not a critical test, it firmly establishes that our model is feasible and can account for the phenomena we sought to explain. Other models such as unequal sister chromatid exchange (*9*) or reintegration of DNA that is deleted in the initial joining step (*8*) have not been similarly supported.

Note added in proof: It has recently been shown that rearrangements in endogenous K genes have properties that imply an inversional joining mechanism (*52*).

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RESEARCH ARTICLE

Function and Autoregulation of Yeast Copperthionein

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Heavy metals play a dual role in biology. Ions such as copper and zinc are essential trace nutrients for all life forms because of their participation in oxidation, electron transfer, and various enzymatic reactions. In contrast, inappropriately high concentrations of the same ions, or of elements such as cadmium and mercury, act as potent inhibitors of cell growth and development. How do living organisms acquire sufficient heavy metal ions to survive yet protect themselves against metal poisoning?

The metallothioneins, and metallothionein-like proteins, are thought to play an important role in this homeostatic process. These small, cysteine-rich polypeptides chelate heavy metal ions through thiolate complexes and are present in many kinds of organisms including vertebrates, invertebrates, plants, fungi, and even prokaryotes. The amino acid sequences of the metallothioneins from higher eukaryotes have been strongly conserved during evolution, while those from lower forms show less homology and may represent the products of either convergent or divergent evolution. In vertebrates, metallothioneins are expressed in various tissues and cell types, the greatest accumulation occurring in the liver and kidney. Metallothionein synthesis is inducible, at the transcriptional level, by the same heavy metal ions to which the proteins bind. The metal content of a metallothionein thus depends upon the species, heavy metal exposure, and cell type from which it is isolated (*1*).

The precise role of metallothioneins in heavy metal metabolism has been debated ever since their discovery more than a quarter of a century ago. It is clear that they can protect cells against heavy metal poisoning since cells that overproduce metallothionein, as a result of gene amplification, are unusually resistant to heavy metal poisoning, whereas cells that synthesize low levels of these proteins are unusually sensitive (*2*). The role of metallothioneins in normal metal metabolism is less certain. It has been suggested that they may participate in heavy metal storage, transport, or the activation of metalloenzymes. However, the evidence rests largely on experiments in which metallothionein synthesis is altered by nonspecific treatments, such as food restriction or injection with actinomycin D, that could affect metal metabolism by other routes. It has also been speculated that metallothioneins might participate in sulfur or nucleotide metabolism, control of the intracellular redox potential, amino acid transport, cellular differentiation, or the regulation of their own expression (*1, 3*). None of these possibilities has been experimentally tested.

One direct way to determine the actual function or functions of metallothionein is to study the behavior of organisms that

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Abstract. *The* CUP1 *gene of yeast encodes a small, metallothionein-like protein that binds to and is inducible by copper. A gene replacement experiment shows that this protein protects cells against copper poisoning but is dispensable for normal cellular growth and development throughout the yeast life cycle. The transcription of* CUP1 *is negatively autoregulated. This feedback mechanism, which is mediated through upstream control sequences, may play an important role in heavy metal homeostasis.*

have been genetically manipulated so that they are incapable of metallothionein synthesis. The yeast *Saccharomyces cerevisiae* is ideal for such experiments because its genome can be altered through DNA transformation and homologous recombination (*4*). The *CUP1* gene of this organism encodes a small, metallothionein-like protein that binds to and is inducible by copper (*5, 6*). By constructing strains that lack the *CUP1* gene, we have been able to test directly the involvement of this protein in heavy metal detoxification, normal cell growth, and the regulation of its own expression.

The CUP1 *locus*. This locus, discovered in 1955, is located on chromosome VIII of yeast, 42 centimorgans (cM) distal to the centromere (*7*). The nucleotide sequence of the cloned structural gene shows that it encodes a protein similar to other metallothioneins in its short length (61 amino acids), high cysteine content (20 percent), and the presence of a strongly conserved sequence of six amino acids. However, the primary structure is substantially different from that of vertebrate metallothioneins, and the protein is unusual in that it contains two aromatic amino acid residues and binds only to copper in vivo (*5, 6*). The *CUP1* protein has been called either copper chelatin, copper metallothionein, or copperthionein (*8*); we prefer the latter term since the protein belongs to the thionein superfamily of proteins, as defined in (*1*), yet is clearly distinct from vertebrate metallothioneins.

Most laboratory strains of yeast contain multiple copies of the *CUP1* locus, are resistant to 0.3 m*M* $CuSO_4$ in synthetic complete medium, and are designated $CUP1^R$. The copperthionein structural gene in such strains is present on a 2-kilobase (kb) segment of DNA that is tandemly reiterated ten or more times. The reiteration unit also contains a transcribed open reading frame, located upstream of the copperthionein coding sequences, that is referred to as "gene X" since its function is unknown. Other yeast strains contain a single copy of the *CUP1* locus, are sensitive to 0.3 m*M* $CuSO_4$, and are designated $cup1^S$ (*5*). To facilitate the gene replacement experiments described below, we cloned a 5.2-kb fragment of genomic $cup1^S$ DNA that includes the complete reiteration unit together with 0.8 kb of single-copy 5′ flanking DNA and 2.4 kb of single-copy 3′ flanking DNA. This was accomplished by screening an enriched plasmid library with a previously isolated $CUP1^R$ DNA probe (*6*). The resulting cloned fragment had a restriction map identical to that of the genomic locus as determined by Southern blotting (*9*).

