wild-type *CUP1* and *ACE1* grown on glucose. Growth on glycerol-ethanol (nonfermentable carbon sources) also demonstrated a role for *CUP9* in copper resistance; DTY86 grew at 10 μ M, whereas DTY157 could not (Fig. 6B). These results, in conjunction with Southern hybridization results, demonstrate that the genomic *CUP9* allele was insertionally disrupted to give a copper sensitivity phenotype and that the *CUP9* gene confers a growth advantage to cells on both fermentable and nonfermentable carbon sources in the presence of copper. Furthermore, under respiratory conditions, *CUP9* plays a critical function in copper detoxification in cells that also express the *CUP1* and *ACE1* proteins.

Effect of *CUP9* on intracellular copper concentration. The data described above demonstrate that the *CUP9* gene, encoding a putative homeobox domain protein, plays an important role in protecting yeast cells from copper toxicity, but not other metals, particularly when cells are grown on nonfermentable carbon sources. This may occur via the expression or function of genes which limit copper accumulation during these conditions, which sequester intracellular copper, or which protect or repair cellular damage caused by intracellular copper in the presence of oxygen-derived radicals resulting from respiration (22). To investigate whether the functional status of *CUP9* altered cell-associated copper levels, we compared total cellular copper accumulation in strains DTY86 (*CUP9*) and DTY155 (*cup9-1*). The cells were grown on SC and SCL liquid media, and total cellular copper concentrations were measured by atomic absorption spectroscopy, subsequent to a 45-min incubation in the presence of CuSO_4 . Figure 7 shows the results of copper measurements from five independent experiments. Statistical evidence for a difference in the intracellular copper concentrations that was dependent on *CUP9* was not observed for cells grown in SC or SCL medium. The carbon source, however, had a significant ($P < 0.05$) effect on copper accumulation. Cells grown in SCL medium accumulated two- to threefold more copper than cells grown in SC, when no exogenous copper was added. This might reflect an increase in the synthesis of copper-requiring enzymes involved in oxidative respiration such as cytochrome *c* oxidase (52), or copper-dependent protective enzymes such as Cu-Zn superoxide dismutase (22). In contrast, when 100 and 250 μ M exogenous copper was added to cells grown in SC medium, these cells contained 3- to 5-fold and over 40-fold-more copper, respectively, than cells grown in SCL medium and then exposed to an equal amount of copper. The copper accumulation observations are supported by radiolabeled ^{64}Cu uptake experiments, in which the rate of copper uptake was not affected by *CUP9* (unpublished data). In summary, these results suggest that *CUP9* does not affect total accumulation of copper in these cells or the rate of copper uptake.

Effect of copper and carbon source on *CUP9* mRNA levels. The *S. cerevisiae* *CUP1* and *C. glabrata* MT genes and the *C. glabrata* *AMT1* copper metalloregulatory gene are key components in yeast cell copper detoxification. These genes are biosynthetically regulated at the level of transcription by copper (74). Because *CUP9* is involved in copper homeostasis, it is possible that the gene itself is regulated by copper, perhaps in a manner similar to that of *CUP1*, whose expression is increased via the copper-dependent transcription factor *ACE1* (21, 61, 64). Alternately, like *ACE1* (61), *CUP9* may be constitutively expressed and unaffected by alterations in environmental copper concentrations. To investigate this and to determine the size of the *CUP9* mRNA, strains DTY86 (*cup1Δ ace1Δ*) and DTY3 (*CUP1 ACE1*) were grown to logarithmic

FIG. 6. Copper sensitivity of *cup9::URA3* strains on fermentable and nonfermentable carbon sources. (A) Strains DTY86 (*cup1Δ ace1Δ CUP9*), DTY157 (*cup1Δ ace1Δ cup9::URA3*), DTY3 (*CUP1 ACE1 CUP9*), and DTY158 (*CUP1 ACE1 cup9::URA3*) were streaked onto SC or SCL agar to which copper sulfate had been added at the indicated final concentrations. SC plates were incubated for 4 days at 30°C, and SCL plates were incubated for 6 days at 30°C. The diagram indicates the strain (with relevant genotype) streaked onto each quadrant of the plate. (B) Strains DTY86 and DTY157 were streaked onto SC agar medium containing 3% ethanol and 3% glycerol as the carbon sources and supplemented with copper sulfate at the indicated concentrations. The diagram indicates the relevant strain streaked onto each half of the plate.

