A genetic approach was taken to test the function of yeast metallothionein in metal ion detoxification. A yeast strain was constructed in which the metallothionein locus was deleted (cup1 Δ). The cup1 Δ strain was complemented with normal or mutant metallothionein genes under normal or constitutive regulatory control on high copy episomal plasmids. Metal resistance of the cup1 Δ strain with and without the metallothionein-expressing vectors was analyzed. The normally regulated metallothionein gene conferred resistance only to copper (1000-fold); constitutively expressed metallothionein conferred resistance to both copper (500-fold) and cadmium (1000-fold), but not to mercury, zinc, silver, cobalt, nickel, gold, platinum, lanthanum, uranium, or tin. Two mutant versions of the metallothionein gene were constructed and tested for their ability to confer metal resistance in the cup1 Δ background. The first had a deletion of a highly conserved amino acid sequence (Lys-Lys-Ser-Cys-Cys-Ser). The second was a hybrid gene consisting of the sequences coding for the first 20 amino acids of the yeast protein fused to the monkey metallothionein gene. Expression of these genes under the CUP1 promoter provided significant protection from copper, but none of the other metals tested. These results demonstrate that there is significant flexibility in the structural requirements for metallothionein to function in copper detoxification and that yeast metallothionein is also capable of detoxifying cadmium under conditions of constitutive expression.

Metallothioneins are low molecular weight cysteine-rich metalloproteins that are ubiquitous in eukaryotes. A high degree of sequence homology is evident in metallothioneins from a variety of mammalian sources, and to a lesser extent, from such diverse organisms as crab, Drosophila, sea urchin, and yeast (1). The function of metallothionein has been the subject of much speculation and experimentation. Metallothionein has been proposed to be involved in metal storage and detoxification, development, differentiation, control of cellular metabolism, protection from free radical toxicity, and in the UV response (reviewed in Ref. 2). The evidence that metallothionein is involved in these activities tends to be correlative, i.e. enhanced synthesis of mRNA or protein is observed during some phase of development or differentiation, or in response to a certain chemical or physical assault on the cells. Indeed, a vast variety of organic and inorganic reagents as well as several types of radiation have been shown to induce metallothionein synthesis in numerous experimental systems (3, 4). Thus, it is clear that in many cells, metallothionein synthesis is part of a pleiotropic response to acute stress, but these correlations do not necessarily prove that metallothionein has any protective or restorative function in response to a particular stimulus. The function proposed for metallothionein that has the most experimental evidence is protection against heavy metal ion toxicity. However, even in this case, it has been shown that true protection from heavy metal toxicity by mammalian metallothionein occurs for only a small subset (cadmium, copper, zinc, mercury, bismuth) of all the metals that are known to induce the gene (5).

In Saccharomyces cerevisiae, copper resistance is mediated by the CUP1 locus which encodes a small cysteine-rich copper-binding protein, yeast metallothionein (6, 7). Yeast metallothionein has little primary sequence homology with mammalian metallothionein except for a highly conserved region (Lys-Lys-Ser-Cys-Cys-Ser). We have taken a genetic approach to identify the metal detoxification function of yeast metallothionein. We constructed a yeast strain in which the chromosomal metallothionein gene is deleted. In this metallothionein-deleted genetic background, we introduced a series of high copy plasmids that contained normal or mutant metallothionein coding sequences under normal or constitutive regulatory control. This allowed us to directly compare metal resistance in cells that had no metallothionein with cells that had approximately 50 episomal copies of the normal or mutant gene, but were otherwise isogenic. Expression of yeast metallothionein coding sequences under the control of a constitutive promoter (TDH3) has allowed us to study protein function independent of gene induction and test metallothionein protection against metals that do not induce the gene.

