Autoregulation of the Yeast Copper Metallothionein Gene Depends on Metal Binding*

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The yeast 

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teine residues are not functionally equivalent.

MATERIALS AND METHODS

Plasmids and Strains Used—The plasmid YEp36 has been described previously (Butt et al., 1984b) and consists of the yeast 2μ and LEU2 sequences, pBR322 sequences, and a 1.1-kb fragment of DNA containing the CUP1 coding and flanking sequences. The plasmid 336ASat is a derivative of YEp36 in which an approximately 400-base pair SstII fragment was deleted between two SstII sites which were introduced 5’ and 3’ of the CUP1 coding sequence. The plasmid RC4 has also been described previously (designated pYSK in Butt et al., 1984a) and contains a CUP1:galK fusion gene on a vector that contains the TRP1 gene as a selectable marker and ARS1 and CEN3 sequences to promote stable low copy number replication.

All yeast transformations were done using the lithium acetate protocol (Ito et al., 1983) into strain 556B (Mata, trp1-1, leu2-3,112, gal1, ura3-50, His*, cup1Δ:URA3).

Preparation of the Lysine-to-Alanine Substitutions—The double lysine-to-alanine substitution was constructed by oligonucleotide-directed mutagenesis as follows: an approximately 1.1-kb BamHI fragment of DNA containing the CUP1 coding and flanking sequences was gel-purified and mixed in a ligation reaction with BamHI-cleaved, phosphatase-treated M13mp8 DNA. The ligation reactions were transfected into E. coli strain JM101 and plated. Plaques were picked and single-stranded DNA prepared by standard procedures (Sanger et al., 1980). The single-stranded DNA was examined by restriction analysis to determine the orientation of the insert. Single-stranded DNA was prepared from a clone which contained the CUP1 noncoding strand and annealed to an oligonucleotide with the sequence 5’-TCTGAAGGAAAACCGGCGGCTCATGTCGCTCT-3’. This oligonucleotide contains four mismatches with the wild-type CUP1 template (underlined above) creating a SstII site in the DNA and converting lysine residues 54 and 55 of CUP1 to alanine residues. Annealing and extension reactions and purification of closed-circular duplex DNA was performed as described by Winter et al. (1982). The closed-circular duplex DNA was then transfected into E. coli strain JM101 and DNA from the resulting plaques screened by restriction analysis for those containing the desired base changes. After purification, single-stranded DNA from a clone containing an SstII site was also subjected to sequence analysis to assure that no extra base changes were present in the CUP1 coding sequence. Duplex DNA from these plaques was isolated, digested with BamHI, and mixed in a ligation reaction with BamHI-cleaved, phosphatase-treated YEp36 DNA. The ligation mixture was transformed into E. coli strain HB101 and the resulting clones screened by restriction analysis for the presence and orientation of the insert. The resulting plasmid is identical to YEp36 except for the base changes in the CUP1 coding sequence.

Preparation of Truncation and Cys-to-Ser Mutants—The preparation of M13mp10 vectors containing premature translational termination codons and the cysteine-to-serine changes in the CUP1 coding sequence have been described previously (Wright et al., 1986). These mutations were subcloned into the plasmid YEp36 by digestion with SstII and mixing in a ligation reaction with 336ASat digested with SstII and phosphatase-treated. The orientation of the insert was determined to be the same as the CUP1 gene in YEp36 by restriction enzyme analysis.

These mutations were further subcloned onto single copy vectors as follows: the YEp36 derivatives were digested with BamHI, and the resulting 1.1-kb fragment containing the CUP1 coding and flanking sequences was gel-purified and mixed in a ligation reaction with RC4 digested with BgIII. The ligation reactions were digested with BgIII before transformation into E. coli strain MC1061. The resulting clones were screened by restriction analysis to determine the presence and orientation of the insert. These manipulations created a series of plasmids that contain the yeast TRP1-ARS1 sequence, pBR322 sequences, a trid I fusion consisting of the CUP1 promoter driving the galK structural gene and containing the CUP1 coding sequences, and a 1.1-kb fragment containing the CUP1 structural gene and flanking sequences.

