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The *CUP2* gene product regulates the expression of the *CUP1* gene, coding for yeast metallothionein

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The yeast *CUP1* gene codes for a copper-binding protein similar to metallothionein. Copper sensitive *cup1*^{s} strains contain a single copy of the *CUP1* locus. Resistant strains (*CUP1*^{r}) carry 12 or more multiple tandem copies. We isolated 12 ethyl methane sulfonate-induced copper sensitive mutants in a wild-type *CUP1*^{r} parental strain, X2180-1A. Most mutants reduce the copper resistance phenotype only slightly. However, the mutant *cup2* lowers resistance by nearly two orders of magnitude. We cloned *CUP2* by molecular complementation. The smallest subcloned fragment conferring function was ~2.1 kb. We show that *CUP2*, which is on chromosome VII, codes for or controls the synthesis or activity of a protein which binds the upstream control region of the *CUP1* gene on chromosome VIII. Mutant *cup2* cells produced extremely low levels of *CUP1*-specific mRNA, with or without added copper ions and lacked a factor which binds to the *CUP1* promoter. Integrated at the *cup2* site, the *CUP2* plasmid restored the basal level and inducibility of *CUP1* expression and led to reappearance of the *CUP1*-promoter binding factor. Taken collectively, our data establish *CUP2* as a regulatory gene for expression of the *CUP1* metallothionein gene product.

Key words: DNA binding protein/metallothionein regulatory genes/*Saccharomyces cerevisiae*

Introduction

Utilization of the heavy metal ion Cu^{2+} poses a metabolic paradox. On the one hand, copper in low concentrations is an essential element to the growth of *Saccharomyces cerevisiae*; yet, in higher concentrations it functions as a potent fungicide. Since different industrial and wild yeast strains characteristically tolerate a wide range of exogenous Cu^{2+} levels (Welch *et al.*, 1983) these model eukaryotes must encompass a precisely adjustable homeostatic mechanism that poises and regulates the phenotypic expression of the resistance/sensitivity reaction.

The *CUP1* locus, represented by *CUP1*^{r} and *cup1*^{s} alleles that confer resistance and sensitivity respectively, was assigned to chromosome VIII by Hawthorne and Mortimer (1960). Over a period of two decades, extensive tetrad analyses studies bearing on gene conversion (Fogel and Hurst, 1967), crossing over (Hurst *et al.*, 1972), genetic interference (Mortimer and Fogel, 1974) and chromosome apportionment (Rockmill and Fogel, 1988) utilized the *CUP1*^{r} genetic marker located 45 cM distal to the centromere of chromosome VIII.

To understand the molecular mechanisms by which the homeostatic copper resistance/sensitivity reactions are mediated, we cloned *CUP1* (Fogel and Welch, 1982) and demonstrated that it encodes a specific copper-binding protein known as either copper-chelatin (Karin *et al.*, 1984) or yeast metallothionein (MT) (Winge *et al.*, 1985). The *CUP1*^{r} allele was found to comprise 10–12 tandemly repeated 2.0 kb units, while the *cup1*^{s} allele contained a single repeat unit (Welch *et al.*, 1983; Karin *et al.*, 1984). In general, the phenotypic resistance level is proportional to the copy number of the tandemly amplified unit (Fogel *et al.*, 1983), which may increase or decrease spontaneously both in meiosis and mitosis via a non-reciprocal recombination process essentially indistinguishable from classical gene conversion (Welch *et al.*, 1987). Each of the 2.0 kb repeats contained a 0.5 kb *CUP1* transcription unit and an as yet uncharacterized adjacent gene whose co-amplification with the *CUP1* gene was probably a fortuitous event (Welch *et al.*, 1983). Transcription of the 0.5 kb *CUP1* gene is stimulated 10–20-fold in response to elevated copper concentrations, while transcription of the adjacent co-amplified gene is not affected (Karin *et al.*, 1984). Dissection of the *CUP1* promoter region led to the identification of two *cis* elements capable of conferring copper-responsiveness upon heterologous promoters (Thiele and Hamer, 1986). These features of the *CUP1* gene are highly similar to those of the mammalian MT genes, which also encode low mol. wt heavy metal binding proteins, and whose transcription is also induced by heavy metal ions (Karin, 1985; Hamer, 1986).