CUP1 *is a nonessential gene*. To study the physiological role of copperthionein, we constructed yeast strains lacking the endogenous copperthionein gene. This was accomplished by the one-step gene replacement method in which homologous recombination leads to the substitution of a cloned, manipulated gene for its chromosomal counterpart (*4*). We constructed a plasmid in which the complete *CUP1* gene, together with approximately 350 base pairs (bp) of 3′ flanking sequences, is replaced with the yeast *URA3* gene (Fig. 1, left). This plasmid was digested with Kpn I, a restriction enzyme with sites in the single-copy 5′ and 3′ flanking sequences. The resulting linear molecules, containing termini available for recombination, were introduced into haploid $cup1^S$ and diploid $cup1^S/cup1^S$ strains carrying the *ura3–50* mutation. Transformants were readily isolated as uracil prototrophs in both types of recipients.

The structure of the *CUP1* locus in the transformants was examined by a Southern blot analysis (*9*) of Eco RI–digested chromosomal DNA. The parental strain gave a 5.2-kb band that encompasses the locus (Fig. 1, right). This band was missing in the haploid transformant, verifying that a gene replacement event had occurred. In its place were two smaller bands, of 3.5 and 2.3 kb, as was expected because the inserted *URA3* gene introduces a new Eco RI site into the locus. The diploid transformant gave a mixture of the 5.2-, 3.5-, and 2.3-kb bands as anticipated for a heterozygote containing one normal and one replaced gene. In an amplified strain, the *CUP1* gene migrat-

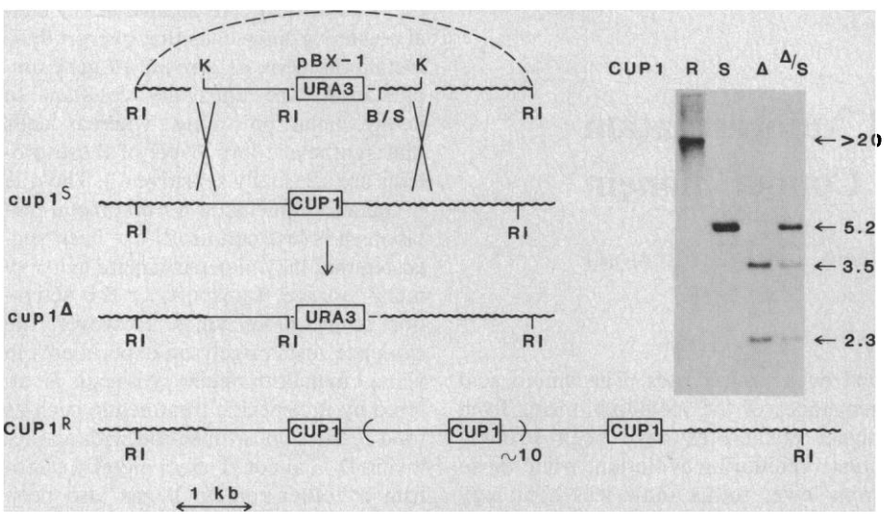


Fig. 1. Replacement of the *CUP1* locus. (Left) Structures of the replacement plasmid pBX-1 and of the chromosomal single-copy locus $cup1^S$, the replaced and deleted locus $cup1^\Delta$, and the amplified locus $CUP1^R$. Dashed lines represent plasmid DNA, wavy lines represent single-copy flanking DNA, and straight lines represent reiterated DNA. Restriction endonuclease sites are indicated as RI, Eco RI; K. Kpn I; B, Bam HI; and S, Sau 3A. After the $cup1^S$ locus was cloned and the plasmid pBX-1 was constructed (*18*), the plasmid was cleaved with Kpn I. The cleaved plasmid was used to transform the haploid yeast strain 19.10B (*MATα trp1-1 gall met13 can1* $cup1^S$ *ura3-50* Ade^- His^-), and the diploid strain XY9481305B (*MATa/MATα met13/met13 can1/CAN1 his2/HIS2 THR1/thr1* $cup1^S$ $cup1^S$*ura3-50/ura3-50* Ade^-/Ade^-). The cells were plated on synthetic complete agar lacking uracil to select for *URA3* transformants (*11*). (Right) A Southern blot analysis of total DNA from strains of the indicated *CUP1* genotype. The DNA was extracted, cleaved with Eco RI, subjected to electrophoresis through a 1 percent agarose gel, transferred to nitrocellulose, and hybridized to the 5.2-kb Eco RI $cup1^S$ fragment labeled with ^{32}P by nick translation (*9*). Each lane contained 5 μg of DNA with the exception of the $CUP1^R$ lane, which contained 1 μg. The arrows to the right indicate the sizes of the bands in kilobases as determined by comparison to a standard lane of phage λ DNA digested with Hind III. Strains used were 19.3C (*MATa trp1-1 gall met13 can1* $CUP1^R$ *ura3-50* Ade^- His^-), 19.10B ($cup1^S$), 51.2-2 ($cup1^\Delta$), which is the same as 19.10B transformed with pBX-1, and 51.5-1 ($cup1^\Delta/cup1^S$), which is XY9481305B transformed with pBX-1.