phase in SC and SCL liquid media and incubated in the absence and presence of copper for 45 min. RNA was isolated, and *CUP9* mRNA was analyzed by RNA blot hybridization with a ^{32}P -radiolabeled 0.7-kb *ScaI-NsiI* restriction DNA fragment encompassing the majority of the *CUP9* ORF. This probe detected a single RNA species of approximately 1 kb, consistent with the size of the *CUP9* ORF for translation. The steady-state level of this mRNA species was dramatically decreased when strain DTY86 was grown in SC medium in the presence of 10 μM exogenous copper and was not detectable at 100 μM copper (a level that has no effect on the rate of growth during the incubation time) (Fig. 8A). *CUP9* mRNA levels were also repressed in DTY3 cells exposed to 10 μM copper but were still detectable with up to 250 μM copper (a nontoxic level) (Fig. 8B). This could be due to the efficient binding of copper by the yeast CUP1 protein expressed in this strain. Since the copper-dependent down regulation of *CUP9* mRNA observed was in an $\Delta ace1$ background, the down regulatory effect that copper has on *CUP9* mRNA steady-state levels in cells grown on a glucose carbon source is independent of *ACE1*, the copper metalloregulatory transcription factor. In contrast, exogenous copper had

no effect on *CUP9* mRNA levels in DTY86 and DTY3 cells that had been grown in SC medium with lactate as the sole carbon source, even at copper levels as high as 1 mM. In the absence of exogenous copper, the levels of *CUP9* mRNA in cells grown in SC and SCL media were similar, with perhaps a twofold elevation in lactate-grown compared with glucose-grown cells. Iron, as ferric ammonium citrate, had no effect on *CUP9* mRNA levels at the concentrations tested (up to 200 mM) (data not shown). *ACT1* mRNA steady-state levels were decreased in cells that had been grown on the lactate carbon source compared with cells that had been grown on the glucose carbon source. The *ACT1* mRNA level, however, was unaffected by copper concentration in cells that had been growing with lactate or glucose as the sole carbon source, indicating that the down regulatory effect of copper on *CUP9* mRNA in cells grown with a glucose carbon source is not a general effect on all mRNA levels. The faint hybridization signal observed above the primary signal for *ACT1* in cells grown on lactate corresponds to the migration position of the 18S rRNA and may represent nonspecific cross-hybridization on this blot.

FIG. 7. Accumulation of copper in strains bearing functional or nonfunctional *CUP9* alleles. Strains DTY86 (*CUP9*) and DTY155 (*cup9-1*) were grown to an optical density at 650 nm of 0.8 to 1.5 in SC-glucose or SC-lactate liquid medium. Copper sulfate was added at the indicated concentrations, and the cultures were incubated for 45 min at 30°C. Cells were harvested by centrifugation and washed twice with Tris-succinate buffer containing 10 mM EDTA and twice with glass-distilled water. An aliquot of cells was removed for protein estimation, and the remainder was digested by being boiled in concentrated nitric acid. Total cellular copper was determined by atomic absorption spectroscopy. Results are presented as the means and standard deviations (indicated by bars) of five independent experiments.

DISCUSSION

The involvement of *ACE1* and *CUP1* as key players in cellular copper homeostasis in *S. cerevisiae* serves as an important portion of the mechanisms by which yeast cells counteract toxic levels of environmental copper and maintain sufficient copper for essential processes (74). These two proteins are not the sole homeostatic regulators of copper, as the recent identification of the copper transporter *CTR1* (12), the multi-copper oxidase *FET3* (1), and the *ACE1*-like transcription factor *MAC1* (33) demonstrates. The involvement of these genes in both copper and iron homeostasis suggests that the regulation of these redox-active metals requires a number of factors, under a variety of physiological conditions. Indeed, comparison with other species indicates that there are a number of different components involved in copper regulation. The yeast *C. glabrata* harbors a family of MT genes: an *MT-I* gene and two *MT-II* genes, which are all transcriptionally activated by copper, as well as by silver (45, 46). The copper-dependent transcription activator in *C. glabrata* *AMT1* activates *MT-I* and *MT-II* transcription and also rapidly autoactivates transcription in response to high levels of environmental copper, an event essential for full protection against copper toxicity (72, 74). In addition to the family of MTs in humans, two cDNAs encoding putative copper-transporting ATPases, corresponding to genes mutated in Menkes' and Wilson's diseases, have been cloned (5, 8, 47, 63, 69). It is thus apparent that there are a number of cellular factors which must participate in establishing and maintaining copper homeostasis in eukaryotes. Using classical yeast genetics, we have identified a gene, denoted *CUP9*, which is of critical importance in the homeostasis of cellular copper under conditions of active cellular respiration.

