Mammalian Metallothionein Is Functional in Yeast

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Expression of two monkey metallothioneins in yeast leads to complementation of both known functions of the endogenous yeast copperthionein gene, namely copper detoxification and autoregulation of transcription. The metallothionein-like proteins of higher and lower eukaryotes are therefore functionally analogous despite their dissimilar primary sequences.

METALLOTHIONEINS (MT's) ARE A group of small, cysteine-rich proteins that bind heavy metals such as copper, zinc, cadmium, and mercury. The MT's of mammals, from which they were originally isolated, show strong conservation of their primary structure (*1*). In contrast, MT-like proteins of lower eukaryotes, such as the yeast *Saccharomyces cerevisiae*, share little primary sequence homology with mammalian MT despite their similar amino acid composition and metal binding capability (*2–4*). The precise function of MT in heavy metal metabolism is a matter of continuing debate, and the lack of any known enzymatic activity has made classification of these proteins difficult. To determine whether the structurally dissimilar mammalian and yeast proteins are functionally analogous, we expressed monkey metallothionein-I and -II complementary DNA's (cDNA's) in yeast cells lacking their endogenous MT-like copper binding protein. The endogenous yeast protein, copperthionein, is encoded by the *CUP1* locus (*5*).

The construction of *cup1*$^{\Delta}$ yeast strains in which the *CUP1* locus is deleted and replaced by *URA3* has been described (*5*). Such strains grow normally under standard laboratory conditions, but are hypersensitive to copper poisoning. They also show high levels of basal transcription from an episomal *CUP1* promoter in media containing no exogenously added copper. To express monkey MT's in such strains, we inserted monkey MT-I and MT-II cDNA's (*6*) into a transcription cassette containing the promoter and transcription initiation sequences from the yeast *CUP1* gene together with transcription termination and polyadenylation signals from a 5′ terminally truncated yeast *CYC1* gene (Fig. 1). The hybrid genes were joined to yeast–*Escherichia coli* vectors that are maintained in yeast either at low copy number because of the presence of *ARS1* and *CEN3* sequences (YM1 and YM2 in Fig. 1a) or at high copy number because of the presence of 2-μm circle sequences (HYM1 and HYM2 in Fig. 1b). As controls, we also contructed recombinants in which *E. coli* galactokinase (*galK*) coding sequences replaced the monkey MT sequences (RC4 and HCG) or which contained an intact *CUP1* gene in a 2-μm vector [YEp336; see plasmid YEp36 (*7*)]. These plasmids were introduced into appropriately marked *cup1*$^{\Delta}$ yeast strains, and transformants were selected by the *TRP1* or *LEU2* markers on the vectors.