ed as a high molecular weight band because there are no Eco RI sites in the reiteration unit. These results were confirmed by cleavage with Bam HI and Hind III (data not shown). We use the genotype *cup1*$^{\Delta}$ to designate the deleted and replaced allele of the locus.

To verify inactivation of the *CUP1* gene, we analyzed thionein synthesis in *cup1*$^{\Delta}$, *cup1*^{S}, and *CUP1*^{R} strains grown in the presence or absence of various copper concentrations. The soluble proteins, labeled with [^{35}S]cysteine, were carboxymethylated and analyzed on a native gel system that resolves low molecular weight proteins (*10*). The parental strain produced readily detectable quantities of a small, cysteine-containing polypeptide in response to copper concentrations between 5 and 125 μM (Fig. 2). This protein, which was identified as thionein by its overproduction in an amplified strain, was not detected in the replacement strain at any copper concentration tested. Apart from the presence or absence of thionein, no other obvious differences were noted in the proteins of the parental and replacement strains.

The effects of eliminating copperthionein synthesis on cell growth and copper sensitivity are shown in Fig. 3. The *cup1*$^{\Delta}$ replacement strain showed normal growth on standard yeast synthetic agar, which contains approximately 0.1 μM copper, but was hypersensitive to added $CuSO_4$ in the medium; it showed no growth at 75 μM $CuSO_4$ whereas the *cup1*^{S} parent grew normally at this concentration and moderately well at 125 μM. A *cup1*$^{\Delta}$/*cup1*^{S} heterozygote showed intermediate behavior, as was expected for a semidominant mutation, whereas an amplified *CUP1*^{R} strain was resistant to high concentrations of copper. In liquid medium, the *cup1*$^{\Delta}$ replacement strain had the same doubling time as the *cup1*^{S} parent in minimal dextrose (SD), rich dextrose (YPD), and rich glycerol (YPG) broths (*11*). We conclude that copperthionein protects cells against copper toxicity, but is not essential for normal haploid mitotic growth, at least under laboratory conditions.

As a first step in analyzing the role of copperthionein in copper metabolism, we measured soluble (cystolic) and total copper levels in cells grown on standard medium. The soluble copper values obtained (in nanograms per milligram of soluble protein) were 12 ± 2 in the *cup1*^{S} parental strain and 9 ± 2 in the *cup1*$^{\Delta}$ replacement strain. The total cellular copper was 20 ng per milligram of total cell protein for both strains. We also measured the activity of the copper-zinc

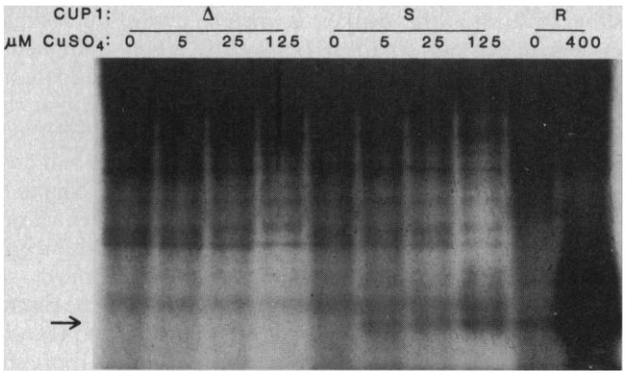


Fig. 2. Thionein synthesis. Strains 19.3C (*CUP1*^{R}), 19.10B (*cup1*^{S}), and 51.2-2 (*cup1*$^{\Delta}$) were grown in synthetic dextrose medium [SD supplemented with methionine, adenine, histidine, uracil, and tryptophan (*11*)] to mid-log phase (A_{650} = 1,10^7 cells per milliliter), treated with the indicated concentration of $CuSO_4$ for 1 hour, then labeled with [^{35}S]cysteine at 250 μCi/ml for 30 minutes. The soluble proteins were extracted, carboxymethylated, and analyzed on a 20 percent acrylamide gel (*6*, *10*). The arrow points to the position of the major species of thionein.

form of superoxide dismutase and obtained values (in units per milligram of soluble protein) of 75 ± 5 for the parental *cup1*^{S} strain and 77 ± 5 for the *cup1*$^{\Delta}$ strain (*12*). We conclude that copperthionein is not required for copper accumulation or activation of a specific copper enzyme in yeast grown on standard laboratory medium.