MATERIALS AND METHODS

Reagents—Auranofin (1-thio-β-D-glucopyranose 2,3,4,6-tetrace- tato-S)(triethylphosphine)gold was obtained from the Department of Medicinal Chemistry, Smith Kline & French Laboratories; cis-platinum, EDTA, superoxide dismutase, xanthine, xanthine oxidase, bathocuproine disulfonic acid, estradiol were obtained from Sigma; CuSO₄, 5H₂O, CdCl₂, ZnCl₂, AgNO₃, La(NO₃)₃·6H₂O, SnCl₂, NiCl₂·6H₂O, Co(CH₃CO₂)₂·4H₂O, Hg(NO₃)₂, H₂O, HgSeO₄ were obtained in the highest quality available (Gold label) from Aldrich.

Yeast Strains and Culture Media—Saccharomyces cerevisiae strains AB17-11D, Mat a, arg4-8, leu2-3, his3-112, his7-2, trp1-289, ura3-52, ade6, cup1 Δ (Seymour Fogel) and the cup1 Δ strain (this laboratory) were used in all experiments. Cells were grown on synthetic complete liquid media (9) on plates containing 2% Phytagar (Gibco).

Plasmids, Transformation, and Gene Disruption—All genetic manipulations were performed by standard methods (9-11). A 5.2-kb1 EcoRI DNA fragment containing a single copy of the yeast metallothionein gene in pUC8 was linearized by cutting at a unique XhoI

1 The abbreviations used are: kb, kilobase(s); bp, base pair(s).

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were then phosphorylated. Buffer and incubated at 85 °C for 4 min, transferred to 65 °C, and allowed to cool to 4 °C over a 60-min period. One-half of the double-stranded DNA fragments were ligated overnight at 15 °C (11). The 5'-hydroxyl groups of the 115-bp DNA fragment A and fragment F were then phosphorylated. This synthetic DNA was then ligated with the transcription terminator sequences, which were originally subcloned as a Dral fragment into puc18 and removed as an SstI-Sphl fragment as shown in the upper right of Fig. 2. A BamHI-Alu 1 fragment which contained the yeast metallothionein promoter and coding sequences for the first 20 amino acids was isolated. These elements were ligated into PBR322 at the BamHI and Sphl sites to generate the plasmid YEP 37 (Fig. 1). The authenticity of fragment orientation and translation frame was confirmed by sequencing. The plasmid YEP 37 was digested with BglII, and the ends were labeled with [γ-32P]ATP. The labeled DNA was subsequently digested with HindIII and Sphl, and the two fragments released were sequenced (14). The HindIII-Sphl cassette was transferred to the HindIII-Sphl sites of the yeast vector YEp 13 to generate YEp 38.

A chimeric plasmid consisting of the yeast metallothionein promoter and a fusion of the yeast and monkey metallothionein structural genes was constructed. The plasmid YEP 37 was digested with BglII and Smal to remove two-thirds of the carboxyl terminus sequence of yeast metallothionein. The monkey MTI cDNA clone (13) was digested with PstI; the Pstl site was rendered flush with T4 DNA polymerase, followed by digestion with BglII. The fragment was then ligated to the BglII-Smal sites of YEP 37. The fusion gene was subsequently transferred to YEp 13 at the HindIII-Sphl restriction sites to generate YEp 40 (Fig. 2).

The construction of plasmid pCD189 containing the intact yeast metallothionein structural gene under regulatory control of the TDH3 promoter (Fig. 2) has been described. We have called this plasmid YEP 42 in the present study. The copy number of the YEP 13-based plasmids was approximately 50 copies/cell determined by LEU2 hybridization (20).

Metallothionein Gene Expression—Total RNA was prepared from 15 ml of cells treated with metals as described in the figure legends. Cells were washed twice with water and suspended in vanadium ribonucleoside complex (Bethesda Research Laboratories). Cells were lysed by vortexing in the presence of 1/4 volume of glass beads (0.5 mm, A. R. Thomas) for 30 s in 1.5-ml Microfuge tubes. The cycle was repeated 4 times, cooling the cells on ice between cycles. The lysed cell suspension was centrifuged for 15 min, the supernatant was extracted 3 times with phenol, and the RNA was precipitated with ethanol. 10 μg of total RNA was analyzed on 1.5% agarose-formaldehyde gels (6). Northern blots were probed with the 680-bp XhoI-KpnI fragment from the CUP1 locus (6).