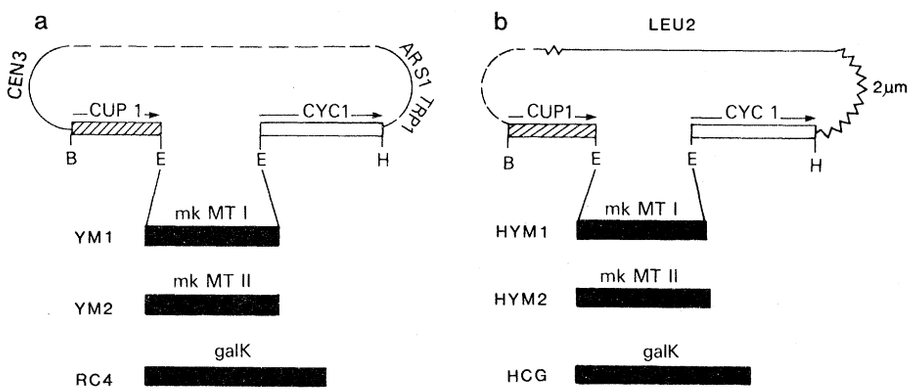

Fig. 1. Structure of low and high copy number vectors in which the expression of heterologous genes is under *CUP1* transcriptional control. Plasmids in (a) are derivatives of RC4 [referred to as pYSK12 (*4*)], a low copy number *TRP1*, *ARS1*, *CEN3* plasmid containing the *CUP1* transcriptional regulatory sequences fused to the *E. coli galK* gene. A 5′ terminally truncated yeast iso-1 cytochrome c gene (*CYC1*) provides transcriptional termination and polyadenylation signals. Monkey MT-I and -II cDNA cloned in pUC9 (*6*) were linearized with Bgl II and Nco I, respectively, which cleave upstream of the translational start codons. The termini were filled with the Klenow fragment of *E. coli* DNA polymerase I, Eco RI linkers were ligated to the termini, and the DNA was digested with Eco RI. The fragments were ligated to RC4, which was cleaved with Eco RI and dephosphorylated by standard techniques. *Escherichia coli* strain MC1061 was transformed, and plasmids containing inserts in the correct orientation were isolated by standard techniques. The high copy number plasmids in (b) were constructed by ligation of the *CUP1*-insert-*CYC1* Bam HI/Hind III unit into the *LEU2*, 2-μm yeast vector YEp13. Solid lines represent yeast sequences as indicated, and broken lines represent pBR322 sequences. Not shown is plasmid YEp336 in which a 1400–base pair Sau 3A fragment containing the intact *CUP1* gene is inserted into the Bam HI site of YEp13 (*7*).

Transformants containing the monkey cDNA's on high copy number vectors were tested for the ability to synthesize monkey MT polypeptides (Fig. 2). Cells grown in the absence or presence of added copper were labeled with [^{35}S]cysteine and soluble proteins of low molecular weight were analyzed by gel electrophoresis. In the absence of exogenous copper, a small amount of polypeptide comigrating with authentic monkey MT was detectable from both HYM1 and HYM2 (Fig. 2, lanes 4 and 7). The addition of $CuSO_4$ to a concentration of 30 μ*M* resulted in an approximately 20- to 35-fold induction of these species (Fig. 2, lanes 5 and 8), whereas 300 μ*M* $CuSO_4$ resulted in a 7- to 17-fold induction, as estimated by densitometry analysis (Fig. 2, lanes 6 and 9) and an inhibition of total protein synthesis (Fig. 2, lane 3). The untransformed *cup1*$^{\Delta}$ parental strain showed no polypeptides comigrating with monkey MT at any $CuSO_4$ concentration (Fig. 2, lanes 1 to 3). We were also unable to detect monkey MT polypeptides in strains transformed with YM1 or YM2, presumably because of the low copy numbers of these plasmids.

We next tested whether monkey MT can protect yeast against copper poisoning (Fig. 3). The *cup1*$^{\Delta}$ parental strain grew normally on unsupplemented synthetic complete agar, which contains approximately 0.1 μ*M* copper, but was not viable on agar containing 25 μ*M* added copper. In contrast, cells carrying the high copy number HYM1 construct were resistant to as much as 1 m*M* $CuSO_4$, and cells carrying HYM2 were resistant to as much as 2 m*M* $CuSO_4$. This difference in protection from copper poisoning by monkey MT-I and MT-II was reproducible for several isolates in three independent transformation experiments. The degree of copper resistance afforded by the high copy number plasmids was intermediate between that of yeast cells containing a single copy of the endogenous copperthionein gene (*cup1*^{s} strain) and cells containing multiple copies of the gene (*CUP1*^{r} strain or *cup1*$^{\Delta}$ strain transformed with YEp336). Cells carrying the low copy number recom-

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binants YM1 and YM2 exhibited little copper resistance, growing as papillae on medium containing 25 μM $CuSO_4$, as expected from the small amount of MT polypeptides synthesized by these strains.

We also tested the ability of monkey MT to complement the autoregulatory function of yeast copperthionein. For this purpose, *cup1*$^{\Delta}$ strains carrying HYM1, HYM2, YEp336, or HCG were grown for 1 hour in the presence or absence of 75 μM added copper, and RNA was isolated and analyzed for insert-specific transcripts by RNA blotting and hybridization with appropriate probes. The transcription of the insert sequences in each of these plasmids is driven by the *CUP1* promoter and upstream regulatory elements. Therefore, we would expect to observe copper inducibility of transcription only if the encoded polypeptide can regulate *CUP1* transcription by feedback (*5*). Consistent with previous results, the recombinant encoding the *E. coli* galactokinase gene (HCG) showed no regulation by added copper (section 4 in Fig. 4) whereas the recombinant encoding authentic copperthionein (YEp336) showed a 3.5-fold induction (section 1), as estimated by densitometry analysis. The recombinants encoding monkey MT (HYM1 and HYM2) showed a 4.5- to 6.5-fold induction by copper (sections 2 and 3), indicating that monkey MT can restore transcriptional control to the *CUP1* promoter. Because each of the *CUP1*-driven transcription units in this experiment contained different translated and 3′ untranslated sequences, the absolute levels of the steady state messages may reflect differences in RNA stability as well as transcription rates. Therefore, to quantitate the autoregulatory competence of the monkey MT's, we measured galactokinase activity in *cup1*$^{\Delta}$ strains co-transformed with the *CUP1-galK* recombinant RC4 and either HYM1, HYM2, or YEp336. The use of the *galK* readthrough assay to determine relative transcription rates has been described (*5*). Each of these plasmids repressed basal galactokinase expression fivefold, whereas the parental vector YEp13 had no effect. Hence the monkey MT's can efficiently repress basal transcription through a *trans*-acting mechanism.