Genetic analysis. To determine whether replacement of the copperthionein gene has any effect on the yeast life cycle, we performed tetrad analyses and mating experiments. Four independent transformed diploids, shown as above to be heterozygous for *cup1*^{S}/*cup1*$^{\Delta}$, were sporulated, dissected to separate the four meiotic products, and germinated. Viable progeny were analyzed for segregation of *CUP1* (inferred by copper sen-

Table 1. Genetic analysis. Diploids were sporulated, dissected, germinated, and analyzed by standard methods (*11*). Plasmid pBX-1′ is identical to pBX-1 except that it lacks the 350 bp of 3′ flanking sequences between the Sau 3A and Kpn I sites. Plasmid pLUC is a *URA3* plasmid lacking *CUP1* flanking sequences. Strain XY958 was obtained by mating two *cup1* Δ spores derived from 51.5–1.

Experiment	Strains	Integrated plasmid	Viable spores per tetrad					Meiotic segregation	
			CUP1	4	3	2	1	Gene pair	Linkage (cM)
1	51.5–1 →	pBX-1	S/Δ	41	3	3	1	*cup1*$^{\Delta}$*URA3*$^{+}$	0*
	51.5–4						1	*cup1*$^{\Delta}$*THR1*	23†
2	XY958	pBX-1	Δ/Δ	14	2	0	0		
3	30.5–1,	pBX-1′	S/Δ′	0	2	46	2	*cup1*^{S}*ura3*	0‡
	30.5–2						1	*cup1*^{S}*THR1*	23§
4	18.12–1	pLUC	S/S	30	1	2	0	*URA3*$^{+}$*CAN1*$^{+}$	39‖
5	XY9481305B	none	S/S	13	2	0	0		

*All 41 complete tetrads contained two *cup1*$^{\Delta}$*URA3*$^{+}$ spores and two *cup1*^{S}*ura3* spores. †*cup1*$^{\Delta}$ was linked to *THR*$^{+}$ in 51.5–2 and 51.5–3 and to *thr1* in 51.5–1 and 51.5–4. Pooled tetrad ratios from 39 complete asci were PD:NPD:T = 21:0:18. Map distance (*Xp*, in cM) was estimated according to Perkins (*19*). ‡All viable spores were *ura3* auxotrophs. §*cup1*^{S} was linked to *THR1*$^{+}$ in both 30.5–1 and 30.5–2 since pooled dyad ratios were PD (2 *THR1*$^{+}$):NPD (2 *thr1*): T (1 *THR1* + 1 *thr1*) = 30:1:15. Three additional transformants exhibited *cup1*^{S} linked to *THR1*$^{+}$ while four others exhibited linkage to *thr1*. ‖*URA3*$^{+}$ was linked to *CAN1*$^{+}$ in both 18.1–1 and 18.1–2. Pooled tetrad ratios from 27 complete asci were PD:NPD:T = 11:1:15.

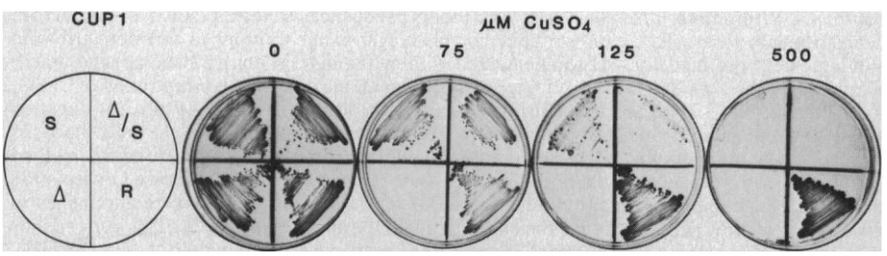


Fig. 3. Effect of the *CUP1* gene replacement on copper sensitivity. Strains 19.3C (*CUP1*^{R}), 19.10B (*cup1*^{S}), 51.2-2 (*cup1*$^{\Delta}$), and 51.5-1 (*cup1*$^{\Delta}$/*cup1*^{S}) were streaked on synthetic complete agar (*11*) containing the indicated concentration of $CuSO_4$ and grown at 30°C for 3 days. The medium without added $CuSO_4$ contains approximately 0.1 μM total copper as determined by atomic absorption spectrophotometry (*12*).

