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The *CUP2* Gene Product, Regulator of Yeast Metallothionein Expression, Is a Copper-Activated DNA-Binding Protein

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***CUP2* is a regulatory gene controlling expression of *CUP1*, which encodes the Cu-binding yeast metallothionein. *CUP2*, which is identical to the *ACE1* gene, encodes a Cu-regulated DNA-binding protein. The CUP2 protein contains a cysteine-rich DNA-binding domain dependent on Cu^+ and Ag^+ ions which bind the cysteine residues and direct the refolding of the metal-free apoprotein. *CUP2* mutant alleles from Cu-sensitive yeast strains have point mutations affecting the DNA-binding activity. These results establish CUP2 as the primary sensor of intracellular Cu^+ in the yeast *Saccharomyces cerevisiae*, functioning as a Cu^+-regulated transcriptional activator.**

In animal cells, the primary regulators of trace metal homeostasis are the metallothioneins (MTs), which are cysteine-rich heavy-metal-binding proteins (9, 12). The genetic control of trace metal metabolism can be studied in the yeast *Saccharomyces cerevisiae*, which requires low amounts of Cu as an essential element for proper growth. However, in high concentration, Cu is a potent and commonly used fungicide. In yeast cells, Cu resistance and sensitivity are controlled by the *CUP1* locus (3, 4, 22). *CUP1* encodes a specific Cu-binding protein known as copper-chelatin (13) or yeast MT (24). In general, the phenotypic resistance to Cu is proportional to the *CUP1* gene copy number (4, 22, 23).

Transcription of the 0.5-kilobase *CUP1* gene is enhanced 10- to 20-fold by elevated Cu (13), a response mediated through two Cu-regulated upstream activation sequences (UASs) (20). Two regulatory genes responsible for induction of *CUP1* were identified: *CUP2* (21) and *ACE1* (18, 19). Nucleotide sequence analysis indicates that the two genes are identical (6; P. Skroch and C. Buchman, unpublished results). Analysis of nuclear extracts of *CUP2*$^+$ and *cup2* cells indicated that *CUP2* either codes for or controls the synthesis or activity of a protein which binds to the UASs of *CUP1* (21). Similarly, the *ace1-1* mutation causes disappearance of Cu-induced protection of one of the UASs of *CUP1* (11). To further understand the mechanism by which *CUP2* acts to regulate expression of *CUP1*, we analyzed the proteins encoded by various *CUP2* alleles. As was previously shown for *ACE1* (6), *CUP2* encoded a protein whose N-terminal half was rich in cysteine residues and functioned as a DNA-binding domain, dependent on either Cu^+ or Ag^+ ions. The *cup2* and *ace1-1* alleles were found to be point mutants affecting two different amino acids in the N-terminal DNA-binding domain of CUP2. While an *ace1-1* fusion protein retained a reduced ability to bind *CUP1* DNA, a *cup2* fusion protein was completely inactive. Taken collectively, our findings indicated that the primary mechanism controlling yeast MT gene expression by Cu involved a conformational change of the N-terminal domain of the CUP2 protein induced by the binding of Cu^+ ions which enabled the CUP2 protein to bind DNA in a sequence-specific manner and thereby activate transcription.

***CUP2* encodes a DNA-binding protein.** Nucleotide sequence analysis has indicated that the *CUP2* gene (21) is identical to *ACE1* (6; Skroch and Buchman, unpublished results). To analyze the CUP2 protein, we cloned *CUP2* into the bacterial expression vector pATH2 (1) to generate pTE · CUP2. *Escherichia coli* cells harboring pTE · CUP2 expressed an induced protein of M_r 60 kilodaltons (Fig. 1A). In cells containing pTE · CUP2Tr, which contained only the first 122 amino acids of CUP2, the inducible protein had an M_r of 45 kilodaltons. These proteins were not present in cells harboring pATH2, which expressed a 37-kilodalton inducible protein derived from the N-terminal portion of trpE.