Our results show that monkey MT, when expressed from high copy number vectors, can complement both the detoxifying and autoregulatory functions of yeast copperthionein. The ability of mammalian MT to protect against copper poisoning is not unexpected since these proteins are capable of tightly binding 12 atoms of copper per molecule (*1*). We have purified yeast copperthionein and found that it contains eight atoms of copper per molecule (*8*).The yeast protein can also bind silver, zinc, and cadmium, but unlike mammalian MT it is not induced by these ions (*3, 4*). An unusual feature of the yeast copperthionein sequence is the presence of an NH_2-terminal, hydrophobic stretch of seven amino acids that are lacking from the purified protein (*8*). The absence of such a sequence from mammalian metallothionein suggests that it is not an absolute requirement for protection against copper poisoning. This is a qualitative conclusion, however, based on complementation data; the sequence may play a role in determining the efficiency of copper detoxification or sequestration.

We have also shown that both monkey

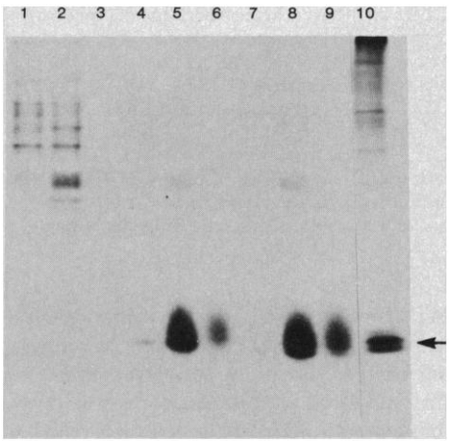

Fig. 2. Analysis of monkey MT polypeptides in *cup1*$^{\Delta}$ transformants. Yeast cultures were grown to mid-logarithmic phase (OD_{650} = 1) in synthetic dextrose medium under conditions to select for plasmid maintenance. Cultures were untreated or incubated in the presence of $CuSO_4$ for 1 hour at 30°C, then labeled with 250 μCi of [^{35}S]cysteine per milliliter for 30 minutes at 30°C. Soluble proteins were extracted by being vortexed with glass beads, carboxymethylated, and analyzed (in equal volumes of each extract) on a 20 percent native polyacrylamide gel (*5*). The fluorographed gel was exposed to Kodak XAR-5 film at −70°C. Extracts from monkey CV1 cells induced with 2 μM $CdCl_2$ were prepared as described (*6*). Lanes 1 to 3 contain extracts from the untransformed parental strain 55.6B (*MAT*α *trp1-1 leu2-3 leu2-112 gal1 ura3-50* His$^-$ *cup1*$^{\Delta}$::*URA3*) grown in the presence of 0, 30, or 300 μM $CuSO_4$. Lanes 4 to 6 contain extracts from HYM1 transformants grown in the presence of 0, 30, or 300 μM $CuSO_4$, and lanes 7 to 9 contain extracts similarly prepared from HYM2 transformants. Lane 10 contains a CV1 cell extract. The arrow indicates the position of the monkey MT polypeptides. The apparent heterogeneity of the MT synthesized in yeast is presumably due to incomplete carboxymethylation; similar heterogeneity is seen in yeast copperthionein preparations that have been shown to be homogeneous by sequencing. The nature of the higher molecular weight species induced by copper in lane 2 is unknown.