To identify putative *trans*-acting regulatory genes whose products may interact with the copper-responsive elements of the *CUP1* promoter to mediate copper resistance and induction, we have screened a collection of copper sensitive mutants induced by ethyl methane sulfonate (EMS) in X2180-1A, a wild-type multicopy *CUP1*^{r} strain (Fogel *et al.*, 1983; Welch *et al.*, 1983), for possible regulatory gene mutations. One recessive isolate, designated as *cup2*, dramatically reduced the phenotypic copper resistance of the extremely resistant parental strain. Compared to X2180-1A, the *cup2* mutant's resistance was diminished by approximately two orders of magnitude. Compared with *cup2*, each of the remaining mutants exerted considerably lesser effects on the expression of copper resistance relative to the parental X2180-1A strain.

The study reported here centers on the isolation, identification and characterization of *trans*-acting loci involved in modulating resistance to external copper. In addition to the characterization of the *CUP2* locus, we also report the linkage relations of five independently assorting and distinctive recessive mutant loci that alter the level of copper resistance/sensitivity. The wild-type allele of the *CUP2*$^{+}$

regulatory locus was cloned by complementation of the recessive *cup2* mutant. We show that the *cup2* gene is a *trans*-acting regulator of *CUP1* expression. *CUP2* appears to encode or regulate the synthesis or activity of a *trans*-acting factor that binds to the *CUP1* promoter region to control its expression.

Results

Isolation of mutants in copper resistance

The standard wild-type X2180-1A *mat a* carries 10–12 tandemly iterated copies of *CUP1*^{r} at its normal chromosomal locus (Fogel *et al.*, 1983). It is resistant to 1.5 mM copper and exhibits a normal growth rate on agar-solidified synthetic complete (SC) medium at this supplementation level. A fresh, overnight, washed culture was mutagenized with EMS to a survival of ~15%. Approximately 150–200 viable cells were deposited on 100 individual SC plates and incubated at 30°C for 4–5 days. After the colonies attained a diameter of ~4 mm, they were replica-plated onto plates containing SC medium and the same medium supplemented with 0.1 mM $CuSO_4$. Colonies that failed to grow on these copper plates were recovered and purified by streaking on SC plates. Each purified isolate was crossed to the isogenic X2180-1B wild-type strain. The hybrids were sporulated, asci were dissected and tetrad analysis was performed. In this manner, each presumptive mutant was obtained in both mating types and hence the various mutants could be tested against each other in all possible combinations for complementation responses.

Taken collectively, 12 complementation groups could be identified. However, of these only five represented individual mutants that yielded tetrad segregation patterns sufficiently clear to establish specific chromosomal locations in subsequent crosses to standard tester strains. The various mutant loci were identified by their original isolation numbers.

For the most part, these five mutants exhibited only modest or slight phenotypic shifts relative to diminished copper resistance compared to the standard wild-type. However, the *cup2* mutant displayed a drastically reduced copper tolerance level. It could not grow on plates with more than 0.02 mM Cu^{2+} and thus exhibited a sensitivity more pronounced than *cup1*^{s} alone, which can grow on SC agar in the presence of 0.15 mM copper. Consequently, *CUP2* was selected for extensive analysis based on the assumption that it functioned as a *trans*-acting regulatory locus for the expression of the *CUP1*^{r} locus that codes for the copper-binding yeast MT. The mapping studies involving the various mutants are summarized in Table I.

Cloning of CUP2

We cloned *CUP2*$^+$ by transforming yeast strain JW1038-4B which carries the markers *CUP1*^{r}, *cup2, ura3-52, trp1-298, arg4-16 thr1* and *leu2-1* with the Nasmyth Sau3A bank of genomic wild-type DNA inserted in YRp7 (Struhl *et al.*, 1979). Thus, the recipient strain carried *cup2*