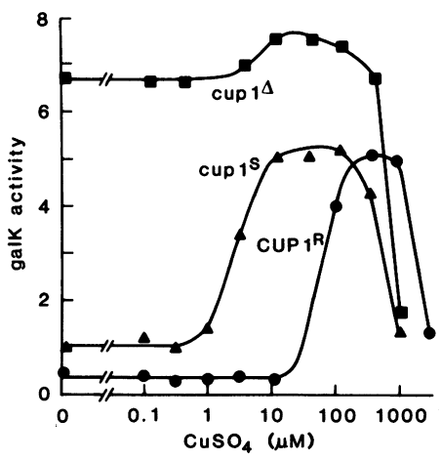
sitivity), *URA3* (the marker in the replacement plasmid), and *THR1* (linked to *CUP1* on chromosome VIII). These diploids gave complete cosegregation of $cup1^{\Delta}$ and $URA3^{+}$, a genetic linkage of approximately 23 cM between the replaced $cup1^{\Delta}$::$URA3^{+}$ locus and *THR1* (Table 1, experiment 1), and the same normal spore viability as the untransformed parental strain (Table 1, experiment 5). This confirms that *URA3* integrated at *CUP1* and that this event was not lethal. We next mated two $cup1^{\Delta}$ spores and observed normal zygote formation. Furthermore, the resulting $cup1^{\Delta}$/$cup1^{\Delta}$ homozygous diploid showed no obvious defects in vegetative growth, sporulation, or spore viability (Table 1, experiment 2). Thus, cells lacking the copperthionein gene can participate in all phases of the yeast life cycle.

We also constructed a plasmid that removes an additional 350 bp of the 3′ flanking DNA downstream of *CUP1*. This plasmid efficiently transformed a $cup1^{S}$/$cup1^{S}$ diploid but not a $cup1^{S}$ haploid. Sporulation of the transformed diploids revealed 2:2 segregation of a lethal mutation with complete correspondence between viability and *ura3* and an approximately 23 cM linkage to *THR1* (Table 1, experiment 3). This lethal mutation was not complemented by overproduction of thionein from an episomal *CUP1* plasmid (data not shown). These results indicate that there is an essential function encoded downstream of *CUP1*. The identity and possible involvement of this gene in copper metabolism are unknown, and there are no known lethal markers at this position on the yeast genetic map. A transformed $cup1^{S}$/$cup1^{S}$ diploid in which the plasmid integrated at *ura3–50*, linked to *CAN1* on chromosome V, gave normal spore survival and no segregation of copper hypersensitivity (Table 1, experiment 4). Thus, the presence of the *URA3* marker per se does not affect viability or copper sensitivity.

Autoregulation. The synthesis of copperthionein is inducible by copper. Previous experiments have shown that is due, at least in part, to an increase in the rate of transcription mediated by *cis*-dominant control sequences upstream of the coding region (*6*), but the *trans*-acting cellular factors responsible for this type of regulation have not been identified. To test the possibility that *CUP1* itself might be involved, we compared transcription from an episomal *CUP1* promoter in cells lacking or retaining an intact chromosomal gene. For this purpose, we used a *CUP1-galK* fusion gene that contains 391 bp of 5′ flanking sequences and 36 bp of 5′ untranslated sequences (but no coding sequences) from the copperthionein gene, the coding sequences of the *Escherichia coli* galactokinase (*galK*) gene, and 3′ sequences from the yeast iso-1-cytochrome c (*CYC1*) gene to provide RNA termination and processing signals. The fusion gene is carried on an *E. coli*–yeast shuttle vector that contains the yeast *TRP1* gene, as a selectable marker, together with *ARS1* and *CEN3* sequences to promote stable, low copy number replication (*6*, *13*). This plasmid was transformed into $CUP1^{R}$, $cup1^{S}$, and $cup1^{\Delta}$ strains carrying the *trp1-1* mutation, to allow selection, and the *gal1* mutation to inactivate the endogenous yeast galactokinase gene. Transformants were grown to mid-log phase, treated or not treated with various concentrations of $CuSO_4$ for 1 hour, then assayed for *E. coli* galactokinase to evaluate transcription from the copperthionein gene promoter.

The $CUP1^{R}$ strain, containing multiple copies of the chromosomal structural gene, gave a low but readily detectable basal level of galactokinase expression at 0 to 10 μM added $CuSO_4$ (Fig. 4). At 100 to 1000 μM $CuSO_4$, expression was induced by a factor of 10 (maximum), and at 3000 μM $CuSO_4$ expression declined as a result of a general inhibition of RNA and protein synthesis. The $cup1^{S}$ strain, carrying a single copy of the structural gene, gave a qualitatively similar curve with two quantitative differences. First, optimal induction occurred at a tenfold lower copper concentration, and second, the basal level of activity was two times higher, thus giving a maximum induction ratio of 5.5 as compared to 10. A strikingly different result was obtained in the $cup1^{\Delta}$ strain in which the endogenous structural gene for copperthionein has been deleted. Even in the absence of added $CuSO_4$, there was a high level of *galK* expession, in fact, higher than the optimally induced levels in the $cup1^{S}$ or $CUP1^{R}$ strains. When $CuSO_4$ was present at 3 μM to 100 μM, there was a slight increase in activity, but the maximum induction ratio was only 1.2. The increased basal expression of the *CUP1-galK* fusion gene in a $cup1^{\Delta}$ strain could also be visualized by streaking the cells on a bromthymol blue galactose indicator plate (see cover). Thus deletion of the endogenous copperthionein gene leads to a high, constitutive level of expression of the episomal *CUP1-galK* fusion gene.