RNA Blot Analysis—Yeast cultures were grown in synthetic dextrose medium, induced with 0.05 mM copper, and RNA extracted as previously described (Hamer et al., 1985) except that total cellular RNA was used. Blots were probed with one or more of the following 32P-labeled probes: a 700-base pair XbaI/KpnI fragment containing the CUP1 coding sequences; a 1.1-kb EcoRI fragment containing the galK coding sequences; a 2.5-kb BglII fragment containing the yeast LEU2 coding sequences; or a plasmid containing the yeast PYK1 coding sequences.

Galactokinase Assays—Yeast cultures were grown in synthetic dextrose medium, induced with 0.05 mM copper, and galactokinase assays were performed as described previously (Hamer et al., 1985).

RESULTS

We used oligonucleotide-directed mutagenesis in a CUP1-lacZ construct (Wright et al., 1986) to generate three classes of mutants: 1) carboxyl-terminal truncation mutations in which translational stop codons were introduced into the CUP1 coding sequence at positions 17, 32, 44, and 57 (see Fig. 1); 2) amino acid substitutions in which pairs of cysteine residues at positions 17 and 19, 32 and 44, 46 and 57 and 58 were converted to serine residues; and 3) double lysine-to-alanine substitution in the carboxyl-terminal hexapeptide sequence Lys-Lys-Ser-Cys-Cys-Ser which is highly conserved in all metallothioneins (Butt et al., 1984a).

All mutations were transformed into a yeast strain lacking a chromosomal copy of CUP1 (cup1Δ), allowing us to study copper detoxification and regulation of the mutant proteins in a background lacking the wild-type protein.

Copper Detoxification by Mutants—The CUP1 coding and flanking sequences from all of the mutations were transferred from the M13 vectors on which they were constructed to the high copy number yeast vector YEp13, which contains pBR322 sequences, the yeast LEU2 gene, and sequences from the yeast 2μ circle. The resulting plasmids were transformed into a cup1Δ yeast strain. To determine the ability of the mutant CUP1 genes to support metallothionein synthesis, [35]S-cysteine-labeled cell lysates from the yeast transformants grown in the presence of copper were run on 20% polyacrylamide gels. The CUP1 gene product was identified by its mobility and by its intense labeling with this isotope (Hamer et al., 1985). The results from this analysis (data not shown) showed that the stop 44 and stop 57 mutants and all of the substitution mutants were expressed at approximately the same level as the wild-type CUP1 gene. The peptides produced from the shortest truncations, stop 17 and stop 32, were not visible on this gel; whether this resulted from inactivity of these proteins or whether these peptides were simply not retained on the gel was not determined. Next, the transformants were streaked onto a series of plates containing different concentrations of copper. As can be seen in Fig. 1, the degree of copper protection, which presumably reflects the ability of the mutant protein to bind to copper, varied over a wide range. The truncations showed an essentially linear response with the longest, stop 57, conferring wild-type protection to copper; the two shorter truncations, stop 17 and stop 32, conferring very little resistance; and stop 44 conferring an intermediate resistance. The amino acid substitutions also followed a pattern in their ability to impart copper resistance. The substitutions at the carboxyl terminus of the molecule did not greatly affect the ability of the protein to detoxify copper. In contrast, the substitutions at the amino terminus of the molecule, in particular the ser 32/34 double mutant, were noticeably more copper-sensitive than yeast transformed with the wild-type CUP1 gene. These results suggest that all of the Cys residues do not participate equally in copper binding since these mutants, which all contain the same number of Cys residues, have different degrees of copper resistance. The double lysine-to-alanine substitution in the conserved cysteine tripeptide was functionally equivalent to the wild-type with respect to copper detoxification. Therefore, this conserved hexapeptide sequence, at least in yeast, does not seem to play an important role in the ability of metallothionein to bind metal.
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High copy vectors  Low copy vectors

| Added copper concentration allowing confluent growth (mM) |
|----------------------------------------------------------|
| High copy vectors | Low copy vectors |
|-------------------|-----------------|
| 3 | 0.1 |
| 3 | 0.075 |
| 0.5 | 0.025 |
| 0.025 | 0 |
| 0.025 | 0 |
| 3 | 0.1 |
| 2 | 0.1 |
| 1 | 0.05 |
| 1.5 | 0.05 |
| 3 | N/D |

FIG. 1. Summary of mutants introduced into the yeast CUP1 gene. The amino acid sequence of the wild-type and mutant CUP1 genes is shown inside the stippled box. The stop codons of the truncation mutations are represented by asterisks; the amino acid replacements of the substitution mutants are represented by breaks in the stippled box. Shown in the right of the figure is the copper sensitivity of a cupZA yeast strain transformed with high or low copy number vectors containing the wild-type or mutant CUP1 genes; copper sensitivity was monitored by streaking the transformants on synthetic dextrose plates containing increasing increments of added CuSO. N/D, not determined.