Table I. Chromosome assignment and linkage relations of various copper mutants

Mutant	Chromosome	Interval	PD : NPD : T	Map distance in cM	Maximal tolerable Cu^{2+} (mM)
cup2	VII	*cup2–aro2*	77 : 1 : 34	18	0.02
		cup2–met13	34 : 1 : 54	34	0.02
		cup2–lys5	82 : 0 : 12	6.4	0.02
cup3	XII	*cup3–ura4*	73 : 2 : 31	20	0.5
		cup3–car2	14 : 0 : 6	15	0.5
cup5	V	*cup5–ura3*	130 : 0 : 36	10.8	0.6
		cup5–can1	56 : 3 : 100	37	0.6
		cup5–hom3	33 : 9 : 127	52	0.6
cup14	IV	*cup14–ade8*	68 : 3 : 73	32	0.8
		cup14–trp4	42 : 8 : 106	49	0.8

An additional mutant, *cup7*, exhibits clear centromere linkage indicated by a PD : NPD : T ratio of 12 : 9 : 0 relative to *trp1*. However, it has not been assigned to a specific chromosome. With the clear exception of *cup2*, the various mutants exert only minor effects on copper resistance levels. Accordingly, they are tentatively viewed as altering reactions other than a defect in the primary transcriptional regulation of *CUP1*^{r}. However, their further analysis will undoubtedly allow for a more comprehensive genetic and physiological dissection of the metabolic reactions involved in various aspects of copper metabolism. We are aware that such effects on resistance may be secondary. The various mapped genes are reported in Mortimer and Schild's (1988) Genetic map of *S.cerevisiae*, Edition 9. Another group of mutants selected for analysis are *cup2* revertants designated JW1038-4B-1R to 31R. Crossed to a *cup2 lys5* strain 12 such mutants yielded tetrad data indicating that the reversion is at or is closely linked to the *cup2* site.

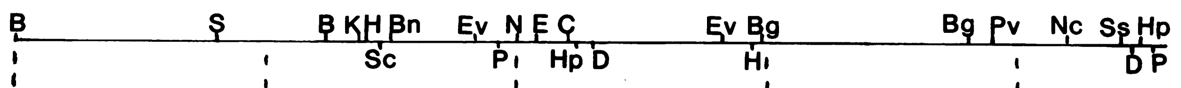

Fig. 1. Restriction map of *JW16*, a 4.6 kb genomic DNA segment cloned in YRP7. Dashed lines below the map are 1 kb intervals. B, *Bam*HI; Bg, *Bgl*II; Bn, *Ban*II; C, *Cla*I; D, *Dra*I; E, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; N, *Nhe*I; Nc, *Nco*I; P, *Pvu*I; Pv, *Pvu*II; S, *Sac*I; Sc, *Sca*I; Ss, *Ssp*I. The functional gene lies within the *Dra*I–*Dra*I 2.1 kb segment. The subcloned *Bam*HI–*Pvu*II fragment does not give function and deletion of the *Bgl*II–*Bgl*II fragment from *JW16* causes loss of function.

at the chromosome VII site linked to *lys5* (6.5 cM) and 12 copies of the 2.0 kb repeat unit harboring the *CUP1* gene at chromosome VIII. About 2000 *TRP*$^+$ transformants were isolated and tested for copper resistance on SC plates containing 0.1 mM $CuSO_4$. Plasmids isolated from four independent colonies resistant to this copper level were retransformed into *Escherichia coli*. The shortest cloned DNA segment conferring the wild-type *CUP2*$^+$ resistance function was a 4.6 kb fragment (Figure 1). Subsequently, we selected several stable yeast transformants in which the plasmid had integrated into the genome. One of these was crossed to a *cup2* mutant haploid strain carrying other chromosome VII markers to determine whether or not integration had occurred at the *cup2* chromosome VII site. After sporulation and dissection of asci generated from the hybrids, we observed the *CUP2*$^+$ and *TRP1*$^+$ cosegregated and yielded the following linkage data relative to *lys5*; PD : NPD : T = 20 : 0 : 4. This linkage estimate agrees closely with the previously determined map distance for the