To determine whether this was due to a change in messenger RNA (mRNA) synthesis, we performed a Northern blot analysis (*14*) on RNA from the $cup1^{S}$ and



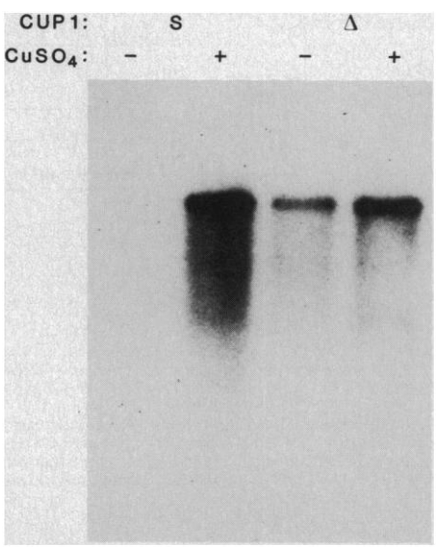


Fig. 4 (left). Expression of a *CUP1-galK* fusion gene. Strains 19.3C ($CUP1^{R}$), 19.10B ($cup1^{S}$), and 51.2–2 ($cup1^{\Delta}$) were transformed with a *TRP1*, *ARS1*, *CEN3* plasmid containing the *CUP1* promoter fused to *E. coli galK* coding sequences [referred to as pYSK12 in (*6*)]. Transformants were selected on synthetic complete agar minus tryptophan and were grown in synthetic dextrose medium (SD supplemented with methionine, adenine, histidine, and uracil) to mid-log phase (*11*). Samples (1 ml) were incubated with the indicated concentration of $CuSO_4$ for 1 hour at 30°C, collected by centrifugation, resuspended in 50 μl of 40 percent dimethyl sulfoxide, and agitated at room temperature for 30 minutes to permeabilize the cells. Portions were directly assayed for *Escherichia coli* galactokinase activity by a standard method (incubation with [^{14}C]galactose and excess adenosine triphosphate (ATP) followed by isolation of the [^{14}C]galactose 1-phosphate product on DE81 filter disks (*6*). Results were normalized to give a relative *galK* activity of 1 for the $cup1^{S}$ strain in the absence of $CuSO_4$. This corresponds to 2.2 pmol of galactose phosphorylated per 5 μl of extract per 30 minutes. Fig. 5 (right). Analysis of fusion mRNA. Strains 19.10B ($cup1^{S}$) and 51.2-2 ($cup1^{\Delta}$) transformed with the *CUP1-galK* fusion plasmid were grown in the presence or absence of 100 μM $CuSO_4$ for 1 hour. Polyadenylated RNA was extracted, subjected to electrophoresis through a formaldehyde-agarose gel, transferred to nitrocellulose paper, and hybridized to the 1.2-kb Eco RI *galK* fragment labeled with ^{32}P by nick translation (*14*).

cup1$^{\Delta}$ strains grown in the presence or absence of copper. The *cup1*^{S} transformant gave a low level of *CUP1-galK* fusion mRNA in the absence of added copper (the band could only be seen in a longer exposure of the autoradiogram) (Fig. 5), and this was strongly induced by 100 μM $CuSO_4$. In contrast, the *cup1*$^{\Delta}$ strain cultured in the absence of added copper contained a readily detectable basal level of *CUP1-galK* mRNA (at least ten times higher than in the *cup1*^{S} parent), and this was only slightly induced by added $CuSO_4$. While the induction ratios at the RNA level were substantially higher than those from enzyme levels, possibly due to differences in the stability of fusion mRNA and galactokinase or to some form of posttranscriptional regulation, the increased basal expression in the *cup1*$^{\Delta}$ strain is clear. In addition, nuclease S1 mapping showed that the *CUP1-galK* fusion RNA had the expected 5′ end and that this was not affected by the status of the chromosomal locus (data not shown). We conclude that the high basal level of *galK* expression in the *cup1*$^{\Delta}$ replacement strain is due to an increase in mRNA production.