Extracts of *E. coli* cells harboring pATH · CUP2 grown in the presence of 1 mM Cu^{2+} but in the absence of tryptophan were incubated with a ^{32}P-labeled *CUP1* probe containing the Cu-responsive UASs (20, 21) and were assayed by gel retardation (5, 7). Two protein-DNA complexes, B1 and B2, were detected (Fig. 1B). These complexes were not formed by incubation with extracts of cells harboring pATH2 grown in the presence of Cu^{2+} (Fig. 1C). Formation of both complexes was inhibited by incubation with excess *CUP1* promoter region DNA but not by pBR322 DNA, indicating that the complexes were attributable to sequence-specific interaction (Fig. 1B). Since other trpE · CUP2-containing extracts gave rise to only a single major complex (for example, see Fig. 1C), it is likely that complex B2 was due to binding of the probe to a degradation product of trpE · CUP2. The truncated fusion protein trpE · CUP2Tr also bound specifically to the *CUP1* probe to generate a protein-DNA complex migrating faster than the one formed by trpE · CUP2 (Fig. 1C). In sum, the results indicated that the N-terminal 122 amino acids of CUP2 contained its DNA-binding domain.

CUP2 is a Cu-dependent DNA-binding protein. In addition to abundance of basic residues, the N-terminal half of CUP2 also contains a large number of cysteines (6). The abundance and the arrangement of these residues as Cys-X-Cys and Cys-X_2-Cys clusters raised the possibility that in addition to DNA, this part of CUP2 also bound Cu. Binding of Cu to CUP2 was likely to affect its DNA-binding activity, providing a mechanism for Cu-regulated transcription. The binding activity of trpE · CUP2 was greatly reduced when the protein was extracted from *E. coli* cells grown in the absence of Cu (Fig. 2A). These extracts still contained substantial amounts of the trpE · CUP2 protein, as shown by sodium

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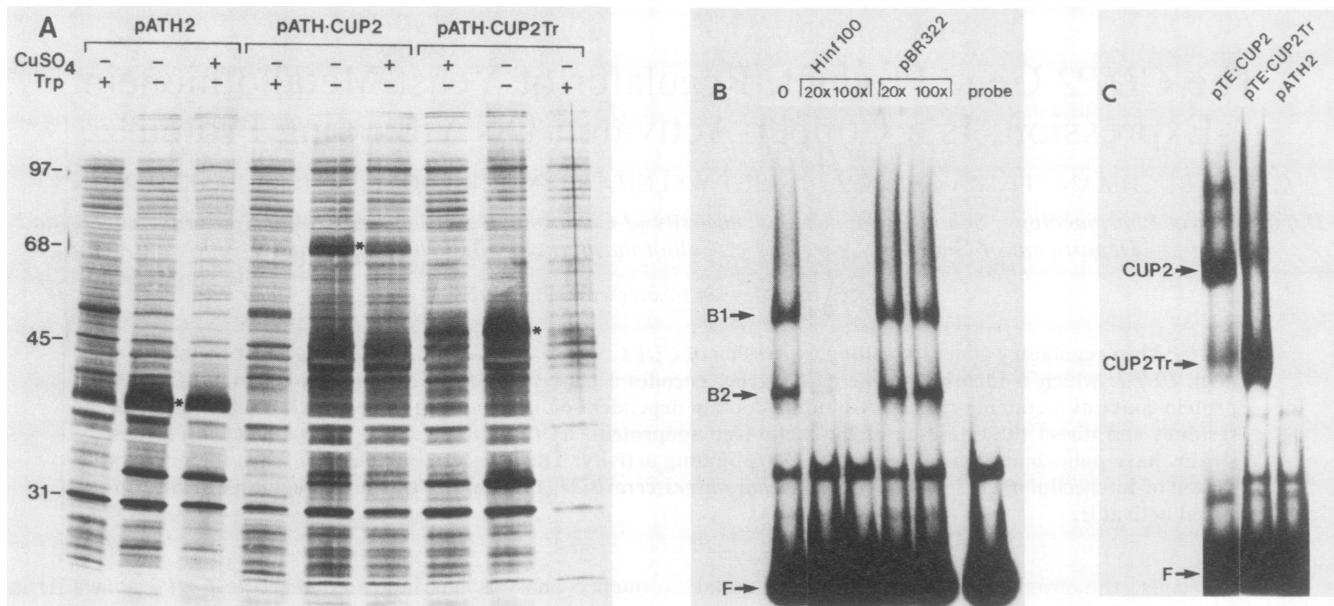