Protein Expression—Cells were pulse labeled with 100 μCi of [35S] cysteine (1000 Ci/mmol) for 1 h at 30 °C. Total soluble proteins were prepared from 15 ml of cells treated with metals as described in the figure legends. Cells were washed twice with H2O and resuspended in 500 μl of 10 mM Tris, pH 7.6, 10 mM dithiothreitol, 1 mM phenyl-methylsulfonyl fluoride, 1 mM 1,10-phenanthroline-2-phenylthethyl chloromethyl ketone. Cells were lysed as described above for RNA isolation. 50,000 DPM of trichloroacetic acid-insoluble material was loaded per well on an 18% nondenaturing polyacrylamide gel as previously described (6), but with the addition of 50 mM thioglycolic acid in the upper chamber buffer.
_____ Metallothionein Gene Disruption and Replacement—We disrupted the metallothionein gene in the haploid strain AB17-11D, which has a single copy of the gene. Disruption of metallothionein in URA3 transformants was confirmed by the following criteria: 1) increased sensitivity to copper toxicity in the disrupted over the parent strain; the parent strain tolerated up to 25 \( \mu \text{M} \) CuSO\(_4\) while the disrupted strain tolerated only 1 \( \mu \text{M} \) CuSO\(_4\) (Table II); 2) lack of detection of metallothionein mRNA or protein in the disrupted strain (Figs. 3 and 4); 3) integration of the URA3 gene at the \( \text{CUP}1 \) locus accompanied by the expected mobility shift from 5.2 to 5.7 kb on a Southern blot of an EcoRI genomic digest (not shown); 4) lack of hybridization to a probe consisting of yeast metallothionein coding sequences; the probe hybridized to a prominent band at 5.2 kb on an EcoRI genomic blot of the parent strain (not shown).

Having constructed a yeast strain with no metallothionein gene (\( \text{cup}1^{+} \)), it was possible to analyze metal resistance and gene induction under conditions where the plasmid-encoded metallothionein served as the only source of metallothionein to these cells. The plasmids employed are described under "Materials and Methods," and their salient components are summarized in Table I. Plasmid YEp 36 contains the normal yeast metallothionein structural gene under the control of its own promoter, \( \text{CUP}1 \). Plasmid YEp 38 is the same as YEp 36 except for the mutations in the coding sequences. Plasmid YEp 40 has the first 20 amino acids of the yeast protein fused to the monkey metallothionein (I) structural gene under the control of the \( \text{CUP}1 \) regulatory sequences. Plasmid YEp 42 has the normal yeast metallothionein structural gene under the control of a constitutive promoter (\( \text{TDH}3 \)).

By transforming the deleted strain with high copy plasmids we were able to create strains with multiple copies of each gene. The copy number of the YEp 13-based plasmids was approximately 50 copies/cell. In the absence of exogenous metal there were no apparent differences among the various transformed strains in growth rate, colony size, or morphology.

Metallothionein and Metal Resistance—To test metallothionein protection against the toxic effects of various metals, we supplemented agar plates with a variety of metal ions, metallo-drugs, or other compounds and then plated yeast transformed with the various metallothionein-expressing vectors described in Table I. This directly compared metal resistance in yeast that had no metallothionein gene (\( \text{cup}1^{+} + \text{YEp} 13 \)) with yeast that had about 50 copies of the normal metallothionein gene (\( \text{cup}1^{+} + \text{YEp} 36 \)). The strain with the episomal plasmid YEp 38 was used to probe the function of the conserved amino acid sequence (Lys-Lys-Ser-Cys-Cys). The episomal plasmid YEp 40 was used to test in vivo the level of resistance bestowed by the yeast-mammalian metallothionein fusion. The plasmid YEp 42 allowed us to identify metallothionein protection from metals that do not induce synthesis of the gene. Finally, the parent strain transformed with the control plasmid (\( \text{cup}1^{+} + \text{YEp} 13 \)) allowed us to compare the resistance conferred by a single chromosomal copy of the gene versus the multicopy episomal versions.