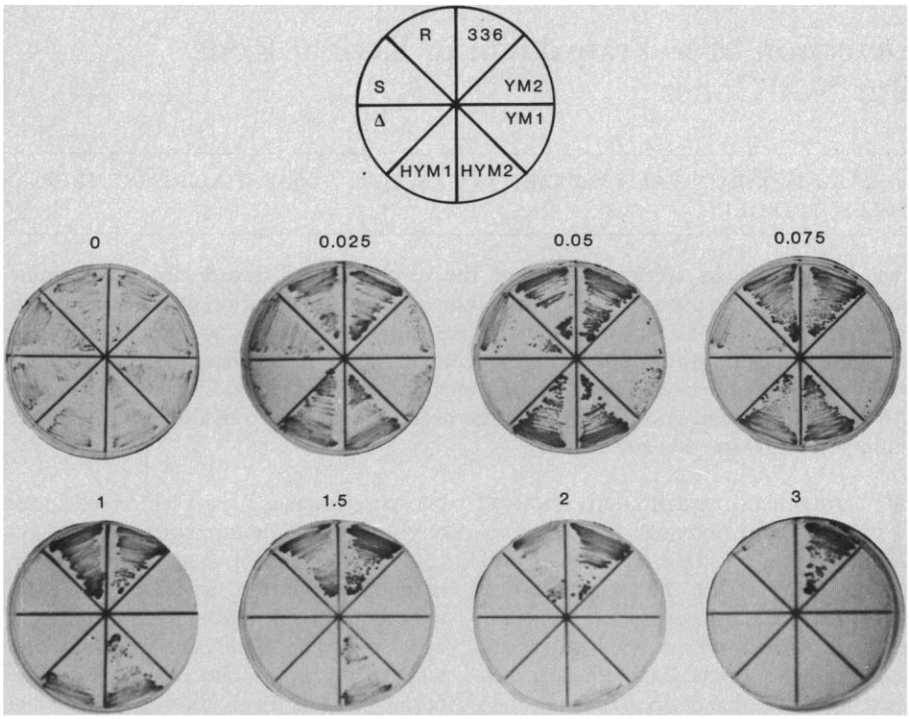

Fig. 3. Complementation of copper sensitivity by monkey MT. Transformants of the strain 55.6B (*cup1*$^{\Delta}$) and control strains were inoculated onto synthetic complete agar with the indicated concentrations of $CuSO_4$ (in millimolar units) and grown at 30°C for 3 days. The key indicates the plasmid transformed into 55.6B (Fig. 1). R indicates strain BR10 (*MATa trp1-1 CUP1*^{R} His$^-$ Ade$^-$), which contains approximately ten copies of the *CUP1* locus amplified in tandem on chromosome VIII. S indicates strain 50.L4 (*MAT*α *trp1-1 leu2-3 leu2-112 gal1 ura3-50* His$^-$ *cup1*^{S}) which contains a single copy of the *CUP1* locus and is the parent of strain 55.6B.

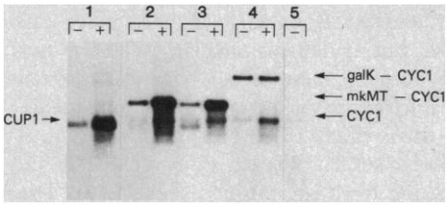

Fig. 4. Analysis of RNA in *cup1*$^{\Delta}$ transformants. Transformants were grown to mid-logarithmic phase in synthetic dextrose medium under conditions to select for plasmid maintenance. One half of each culture was then induced with 75 μM $CuSO_4$ for 60 minutes at 30°C. Cells were harvested and RNA isolated as previously described (*5*). Polyadenylated RNA was enriched by oligo(dT)-cellulose chromatography. One microgram of each RNA sample was subjected to electrophoresis on a 1.5 percent formaldehyde-agarose gel, transferred to nitrocellulose, and hybridized with either a ^{32}P-labeled nick-translated *CUP1* 1.1-kilobase Xba I/Kpn I fragment (section 1) or a *CYC1* 597-base pair Eco RI/Hind III fragment (sections 2 to 5). Transcripts originating at the *CUP1* promoter in the constructs described in Fig. 1 share the common *CYC1* region. Autoradiography was carried out at −70°C with an intensifying screen. The RNA samples were purified from the following plasmid transformants of 55.6B (*cup1*$^{\Delta}$): YEp336 (section 1); HYM1 (section 2); HYM2 (section 3); HCG (section 4); untransformed BR10 (section 5). The symbols − or + indicate growth in the absence or presence of 75 μM $CuSO_4$, respectively. CUP1 indicates the *CUP1* mRNA (500 nucleotides); CYC1 indicates the chromosomal *CYC1* transcript (630 nucleotides); mkMT-CYC1 indicates the monkey MT-I or MT-II *CUP1–CYC1* transcripts (approximately 800 nucleotides); and galK-CYC1 indicates the *E. coli* galactokinase–*CYC1* mRNA (1700 nucleotides). The increase in *CYC1* mRNA was not reproducible in different RNA analyses and the *CYC1* promoter is not inducible by copper (*5*).