FIG. 1. DNA-binding of the trpE · CUP2 and trpE · CUP2Tr fusion proteins expressed in *E. coli*. (A) Expression of fusion proteins. *E. coli* DH5 cells transformed with pATH2, pTE · CUP2, or pTE · CUP2Tr were grown in the presence (repression) or absence (induction) of tryptophan (Trp) and 1 mM $CuSO_4$, as indicated. Cells were pelleted, solubilized in SDS sample buffer, incubated for 10 min at 95°C, and analyzed on a 10% SDS-polyacrylamide gel using silver staining, as previously described (1). The bands that migrated at the predicted positions for trpE and the fusion proteins are marked by asterisks. Migration positions of molecular weight standards are shown at the left. (B) Specific DNA binding of trpE · CUP2 fusion proteins. Protein extracts (2 μg) containing trpE · CUP2 were incubated with the *CUP1* probe (a fragment containing CUP1 sequences from −183 to −83) and analyzed by gel retardation as previously described (21). The formation of the retarded complexes, B1 and B2, was specifically inhibited by the plasmid bearing the region from −183 to −83 of the *CUP1* promoter (Hinf100) used at either 20- or 100-fold molar excess, but not by pBR322 DNA. (C) Binding of truncated and full-length fusion proteins. Extracts (2 μg of protein) from *E. coli* transformed with pTE · CUP2, pTE · CUP2Tr, or pATH2 and grown in the absence of tryptophan and in the presence of Cu^{2+} were incubated with the *CUP1* probe and analyzed by gel retardation. Both pTE · CUP2 and pTE · CUP2Tr formed protein-DNA complexes not formed with pATH alone. F, Free probe. pTE · CUP2 was generated by cloning of a *Bam*HI-*Hin*dIII fragment of polymerase-chain-reaction amplified *CUP2* DNA (see legend to Fig. 3) into pATH2 (1). pTE · CUP2Tr was generated by digestion of pTE · CUP2 with *Bgl*II and religation, which led to removal of the fragment encoding the C-terminal half of CUP2.

dodecyl sulfate (SDS)-polyacrylamide gel analysis (Fig. 1A). In addition, Cu added to these extracts in the form of Cu^{+} restored the DNA-binding activity of the protein (Fig. 2A). Cu^{+} was used in these experiments instead of the Cu^{2+} ion used for in vivo induction because Cu^{+} and not Cu^{2+} is the natural ligand of yeast MT (2, 8, 24). Indeed, while the DNA-binding activity of trpE · CUP2 was also restored by Ag^{+}, which is electronically similar to Cu^{+} (Fig. 2A), reactivation of Cu^{2+} was much less efficient (data not shown).

The results presented here imply that growth in the presence or absence of Cu controlled the activity of the trpE · CUP2 protein made in *E. coli*. As a further proof that the DNA-binding activity associated with trpE · CUP2 depends on bound Cu^{+}, we incubated this protein preparation with KCN, a chelator capable of removing Cu^{+} ions from yeast MT (2). As expected, KCN led to loss of the DNA-binding activity, which could be restored upon addition of excess Cu^{+} (data not shown) or Ag^{+} (Fig. 2B). No binding activity appeared upon addition of Zn^{2+}, Cd^{2+}, or Hg^{2+} ions (data not shown).

Analysis of *CUP2* mutants. JW1038-4B (*cup2*) and DTY20 (*ace1-1*) were independently isolated as ethyl-methanesulfonate-induced copper-sensitive mutants from their copper-resistant parental strains (19, 21). To determine the molecular basis for the failure of these strains to induce *CUP1* in response to Cu (19, 21), we isolated molecular clones of the two mutant alleles after their amplification by the polymerase chain reaction (15, 16). Sequence analysis of at least two different isolates of each of the mutant alleles revealed that each of them differed from the wild-type allele by a single point mutation (Fig. 3). The *cup2* allele contained a G-to-A transition converting Gly-37 to a glutamic acid residue, while Cys-11 was converted to a tyrosine residue by a G-to-A transition in the *ace1-1* strain. Both of these mutations agreed with the known specificity of ethyl methanesulfonate (17).