FIG. 2. CUP1 mRNA levels in a cup1Δ yeast strain transformed with a multicopy vector containing wild-type or mutant CUP1 genes. Autoradiograph of RNA blot analyses in which 7 µg of total RNA prepared from yeast cells was loaded onto a 1.5% agarose gel containing formaldehyde, transferred to nitrocellulose, and hybridized to a probe for the CUP1 transcript (bottom band). Cells used to prepare the RNA were grown to log phase in synthetic dextrose medium and then either incubated in the presence (+) or absence (–) of 0.5 mM CuSO₄ for 1 h at 30 °C. The transformant containing the wild-type CUP1 gene is designated YEp36, other strains contain mutant CUP1 genes, as designated in Fig. 1, on this same vector. After hybridization to the CUP1 probe, the blot was washed and subsequently hybridized to a probe for the yeast LEU2 sequences (data not shown). Scanning laser densitometry of this blot revealed that approximately the same amount of RNA was present in each lane of the gel. Also, since the LEU2 gene is present on the multicopy vector, this hybridization suggests that there is no significant variation in copy number of this plasmid in the various transformants.

Regulation of Mutant Gene Expression—We next analyzed RNA from yeast cells transformed with the wild-type or mutant plasmids to investigate the regulation of CUP1 mRNA. As can be seen in Fig. 2, cells containing the wild-type CUP1 gene (YEp36) express CUP1 mRNA at a low basal level which is induced 10–20-fold by the addition of exogenous copper to the growth medium. In general, cells transformed with the mutant CUP1 genes exhibited an inverse relationship between the basal level of CUP1 mRNA and the ability to detoxify copper. For example, the stop 17 mutant protein, which confers little or no copper detoxification, also showed the highest level of basal CUP1 mRNA expression. Conversely, the double lysine-to-alanine substitution (ala 54/55), which had wild-type ability to detoxify copper, also had a normal low basal level of CUP1 mRNA. The one exception to this pattern was the stop 32 protein which conferred no copper resistance yet had a low basal level of expression. However, it was difficult to interpret this result because the induced level of expression in this mutant was also low, presumably reflecting instability of the mRNA encoded by the mutant gene.

Use of a Fusion Gene to Quantitate Autoregulation—To more carefully quantify the ability of the mutant proteins to regulate CUP1 gene transcription, we tested their effects on the expression of a CUP1::galK fusion gene (Butt et al., 1984a). For this purpose, the mutated genes were subcloned into a low copy number yeast vector containing CEN3 sequences and a fusion gene between the CUP1 promoter and the E. coli galactokinase coding sequences (Fig. 3). The ad-
vantage of this strategy is that the effect of each mutated protein is measured in trans on the same transcription unit rather than on different, mutated genes. Another advantage was the ability to monitor the copper detoxification of the mutant proteins on a low copy number vector. The copper resistance results are shown in Fig. 1 and agree qualitatively with the results obtained with the high copy number vectors. As expected, however, these cells are all more copper-sensitive than cells transformed with the multicopy vectors.

The basal galactokinase activity in extracts of cells grown in the absence of added copper and transformed with the above plasmids are shown in Table I. In addition, galactokinase activity was measured in a strain transformed with a plasmid containing only the CUP1::galK fusion (RC4); no structural gene for CUP1 is present in these cells. In agreement with previous results (Hamer et al., 1985), galactokinase activity is expressed at a high level in these cells, even in the absence of added copper in the medium.Cells in which the wild-type CUP1 gene is also present on the fusion plasmid (RCG24) repress this activity approximately 8-fold. The mutant proteins showed varying degrees of ability to repress this constitutive galactokinase activity. For the truncations, stop 17 repressed activity about 20% as well as wild-type, stop 32 did not repress galK activity to any appreciable extent; stop 44 repressed activity about 25% as well as wild-type; and stop 57 was able to repress the activity about 60% as well as the wild-type. Of all the mutants, ser 57/58 was by far the best able to cause repression of CUP1 transcription; galactokinase activity in these cells was only 2-fold above the repression seen with the wild-type protein. For the other point mutants, ser 44/46 repressed activity about 60% as well as wild-type; ser 17/19 repressed 40% as well as wild-type; and ser 32/34, which confers the least copper resistance, only repressed 30% as well as wild-type.