Metal resistance was compared by plating the same number of cells of all six strains on different sections of the same plate (Table II). All of the metallothionein-expressing plasmids conferred dramatic copper resistance to the \( \text{cup}1^{+} \) strain. The difference in the maximally tolerated copper dose between the metallothionein-deleted strain with the control plasmid (\( \text{cup}1^{+} + \text{YEp} 13 \)) and the same strain carrying the intact yeast-metallothionein-expressing plasmid (\( \text{cup}1^{+} + \text{YEp} 36 \)) was approximately 1000-fold. The yeast-mammalian fusion protein (\( \text{cup}1^{+} + \text{YEp} 40 \)) and the mutant yeast protein (\( \text{cup}1^{+} + \text{YEp} 38 \)) each provided approximately 50% of the copper resistance as compared to the natural yeast protein (\( \text{cup}1^{+} + \text{YEp} 36 \)). (While this paper was being prepared a report appeared (17) describing the expression of monkey metallothioneins I and II in yeast; the results were identical to those described here.) The yeast \( \text{TDH}3 \) promoter was less effective than the \( \text{CUP}1 \) promoter in expression of the same gene product, as measured by copper protection (\( \text{cup}1^{+} + \text{YEp} 42 \)). It is evident from the levels of mRNA and protein expression (Figs. 3 and 4) and from the levels of copper protection (Table II) that these promoters cause high rates of transcription of both heterologous and homologous mRNA.
TABLE II

Resistance to various metal ions by yeast containing metallothionein-expressing plasmids (see Table I)

Yeast strains carrying the plasmid constructions were grown in a synthetic medium to \( A_{\text{opt}} = 1 \). Ten microliters of each culture was streaked in the appropriate sector, and plates were incubated for 2 days at 30 °C. Plates were scored as follows: ++, uninhibited growth (equal to the metal-free control plate); +, stressed growth (colonies were notably fewer in number and smaller in size than the control); --, no growth. A blank indicates that this concentration was not tested.

| Genome + plasmid | Concentration |
|------------------|---------------|
| \( \text{CuCl}_2 \) | 1 \( \mu \text{M} \) | 5 \( \mu \text{M} \) | 10 \( \mu \text{M} \) | 25 \( \mu \text{M} \) | 50 \( \mu \text{M} \) | 1 \( \text{mM} \) | 5 \( \text{mM} \) | 10 \( \text{mM} \) | 50 \( \text{mM} \) |
| Cup\(^1\alpha\) + YEpl3 | ++ | ++ | ++ | + | + | - | - | - | - |
| Cup\(^1\beta\) + YEpl3 | + | + | + | + | + | + | + | + | + |
| Cup\(^1\beta\) + YEpl36 | + | + | + | + | + | + | + | + | + |
| Cup\(^1\beta\) + YEpl38 | + | + | + | + | + | + | + | + | + |
| Cup\(^1\beta\) + YEpl40 | + | + | + | + | + | + | + | + | + |
| Cup\(^1\beta\) + YEpl42 | + | + | + | + | + | + | + | + | + |
| \( \text{CdCl}_2 \) | 5 \( \mu \text{M} \) CuSO\(_4\) | + | + | + | + | + | + | + | + |
| Cup\(^1\alpha\) + YEpl3 | + | + | + | + | + | - | - | - | - |
| Cup\(^1\beta\) + YEpl3 | + | + | + | + | + | + | + | + | + |
| Cup\(^1\beta\) + YEpl36 | + | + | + | + | + | + | + | + | + |
| Cup\(^1\beta\) + YEpl38 | + | + | + | + | + | + | + | + | + |
| Cup\(^1\beta\) + YEpl40 | + | + | + | + | + | + | + | + | + |
| Cup\(^1\beta\) + YEpl42 | + | + | + | + | + | + | + | + | + |
| \( \text{ZnCl}_2 \) | 10 \( \mu \text{M} \) Cu\(^2+\) | + | + | + | + | + | + | + | + |
| Cup\(^1\alpha\) + YEpl3 | + | + | + | + | + | - | - | - | - |
| Cup\(^1\alpha\) + YEpl36 | + | + | + | + | + | + | + | + | + |
| Cup\(^1\beta\) + YEpl38 | + | + | + | + | + | + | + | + | + |
| Cup\(^1\beta\) + YEpl40 | + | + | + | + | + | + | + | + | + |
| Cup\(^1\beta\) + YEpl42 | + | + | + | + | + | + | + | + | + |