To examine the effect of these mutations on CUP2 protein function, both alleles were cloned into the bacterial expression vector pATH2. *E. coli* cells harboring pTE · cup2 and pTE · ace1-1 expressed induced fusion proteins which had the same mobility as the wild-type trpE · CUP2 (Fig. 4A). Although the mutant fusion proteins appeared to be as stable as the wild-type fusion protein, trpE · ace1-1 had reduced DNA-binding activity while trpE · cup2 did not exhibit detectable binding to the *CUP1* promoter (Fig. 4B).

The results reported above indicate that the *CUP2* gene product is a Cu^{+}- and Ag^{+}-regulated DNA-binding protein recognizing the Cu-responsive UASs of the *CUP1* gene. The first clue to this function was obtained by sequence analysis of the *CUP2* gene, which had also demonstrated that *CUP2* was identical to the independently isolated *ACE1* gene (6, 18; Skroch and Buchman, unpublished results). According to its nucleotide sequence, *CUP2* had the potential of coding for a 24-kilodalton protein with a highly asymmetric amino acid distribution. Although all the cysteines and most of the basic

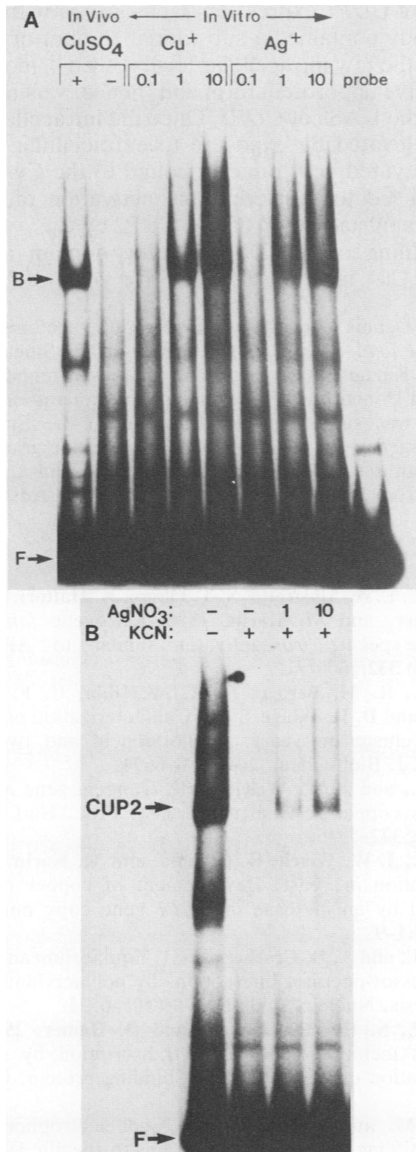

FIG. 2. Cu-dependent DNA binding of CUP2. (A) Cu dependence in vivo and in vitro. Protein extracts (2 μg) of *E. coli* cells harboring trpE · CUP2 grown in the presence (+) or absence (−) of $CuSO_4$ were incubated with the *CUP1* probe and analyzed by mobility shift assays. A specific protein-DNA complex was formed when cells were grown in the presence but not the absence of $CuSO_4$. Incubation of the extract from Cu-depleted cells with Cu^+ (0.1 to 10 μM) or Ag^+ (0.1 to 10 μM) for 1 h at 4°C prior to DNA binding led to reappearance of DNA-binding activity. (B) Binding was reversibly inhibited by KCN. Protein extracts from pATH · CUP2-containing cells were incubated for 1 h in the presence of 50 mM KCN and then for another hour with Ag^+ (1 and 10 μM) and then were analyzed by gel retardation.

residues are concentrated in the N-terminal half of the protein, the C-terminal half contains most of the acidic residues and, therefore, is likely to function as its transcriptional activation domain (10, 14).