The impetus for the work described in this paper stemmed from the observation that yeast strains devoid of *CUP1* and *ACE1* could grow at higher concentrations of environmental

FIG. 8. Effects of copper on *CUP9* steady-state mRNA levels. Yeast strains DTY86 (A) and DTY3 (B) were grown in SC or SCL medium to an optical density at 650 nm of 1.0 to 1.5. The cultures were incubated with CuSO₄ at the indicated concentrations for 45 min at 30°C. Following incubation, cells were harvested and total RNA was isolated and analyzed by RNA blotting and hybridization with a ³²P-labeled *CUP9* gene DNA restriction fragment. The *CUP9* probe hybridized to a single 1-kb mRNA species (upper panels). The same membrane was reprobed with a ³²P-labeled *ACT1* (actin) gene as a control for mRNA levels (lower panels). The results shown are representative of three independent experiments.

copper if the carbon source of the medium was nonfermentable, such as lactate. This suggested that the carbon source effect of resistance to copper toxicity was independent of *CUP1* and *ACE1*; indeed, the 5' noncoding region of *CUP9* does not contain an obvious *ACE1* binding consensus sequence. The genetic nature of the copper resistance phenotype was demonstrated by the isolation of a chemically generated mutant of DTY86, *cup9-1*, that was highly sensitive to copper when grown on nonfermentable carbon sources. The generation of the *cup9-1* mutant (DTY155) allowed us to identify the gene responsible for the copper resistance phenotype by cloning from a single-copy yeast DNA library a fragment of DNA that could restore the copper resistance phenotype to the *cup9-1* mutant strain.

DNA sequencing of the *CUP9* gene revealed an ORF that could encode a protein of 306 amino acids. When the sequence for this protein was compared with those in the databases, no overall homology was found; however, a region of 55 residues with a high degree of identity with the homeobox of the human

PBX proteins was detected. The PBX family of genes encode a novel family of homeodomain proteins (36, 48, 50). The function of these genes is not known; however, a t(1;19) translocation of the homeobox region of PBX with the E2A gene has been associated with human pre-B-cell acute lymphoblastic leukemia (35, 50) and shown to cause acute myeloid leukemia in mice (35). The *CUP9* ORF also showed a high degree of identity in the homeobox region with the fission yeast *mat1-P* locus, baker's yeast *MAT α 2* locus, and *Drosophila* Dpbx gene (2, 18, 37). An amino acid comparison of the putative *CUP9* homeobox with a compilation of homeobox regions of several proteins demonstrated that 7 of the 10 nearly invariant residues present in the homeodomain proteins, including the highly conserved asparagine at position 51, were conserved (40). Taken together, these sequence data strongly suggest that *CUP9* is a homeobox protein and can therefore bind DNA. *CUP9* is not the first homeobox protein identified that is associated with copper metabolism. The *Drosophila* homeobox protein *lab* is required for the differentiation and maintenance of the larval mid-gut copper cell (28). This cell is so termed for its ability to fluoresce orange after excitation with UV light in larvae fed a copper-containing diet and has been postulated to be due to the chelation of copper by porphyrin-like molecules (54). These observations suggest that these cells are involved in the absorption of copper from the gut and that *lab* might modulate the expression of genes whose products are involved in copper homeostasis within these cells (16, 54). Comparison of the *lab* homeobox to the *CUP9* homeobox over a region of 59 amino acid residues shows 41% similarity (40). The identification of *CUP9* in *S. cerevisiae* will allow us to genetically identify additional components of copper metabolism which may be targets for or act in concert with *CUP9* and which may prove to have functional analogs in higher eukaryotes.

The implication that *CUP9* encodes a homeobox protein suggests that *CUP9* regulates another gene(s) that might be involved in copper homeostasis. In this context it could act as either an activator or a repressor of gene expression. Of the two proteins that *CUP9* exhibits the highest similarity to, the yeast protein $\alpha 2$ peptide functions as a gene repressor, and the Pbx1 protein, at least in one context, is unable to activate a chloramphenicol acetyltransferase reporter plasmid though it binds to DNA in vitro (15, 68). Whether *CUP9* acts as a gene repressor is not known. The yeast homeodomain proteins *MAT α 1* and *MAT α 2* interact with the *MCM1* protein in order to repress transcription (15). It is possible that the coiled-coil region of *CUP9* fosters an interaction with a second protein in order to regulate transcription.