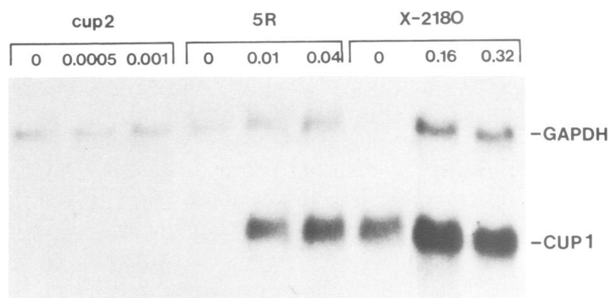

Fig. 2. Analysis of *CUP1* gene expression in various yeast strains. Yeast strains were grown overnight in minimal medium either with no added copper or at the indicated copper concentrations (mM) shown along the top of the figure. These concentrations are the half maximal and maximal concentrations that can be tolerated by each strain during overnight growth. 10 μg of total cellular RNA were applied to each lane, separated by agarose gel electrophoresis, transferred to nitrocellulose and probed with GAPDH and *CUP1* specific probes. The 'O' lane of X2180 contains somewhat less RNA than the other lanes. X2180 is a wild-type strain, *cup2* is the mutant strain. 5R is a *cup2* revertant resistant to a copper level intermediate between *cup2* and wild-type X2180.

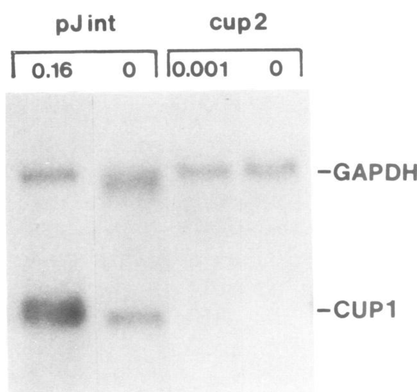

Fig. 3. The cloned *CUP2* plasmid integrated in a *cup2* strain (pJint) restores *CUP1* mRNA expression. Total cellular RNA from *cup2* or pJint (*CUP2*) cells grown in minimal medium either without copper or at maximal tolerable levels of copper were analyzed by Northern blots as described above. The *CUP2* plasmid restores the basal mRNA levels as well as allowing cellular survival at elevated copper concentrations.

CUP2–LYS5 interval, 6.4 cM (see Table I). On tetrad analysis, the strain bearing the integrated *CUP2*$^+$, *TRP1*$^+$ plasmid regularly exhibited 4 : 0 segregation of enhanced copper resistance when it was crossed to a wild-type *CUP2*$^+$ strain. Therefore, we concluded that *CUP2*$^+$ had been successfully cloned. The strain containing the integrated *CUP2* plasmid at the *CUP2* site is referred to in the following discussion as pJint. A restriction map of the *CUP2* gene is presented in Figure 1. Various restriction fragments derived from the original clone were subcloned into the yeast vector YRp7 and tested for their ability to complement the *cup2* mutation and allow growth in 0.1 mM copper. The minimal fragment found to confer the *CUP2* function was 2.1 kb in length as shown in Figure 1.

The CUP2 gene controls the expression of CUP1

As indicated by Figure 2, in the wild-type strain X-2180, the *CUP1* transcript was inducible after growth in elevated copper and there was a relatively high basal level when no added copper was present. When the *CUP1*-specific mRNA was induced by exposure to external copper for 30 min, there was a rapid 20-fold increase in the transcript levels (data not shown, but see Fogel *et al.*, 1983) which later declined and produced the steady state level shown in Figure 2. By contrast, the mutant *cup2* strain produced essentially no detectable *CUP1* transcript in the absence or presence of added copper. In the experiment shown in Figure 2, the cells were exposed only to 0.5 and 1 μM of $CuSO_4$, the half maximal and maximal concentrations tolerated by this strain during an overnight growth in liquid. In other experiments involving a short term incubation (30 min) the *cup2* strain was found to be refractory to induction at any copper concentration (data not shown). However, in a revertant, 5R, obtained by selection for copper resistance from the *cup2* strain, *CUP1*-mRNA was inducible despite the very low basal level. This revertant exhibited partial resistance to copper, as it could grow in up to 0.04 mM copper in liquid cultures. The nature of the reversion leading to increased copper resistance and *CUP1* expression of this strain has not been extensively characterized. Nonetheless, tetrad analysis of crosses involving 5R establish that the reversion lies within, or is immediately adjacent to, the *CUP2* locus.