Metallothionein mRNA Induction

No strain of yeast with constitutively expressed metallothionein, it was surprising that a small amount of cadmium protection was not observed in the strains that had episomal copies of the gene under normal regulatory control. The relatively high basal level of transcription from the \( \text{CUP1} \) promoter in the absence of copper (Fig. 3) might have provided some cadmium protection to these strains compared to the strain that had no metallothionein at all. Moreover, it was not possible to "prime" these strains with an inducing nontoxic dose of copper and observe protection from cadmium toxicity (Table II).

Within the range of concentrations tested in the assay, constitutively expressed metallothionein provided no protection from the toxicity of the following metals: zinc (Table II), cobalt, nickel, silver, mercury, gold (as auranofin), or platinum (as cis-(NH\(_2\))\(_2\)Cl, Pt), lanthanum, tin, or uranium (not shown). In the case of lead citrate, metallothionein-mediated resistance could not be measured because all the strains were resistant to all concentrations of lead, including those where metal precipitation occurred.

The activity of the anticancer drug, bleomycin, is considered to be mediated through DNA damage involving metal ions and oxygen chemistry (15). Also, \( m \)-AMSA has been shown to cause DNA damage via a similar mechanism involving copper (16). However, the metallothionein content of the strains had no apparent effect on the toxicity of these drugs (not shown). Finally, we tested resistance to growth inhibition due to metal chelation by EDTA and bathocuproine sulfonic acid and found no difference in the effect of either compound in the 6 strains (not shown).
Yeast Metallothionein

Regulation of Metallothionein Gene Expression—Metallothionein gene expression induced by metals and other compounds was studied by direct analysis of metallothionein mRNA on Northern blots. Metallothionein mRNA expression was observed in all plasmid-containing strains except the control with no metallothionein gene (cup1Δ YEp13) strain induced with 10 μM CuSO₄. During the second hour, 100 μCi of [35S] cysteine was added. Total protein extracts were prepared, and 50,000 dpm of acid-insoluble radioactivity was loaded in each well.

The metallothionein mRNA levels did not correlate with the protein levels in the cell. For example, metallothionein mRNA levels were approximately the same in the single copy (cup1Δ + YEpl3) strain induced with 10 μM CuSO₄ and in the high copy strain (cup1Δ + YEpl36) without added copper (Fig. 3). However, the protein gel showed only a trace of metallothionein in the latter strain, while in the copper-induced cup1Δ strain metallothionein accounted for about half of the total [35S]cysteine incorporation (Fig. 4). The strain with constitutively expressed CUP1 gene (cup1Δ + YEpl42) had less protein in the absence of metal (Fig. 4) even though the mRNA levels were the same with and without copper (Fig. 3). It is possible that the apoprotein (thionein) is less stable than the metalloprotein, and differences in the rate of protein turnover account for the different steady state protein levels.