To provide further controls for this experiment, and to begin defining the *cis*-acting control sequences that respond to *CUP1*, we compared the expression of three additional *galK* fusion plasmids. A fusion to the promoter of the yeast *CYC1* gene, which is not regulated by copper, showed no induction in the presence of 100 μM $CuSO_4$ (Fig. 6); in fact, there was a decrease in activity, presumably due to the general decline in RNA and protein synthesis at this copper concentration. More important, the *CYC1* fusion behaved identically in the *cup1*^{S} and *cup1*$^{\Delta}$ hosts. We next studied a hybrid promoter that contains *CYC1* sequences between positions −178 and +83 preceded by *CUP1* sequences between positions −391 and −125. (Sequences are consecutively numbered from the most 5′ transcription initiation site, negative numbers for upstream sequences and positive numbers for downstream sequences.) The *CYC1* portion of this construct retains the conserved "TATA" sequence (T, thymine; A, adenine) and the transcription initiation sites but lacks the upstream activator sequences responsible for regulation of this gene (*15*). The *CUP1* portion of the construct contains a copper responsive regulatory region as defined by deletion mapping studies (*16*). This hybrid promoter was inducible by copper in the *cup1*^{S} parent strain but in the *cup1*$^{\Delta}$ replacement strain gave a high level of basal transcription that was only slightly elevated by copper. The abnormal expression of this construct, which contains *CUP1* control sequences but no transcribed mRNA sequences, demonstrates that autoregulation acts at the transcriptional level. Finally, we analyzed a deletion mutant of the *CUP1* promoter that lacks all of the copper regulatory sequences and the most distal "TATA" sequence. This mutant was transcribed at a low level, was not inducible by copper, and behaved identically in both the *cup1*^{S} and *cup1*$^{\Delta}$ backgrounds. Taken together, these results show that the high basal expression of the *CUP1-galK* fusion gene in the absence of an intact chromosomal *CUP1* locus is due to a specific effect on *CUP1* gene transcription, driven by its upstream regulatory sequences, and not an artifact of the fusion gene assay.

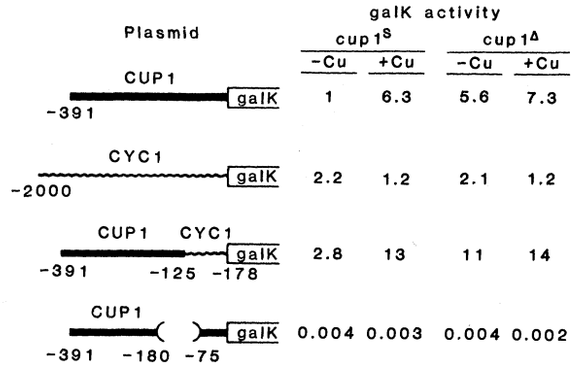


Fig. 6. Autoregulation is controlled through upstream regulatory sequences. Strains 19.10B (*cup1*^{S}) and 51.2-2 (*cup1*$^{\Delta}$) were transformed with *TRP1*, *ARS1*, *CEN3* plasmids carrying the indicated fusion genes. Galactokinase levels were measured in cells grown in the presence or absence of 100 μM $CuSO_4$ as described in Fig. 4. The construction of the *CUP1* (*6*) and *CYC1* (*13*) fusions has been described. The hybrid and deleted promoters were constructed by standard methods involving Bal 31 digestions and recombination between common linker sites (*16, 18*). The numbers under the diagrams to the left refer to nucleotide positions relative to the most 5′ transcription initiation site at +1.

Function of copperthionein. We have constructed yeast strains that lack the single gene for copperthionein, *CUP1*, as a means of testing the function of this metallothionein-like protein. Such strains are hypersensitive to copper poisoning, thus confirming the role of copperthionein in metal detoxification (*5*). However, they grow normally on standard yeast medium and are capable of participating in all phases of the yeast life cycle including haploid and diploid vegetative growth, mating, zygote formation, sporulation, and germination. We conclude that copperthionein does not play an essential physiological role in the normal growth or development of yeast cells. An alternative explanation of our results, that yeast contains a second copperthionein gene, seems less likely since the *CUP1* gene product is the only small copper binding protein that has been identified in biochemical fractionation experiments (*8*). Moreover, *CUP1* is the only gene revealed in Southern blot analyses of yeast genomic DNA hybridized to either the yeast *CUP1* probe (Fig. 1) or to mammalian metallothionein gene probes (*6, 16*). Thus, if yeast does contain a second copperthionein gene, it must be distinctly different from *CUP1*.

Cells lacking the copperthionein gene accumulate normal amounts of total copper and of a copper-dependent enzyme, superoxide dismutase. They also must contain active cytochrome oxidase, a copper enzyme required for respiration, since they can grow on nonfermentable carbon sources such as glycerol. These results indicate that copperthionein is not required for the normal metabolism of copper at the approximately 0.1 μM concentration found in standard yeast medium. We assume that other cellular factors are responsible for copper transport, storage, and enzyme activation under these conditions. We have isolated a series of mutants, unlinked to *CUP1*, that are either hypersensitive or partially dependent on added copper in the medium (*16*). These mutants may be useful in identifying and isolating such factors.

Given the dispensability of copperthionein for normal cell growth in standard media, it is interesting that many laboratory and industrial fermentation strains of yeast contain amplified copies of the *CUP1* locus (*5*). One possible explanation is that copperthionein confers a growth advantage in nature that is not detected in the laboratory. Alternatively, exposure to high copper concentrations may occur in the natural habitat of yeast, perhaps due to release from decomposing fruit. Finally, it should be noted that brewers' and wine-making yeasts are frequently exposed to copper due to the use of copper fermentation vats and bordeaux mixture.