We also analyzed the steady-state levels of galactokinase mRNA in the various transformants. As can be seen in Fig. 4, the basal levels of galK message correlate well with the levels of galactokinase enzyme activity. In particular, the stop 32 mutant shows a high basal level of galK mRNA which confirms the result obtained by assaying galactokinase activity but contrasts with the extremely low basal level of CUP1 mRNA expressed from the stop 32 mutant gene. A possible explanation for this is that the base mutations introduced into the CUP1 coding sequence of this mutant causes the mRNA to be labile and therefore gives the appearance of a low basal message level.

**DISCUSSION**

Using oligonucleotide-directed mutagenesis we have introduced four truncation mutations, four pairs of cysteine-to-serine substitutions, and a double lysine-to-alanine substitution into the CUP1 gene product. Characterization of these mutants for their ability to detoxify copper and down-regulate CUP1 basal transcription has elucidated two points: 1) auto-regulation occurs via a copper chelation mechanism; and 2) the cysteine residues of yeast metallothionein do not participate equally in metal detoxification and, presumably, metal binding.

Previous experiments had shown that the CUP1 gene product is necessary for transcription from the CUP1 promoter to be repressed at a low basal level. However, these experiments did not address the mechanism of this regulation. Two models which were proposed were either that the CUP1 protein is a transcriptional repressor (direct or indirect) or that the CUP1 protein simply controls the levels of intracellular copper by chelation. The latter model requires that other transcriptional factors bind to the CUP1 promoter to stimulate CUP1 transcription in the presence of copper. We reasoned that if we impaired the ability of yeast metallothionein to bind copper by removing cysteine residues from the protein, we could discriminate between these two models. For example, if copper chelation is the autoregulatory mechanism, then a decreased ability to bind copper will necessarily lead to a decreased ability to regulate transcription. On the other hand, if the CUP1 protein modulates transcription by a direct DNA binding mechanism, we might expect to isolate mutants that separate the ability to bind copper and to autoregulate.

The results obtained with the various mutations described above support the copper chelation model of autoregulation. The protein truncations showed a linear response in ability to detoxify copper as well as ability to cause repression of CUP1 basal transcription. The longest truncation peptide,
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containing 10 Cys residues, was the best able to confer copper resistance and restore autoregulation. The two shortest truncations, neither of which could confer an appreciable degree of copper resistance, showed an approximately equal inability to repress basal transcription. The chelation model of autoregulation is perhaps best supported by the Cys-to-Ser substitution mutations since these are specific for the residues known to participate in metal binding. In all cases the ability to detoxify copper correlated directly with the ability of the protein to cause repression of transcription from the CUP1 promoter.

Additional evidence for the copper chelation mechanism comes from the result with the double lysine to alanine substitution in the conserved Lys-Lys-Ser-Cys-Cys-Ser sequence. It had been previously demonstrated that monkey metallothionein can complement both copper detoxification and autoregulation in a cup1Δ yeast strain (Thiele et al., 1986). Because monkey and yeast metallothionein share little sequence homology, and because the monkey and yeast metallothionein control sequences are unrelated, this result was interpreted to favor the copper chelation model. However, because the yeast and monkey genes share the conserved hexapeptide, an alternate explanation was that this sequence was responsible for DNA binding. Our demonstration that a mutant in this sequence exhibits normal regulation excludes this latter hypothesis.

A surprising result from the experiments presented here is that proteins which contain the same number of cysteine residues have different abilities to detoxify copper and to regulate transcription. It has been proposed that copper ions would bind to yeast metallothionein in a symmetrical fashion such that all cysteine residues would participate equally in a trigonal metal binding geometry. However, our results suggest that the cysteine residues may not be functionally equivalent as substitutions at the amino terminus of the molecule are less able to detoxify copper and repress basal transcription than substitutions at the carboxyl terminus of the molecule. One possibility is that the amino terminus serves as a nucleation center to which copper ions bind and thereby initiate the folding of the rest of the molecule.

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