Alternatively, the rate of translation may be influenced by the presence of metal because metals may bind to the protein during the process of translation. Although expression of the normal metallothionein gene is tightly regulated by copper, there is no apparent detrimental effect of having high levels of unregulated expression of thionein (cup1Δ + YEp42) in the absence of metal.

Discussion

It is generally accepted that one important function of metallothionein is to protect cells from heavy metal toxicity (2). In mammalian cells, metallothionein mRNA is induced by a wide range of metal ions (5). In vitro binding studies have shown that the purified apoprotein binds many different metal ions with high affinity and little specificity (18, 19). However, in most cases, the metal ions that induce the gene have not been shown to actually bind to the newly synthesized thionein in vivo. Duram and Palmiter (5) have recently addressed this question by selecting a hepatocyte cell line for cadmium resistance. As previously observed with other cell lines, cadmium-resistant clones had amplified metallothionein genes, optimally synthesized 20-fold more metallothionein mRNA, and were resistant to 7.5-fold more cadmium than the parental cell line. However, the cadmium-resistant cell line with amplified metallothionein capacity was not cross-resistant to many of the metal ions that induced metallothionein mRNA in these cells. Significant cross-resistance was observed only for zinc, copper, mercury, and bismuth; and of these, greater than 2-fold resistance was observed only for zinc. These authors suggested that some metal ions are “gratuitous inducers” of metallothionein and are not detoxified by it. However, in another study, cadmium-resistant cells were only cross-resistant to mercury (21). Still other studies have produced results that conflict with these (22).

The inherent limitations in this experimental approach are that metallothionein gene amplification is quantitatively limited and that resistance in metallothionein-amplified cells must be compared with parent cells which still contain metallothionein genes. Moreover, it cannot be ruled out that changes other than metallothionein gene amplification occur during selection for cadmium resistance, and the effects of these changes on cross-resistance to other metals cannot be predicted. We have addressed the question of metallothionein function in yeast, where it is possible to manipulate the genome such that metallothionein is the only varied parameter. We deleted the metallothionein gene from the yeast genome, and then the metallothionein-deleted cells were transformed with metallothionein-expressing high copy plasmids. Metal resistance in these cells was compared with cells transformed with a control plasmid (without a metallothionein gene).

None of the cells that had metallothionein genes under normal regulatory control were protected from cadmium toxicity. However, when the CUP1 coding sequence was removed from normal regulatory control and placed under a constitutive promoter, the expressed yeast thionein protected cells from cadmium toxicity at least 1000-fold greater than cells without thionein. Constitutively expressed thionein protected cells from copper and cadmium but not from the toxicity of the other metals tested, including zinc and silver, which have been shown to bind to the yeast protein in vitro (19). One possible explanation is that the metals that bind thionein in vitro and are not detoxified by thionein in vivo exert damage at a location such as the cell wall, where thionein is not available. However, we consider it unlikely that toxicity from each of these metals is limited exclusively to the cell wall. We conclude that yeast thionein is structurally capable of coordinating and detoxifying both copper and cadmium, but because cadmium does not induce the gene, thionein protection
in yeast is limited exclusively to copper.

Yeast and mammalian metallothioneins have highly divergent primary sequences except for the six-amino acid region Lys-Lys-Ser-Cys-Cys-Ser, which is conserved precisely (1) and is present in all mammalian metallothioneins sequenced thus far. In order to determine if this sequence plays a crucial role in copper detoxification, we expressed a mutant yeast metallothionein that had this sequence deleted. However, copper protection from the normal protein exceeded that of the mutant by no more than a factor of 2.

Thus, the highly conserved sequence is not crucial for copper detoxification. Moreover, the fusion protein consisting of both yeast and monkey metallothionein sequences protected cells from copper at approximately the same level as the partially deleted yeast protein. These results suggest that there is significant flexibility in the structural requirements for metallothionein to function in copper detoxification.

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