Gene replacement experiments of the sort described here are not yet feasible in higher eukaryotes. However, certain

lines of cultured mouse and hamster cells contain hypermethylated metallothionein genes and produce little if any metallothionein in the absence of metal stimulation (*2*). These lines are hypersensitive to heavy metal poisoning but otherwise grow normally and, presumably, contain normal amounts of copper and zinc metalloenzymes. This suggests that mammalian metallothioneins, like yeast copperthionein, are involved in heavy metal detoxification but not in normal cellular metal metabolism. The role of metallothionein in the whole organism—for example, in the storage and release of metals from the kidney and liver—remains to be tested.

Autoregulation and copper homeostasis. The one normal physiological function for *CUP1* is in the regulation of its own expression. In cells lacking an intact chromosomal locus, an episomal *CUP1* fusion gene is expressed at a high level in the absence of added copper. This is due to increased transcription since it is accompanied by an increase in fusion mRNA and was observed when we used a construct that retains no transcribed *CUP1* mRNA sequences. Moreover, the *cis*-acting control sequences responsible for this effect lie in the same general region of the upstream flanking DNA as the copper-responsive regulatory elements (*16*). Noninduced transcription is also slightly higher in cells carrying a single copy of the chromosomal *CUP1* locus than in cells carrying multiple copies. Hence the degree of autoregulation appears to depend on the *CUP1* gene dosage. It is likely that the *trans*-acting negative factor is thionein or copperthionein itself, but it is also formally possible that *CUP1* mRNA or some product encoded immediately downstream of *CUP1* is involved.

The mechanism of this negative autoregulatory system is not yet known. One possibility is that thionein binds directly to the control DNA sequences and represses transcription in the absence of added copper. Deletion analysis of the *CUP1* upstream region has revealed the presence of negatively acting control elements (*16*), but the cellular factors that interact with these sequences have not yet been identified. A second possibility is that thionein, in the absence of added copper, binds to and inactivates a protein that can positively modulate *CUP1* gene transcription. Such complex regulatory circuits are well known in yeast (*17*). Finally, autoregulation may reflect the role of copperthionein in copper metabolism. Yeast cells must contain some intracellular copper in order to survive, and a portion of this is probably chelated by thionein in a normal cell. Hence the elimination of thionein synthesis might simply cause an increase in the concentration of free intracellular copper that is available for inducing transcription from the *CUP1* promoter. We are trying to distinguish between these possibilities by studying the properties of purified copperthionein and by isolating *CUP1* mutants that affect copper binding or autoregulation.

Regardless of the precise mechanism, autoregulation may play an important role in copper homeostasis in yeast. Cells grown on low copper medium need to accumulate sufficient nonchelated intracellular copper to activate copper enzymes, whereas cells exposed to high copper need to protect themselves against metal poisoning. To accomplish this, it would seem desirable for the cell to induce copperthionein synthesis only when the concentration of intracellular copper is close to or exceeds the binding capacity of the available thionein. Having copperthionein itself act as a regulatory factor assures a relation, direct or indirect, between the copper binding curve and the induction curve. Moreover, it provides a simple mechanism for turning the gene off once the excess copper has been chelated. We also note that autoregulation is a particularly economical form of regulation for genes that are frequently amplified, such as *CUP1*, since it ensures parallel overproduction of the regulatory factor.

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18. Recombinant DNA experiments were performed by standard procedures [T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1982)]. Total DNA from yeast strain 19.10B *cup1*^{S} was digested with Eco RI and subjected to electrophoresis through a 1 percent agarose gel. Fragments in the 5.0- to 5.4-kb size range were recovered and ligated to plasmid pUC8 cleaved with Eco RI. The reaction mixture was transformed into *E. coli* strain MC1061, and recombinant colonies were screened by colony hybridization to a *CUP1*^{R} probe (*6*) labeled with ^{32}P by nick translation. Two out of 600 colonies screened contained the *cup1*^{S} gene. The replacement plasmid pBX-1 was derived from the pUC8-*cup1*^{S} recombinant in three steps. The upstream *cup1*^{S} sequences extend from an Eco RI site in the 5′ flanking DNA to a Rsa I site in the 5′ untranslated region (28 bp upstream of the ATG translation start codon). The Rsa I site was joined to an Eco RI site (*6*). The downstream sequences extend from a Sau 3A site, approximately 50 bp 3′ to the reiteration unit, to an Eco RI site in the single copy 3′ flanking DNA. The *URA3* gene is derived from YIP5 (*4*) and is inserted into the plasmid by means of 32 bp of plasmid pBR322 DNA (from Eco RI to Hind III) upstream of *URA3* and 346 bp of pBR322 (from Hind III to Bam HI) downstream of *URA3*.
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