MT isoforms reestablish transcriptional regulation from the *CUP1* promoter by repressing basal transcription to a level similar to that mediated by authentic yeast copperthionein. We have suggested two possible mechanisms for autoregulation of *CUP1* (*5*). (i) The *CUP1* gene product might act as a direct or indirect transcriptional repressor. The present data do not support this idea, because mammalian MT and yeast copperthionein share little primary sequence homology except for a stretch of six amino acids (*3, 4*) and because the upstream heavy metal control sequences of the mammalian and yeast genes are completely divergent (*3, 9–11*). Furthermore, purified yeast copperthionein and apothionein (*8*) exhibit no obvious DNA binding activity. (ii) Copperthionein might be feedback-regulated simply by sequestering intracellular copper that would otherwise be available to activate putative copper-dependent transcriptional factors. Our observation that mammalian MT can replace the autoregulatory function of yeast copperthionein supports this idea since mammalian proteins can chelate copper. Thus, our experiments suggest that yeast copperthionein, mammalian MT, and probably all other MT-like proteins from eukaryotes constitute a superfamily of proteins with closely related functions.

REFERENCES AND NOTES

1. J. H. R. Kagi and M. Nordberg, *Metallothionein* (Birkhauser Verlag, Basel, 1979).
2. S. Fogel and J. W. Welch, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5342 (1982).
3. M. Karin *et al.*, *ibid.* **81**, 337 (1984).
4. T. R. Butt *et al.*, *ibid.*, p. 3332.
5. D. H. Hamer, D. J. Thiele, J. F. Lemontt, *Science* **228**, 685 (1985).
6. C. J. Schmidt and D. H. Hamer, *Gene* **24**, 137 (1983).
7. T. R. Butt, E. Sternberg, J. Herd, S. T. Crooke, *ibid.* **27**, 23 (1984).
8. D. R. Winge, K. B. Nielson, N. R. Gray, D. H. Hamer, *J. Biol. Chem.* **260**, 14464 (1985).
9. M. Karin *et al.*, *Nature (London)* **308**, 513 (1984).
10. A. D. Carter *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7392 (1984).
11. G. W. Stuart *et al.*, *ibid.*, p. 7318.

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Detection of α-Transducin in Retinal Rods But Not Cones

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The distribution in chicken retina of the α subunit of transducin, the guanine nucleotide–binding protein that couples light-dependent activation of rhodopsin with activation of guanosine 3′,5′-monophosphate phosphodiesterase, was determined with the aid of a specific antiserum. α-Transducin was found in rod photoreceptor cells but was not detected in cones. These results show that rods and cones differ with respect to α-transducin content and suggest that the processes of phototransduction may differ correspondingly in rods and cones.

LIGHT IS CONVERTED INTO SYNAPTIC membrane potentials in rod photoreceptor cells by a sequence of reactions that begins with the photoisomerization of the retinal moiety of rhodopsin. This results in the activation of cyclic guanosine monophosphate (cyclic GMP) phosphodiesterase molecules that catalyze the hydrolysis of cyclic GMP, thereby reducing the permeability of rod outer segment plasma membranes to Na^+ ions. Transducin, a guanosine triphosphate (GTP)–binding protein that indirectly couples rhodopsin-mediated effects of light with activation of cyclic GMP phosphodiesterase, plays a central role in the response of rod photoreceptor cells to light (*1–3*). Less is known about the mechanism of phototransduction in cones. There are indications that the exon-intron segments of cone opsin genes are like those of rhodopsin but that the amino acid sequences of cone opsin proteins have diverged somewhat from those of rhodopsin (*4, 5*). Evidence for (*6*) and against (*7*) cyclic GMP phosphodiesterase activity in cones has been reported, and light-dependent regulation of cyclic adenosine monophosphate (cyclic AMP), rather than cyclic GMP, has been proposed for cones (*8*). However, evidence for a light-dependent activation of cyclic GMP phosphodiesterase in cones also has been reported (*9, 10*).

A cyclic GMP–sensitive conductance was found in outer segment membranes of cones as well as rods (*11, 12*). These results suggest that cyclic GMP is the internal transmitter for phototransduction in both types of photoreceptors. The existence of a guanine nucleotide–binding protein that couples cone photopigments to cyclic GMP phosphodiesterase has been postulated (*13*), but there are no data on its identity or relation to rod outer segment transducin.

We conducted immunocytochemical studies on the distribution of α-transducin in chicken retina and on the expression of α-transducin during retina development.

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