As expected from the sequence analysis, the DNA-binding domain of CUP2 was located in its positively charged, cysteine-rich, N-terminal half. The activity of this domain was highly dependent on Cu^+ or Ag^+ ions, suggesting that these specific ions interact with the cysteine residues to

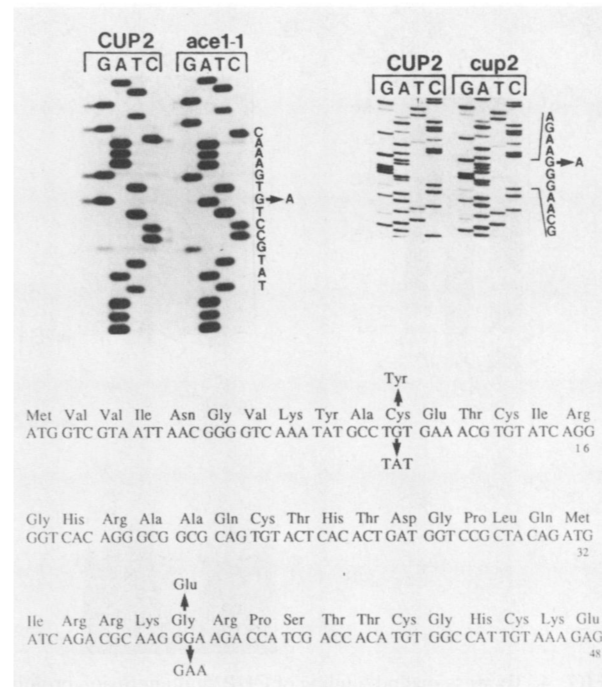

FIG. 3. Sequence analysis of *CUP2* mutants. Autoradiograms of sequencing gels of the wild-type *CUP2* gene and the *ace1-1* and *cup2* mutant alleles in the region containing the identified point mutations are shown. The nucleotide sequence of *CUP2* is indicated on the right, and the changes affected by the mutation are denoted. The nucleotide and amino acid sequences of *CUP2*, with the mutations indicated by arrows, are shown at the bottom. Cys-11-to-Tyr was the *ace1-1* mutation, and Gly-37-to-Glu was the *cup2* mutation. The coding sequences of *cup2* and *ace1-1* were isolated by using the genomic DNA as a template in the polymerase chain reaction procedure with the primers 5′-GGGAAGCTTG GATCCCATGG TCGTAATTAA CGGGGTCAAA T-3′ and 5′-GGGGAAGCTT AGCGGCCGCA GATCTAGATG TCAGATGCAT TATTGTG AAT-3′. Two separate reactions were performed for each strain, and at least two subclones from each reaction in pBluescript were sequenced to ensure that the mutations were not due to errors introduced by the *Taq*I polymerase used for the polymerase chain reaction. The appropriate fragments containing the coding regions of the two mutants were then subcloned into the pATH2 expression vector (see legend to Fig. 1). The primers described above were used to sequence the two mutant alleles by the dideoxy method with Sequenase (U.S. Biochemical Corp.).

direct the folding of the DNA-binding domain. The similar metal ion specificity and distribution of cysteines suggests that the CUP2 protein may form Cu-thiolate clusters resembling those of yeast MT (8). These assumptions are supported by the analysis of *CUP2* mutant alleles. The *ace1-1* mutation entailed a Cys-11-to-Tyr substitution. This mutant protein had a reduced ability to bind *CUP1* DNA, probably because Cys-11 was directly involved in formation of the Cu^+-coordinated DNA-binding domain. While the most likely role of this Cys residue is in the chelating of Cu^+, it is unlikely that a mutation affecting a single Cys residue would abolish Cu^+ binding altogether. The *cup2* mutation was a substitution of a Glu residue for Gly-37. This mutant exhibited no detectable *CUP1* DNA-binding activity. However, unlike the role of Cys-11, the role of the Gly-37 was less obvious, even though this residue was apparently required for DNA binding. One possibility is that the Gly-to-Glu change introduced a negatively charged residue into a cluster