If *CUP9* is a regulator of gene expression for proteins involved in copper homeostasis, one must consider which genes are regulated. There are a number of cellular processes in which cellular copper levels could be regulated. The first is entry into the cell. Our examination of cellular copper levels indicated that there was no detectable difference in copper levels between cells that possessed the wild-type *CUP9* gene and those that harbored the *cup9-1* mutation, regardless of carbon source. This suggests that *CUP9* is not involved in the regulation of copper entry or exit into and from the cell and therefore is probably not a transcriptional regulator of genes involved in copper transport, such as *CTR1* (12). A second means of regulating cellular copper is by chelation. This is the copper detoxification mechanism employed by MT and its copper-dependent transcriptional activator ACE1, discussed in the introduction. It is feasible that *CUP9* might regulate a second copper chelation mechanism. One copper binding protein that might be regulated by *CUP9* is FET3, which is required for ferrous iron uptake in *S. cerevisiae* and encodes a

multicopper oxidase (1). The amino acid sequence of FET3 shows homology to the CopA protein, which is required for copper resistance in bacteria (7); thus, a similar role could be envisioned for yeast cells. At this point, we cannot rule out the possibility that *CUP9* binds copper directly, though this appears unlikely because of the lack of recognized copper binding motifs. A third mechanism to regulate cellular copper is partitioning the copper within the cell. Examination of the cellular distribution of ^{64}Cu in *S. cerevisiae* cells indicates that as much as 86% of newly incorporated copper is associated with the particulate (membranes and organelles) fraction of the cell, and the remainder is recovered in the soluble (cytosol) fraction (41). Keyhani and coworkers (38) have presented evidence that at high environmental copper levels, excess copper in yeast cells is deposited in the mitochondria and that this shift in the distribution of cellular copper might protect cellular proteins from the effects of copper toxicity. It is possible that *CUP9* might be involved in the regulation of a protein(s) that is involved in this partitioning of copper.

We have proposed that *CUP9* is involved in copper homeostasis; if this is so, then it might be expected that in order for *CUP9* to be responsive to changes in environmental copper concentrations, it is itself regulated by copper. Northern (RNA) blot analysis of *CUP9* mRNA revealed that the *CUP9* mRNA steady-state levels were dramatically down regulated by copper, in an ACE1-independent mechanism. This down regulation of *CUP9* mRNA, however, occurred only on the glucose carbon source. Under conditions of respiratory growth, *CUP9* mRNA steady-state levels were not affected by copper. Cells grown with a lactate carbon source accumulate less copper than cells grown with a glucose carbon source (Fig. 7). Thus, the lack of copper-dependent down-regulation of *CUP9* mRNA levels in lactate-grown cells may be due to lower cell-associated copper levels. Alternatively, yeast cells growing on a nonfermentable carbon source derive all their energy from respiration, a process which requires copper as a cofactor, but can be poisoned by high copper concentrations (9). It would be an advantage for respiring yeast cells to be able to express genes, such as *CUP9*, that might participate in combating the toxic effects of copper under these increased stress conditions.

There are a number of proteins in *S. cerevisiae* whose expression is either up regulated or down regulated by the metal which they in turn regulate. MT is up regulated in response to copper by the copper-dependent transcription factor ACE1 (65). In contrast, the yeast gene encoding the high-affinity copper transport protein *CTR1* is down regulated at the level of mRNA by copper (12). In *C. glabrata*, the copper-dependent transcriptional activator *AMT1* is up regulated in an autoregulatory manner by copper (74). The *S. cerevisiae* gene *FRE1*, which encodes ferric and copper reductase, is down regulated in response to increased environmental iron (10). The down regulation of *FRE1* expression by iron was shown to be mediated through a 12-nucleotide repeated sequence in the 5' noncoding region of the gene (11). The mechanism by which copper specifically down regulates *CUP9* steady-state mRNA levels remains to be established. The observation that the mRNA levels of *CUP9* are down regulated by copper on fermentable but not nonfermentable carbon sources is consistent with the more prominent role of *CUP9* in copper tolerance under respiratory growth conditions. The strong similarity of *CUP9* to homeobox proteins suggests that it is a regulator of gene expression for proteins involved in copper homeostasis under conditions of active respiration. Further experiments to identify the target genes and the

mechanism of action of *CUP9* will shed light on the important role this protein plays in copper homeostasis.

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