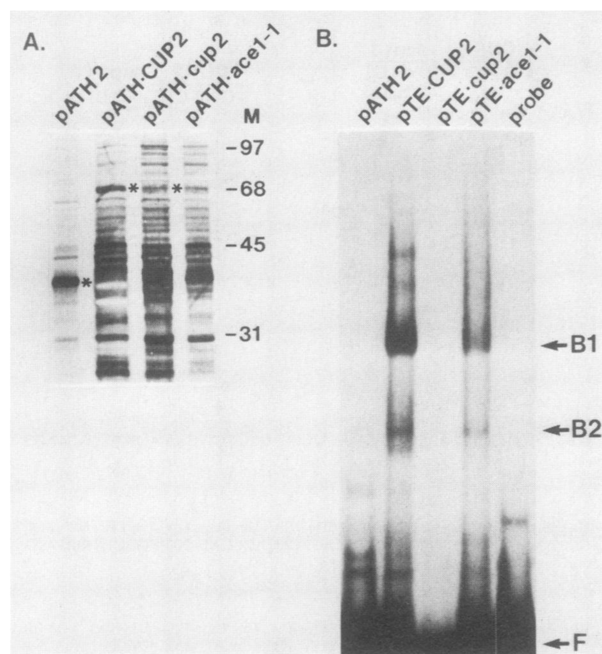

FIG. 4. Expression and binding of CUP2 mutant fusion proteins. *E. coli* DH5 cells transformed with pATH2, pTE · CUP2, pTE · cup2, and pTE · ace1-1 were grown in the absence (induction) of tryptophan and presence of 1 mM $CuSO_4$. (A) Cells were pelleted, solubilized in SDS-sample buffer, incubated at 95°C for 10 min, and analyzed on a 10% SDS-polyacrylamide gel using silver staining, as previously described (1). The bands that migrated at the positions corresponding to trpE and the fusion proteins are marked by asterisks. The migration positions of the molecular weight standards (M) are shown on the right. (B) *E. coli* protein extracts containing equal amounts of the various fusion proteins, as determined by silver staining, were incubated with the labeled *CUP1* probe and analyzed by gel retardation. Both pTE · CUP2 and pTE · ace1-1 formed protein DNA-complexes not formed by pTE · cup2 or pATH2 alone. B1 corresponds to the full-length trpE · CUP2 protein DNA complex, and B2 is probably a degradation product. F, Free probe.

of positively charged amino acids and thereby affected interaction with phosphate groups in the DNA backbone. The other possibility is that the Gly-to-Glu change led to loss of conformational flexibility and interfered with proper folding of the DNA-binding domain. As for the Cys-11-to-Tyr mutation, it is unlikely that the Gly-37-to-Glu mutation affected Cu^+ binding per se.

The ability to express in *E. coli* CUP2 fusion proteins which are capable of binding DNA in a Cu^+-dependent manner strongly suggests that no other yeast protein is required for activating CUP2 in response to Cu^+. This conclusion could not be reached from the analysis of CUP2 activity in yeast extracts. Although genomic footprinting experiments suggest that CUP2 is a Cu-activated DNA-binding protein in vivo (11), the CUP2 protein exhibited constitutive DNA-binding activity when it was analyzed in vitro with yeast extracts (21). The likely explanation for this discrepancy is that during the preparation of yeast extracts, enough Cu^+ is released from intracellular stores to activate the CUP2 apoprotein.

While the DNA-binding activity of the CUP2 protein is highly regulated by Cu, expression of the *CUP2* gene is constitutive (18; Buchman, unpublished results). Taken collectively, these findings suggest the following scheme for regulation of *CUP1* expression. Cells grown with low levels of Cu already contained a substantial amount of CUP2, but because of the low intracellular levels of Cu^+, most of it was in the inactive apoprotein form and, hence, was incapable of binding to the UASs of *CUP1*. Once the intracellular level of Cu^+ was elevated by exposure to extracellular Cu, CUP2 became activated very rapidly, bound to the Cu-responsive UASs, and led to transcriptional activation of the *CUP1* gene. The rapid activation of apo-CUP2 by Cu^+ was likely to involve folding of the DNA-binding domain around the complexed Cu^+ ions, as suggested for yeast MT (8, 24).

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