The results described above indicate that the cause for copper sensitivity of the *cup2* mutant is probably the low level of *CUP1* expression. Furthermore, these results suggest that *CUP2* is a *trans*-regulator of *CUP1*. This assumption was tested by examining the regulation of *CUP1* expression in the pJint strain which contains the cloned *CUP2*$^+$ gene intergrated at the *cup2* site on chromosome VII. As can be seen in Figure 3, both the basal and the copper-induced expression of *CUP1* were restored to essentially wild-type levels in pJint. Accordingly, this strain survived and grew in up to 0.32 mM copper.

A defect in protein–DNA interaction

The results from the RNA blot analysis described above indicate that in *cup2* cells, the *CUP1* gene is expressed only at a very low basal level and is refractory to induction by Cu^{2+} ions. To determine whether this defect was attributable to an altered interaction of regulatory proteins with the *CUP1* promoter in the *cup2* strain, nuclear protein extracts were prepared from both X2180-1A (WT) and otherwise isogenic *cup2* cells. The interaction of proteins

in these extracts with the *CUP1* promoter region was examined by the gel retardation method (Fried and Crothers, 1981; Garner and Revzin, 1981).

Using a *CUP1* probe which spans positions −183 to −83 (relative to the start site of transcription), a region that is thought to contain the copper-responsive elements (Thiele and Hamer, 1986), we found that incubation with extracts of X2180 or *cup2* cells led to formation of a large protein−DNA aggregate which did not enter the gel (Figure 4, lane 0). However, addition of the nonspecific competitor poly(dI-dC) resulted in the appearance of several distinct protein−DNA complexes (B1−B4) upon incubation with these extracts. Complexes B2−B4 were common to both extracts, while complex B1 formed only upon incubation with the X2180 extract. Using a second *CUP1*-derived probe which spans positions −163 to +7, we found that incubation with the X2180 extract led to the formation of at least three distinct protein−DNA complexes. In contrast, only two of these complexes were formed after an incubation with the *cup2* extract (data not shown but see Figure 5B). As a control for the quality of the extracts, we found that both wild-type and *cup2* extracts contained similar levels of the yeast 'AP-1 like' activity (Harshman *et al.*, 1988) which recognizes the AP1 site within the SV40 enhancer (data not shown). In addition, the intensity of the B4 protein−DNA complex was identical between wild-type and mutant extracts.

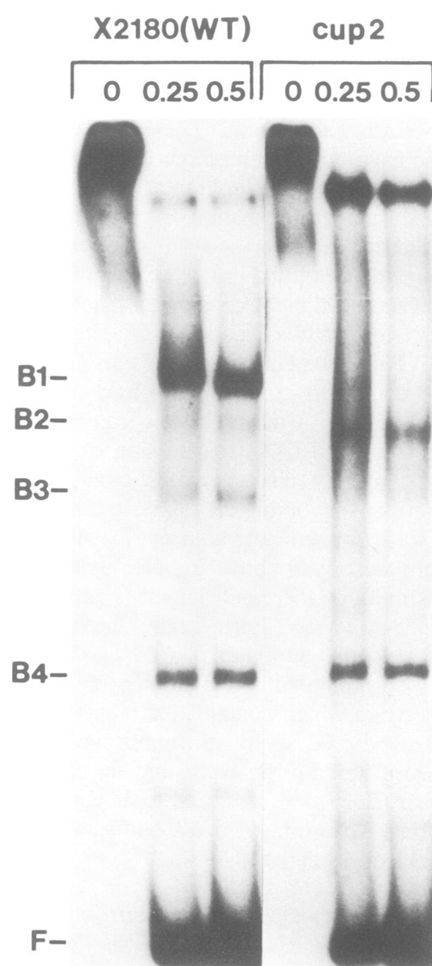

Fig. 4. Detection of nuclear factors that bind to the *CUP1* promoter. 2 μg of nuclear protein extracts of X2180 (WT) and *cup2* cells were incubated with ~0.3 ng of a *CUP1* probe (encompassing positions −183 to −83 from the mRNA start site) in the presence of 0−0.5 μg poly(dI−dC) as indicated above and analyzed by gel retardation. B1−B4, protein−DNA complexes; F, free probe; '−' lane contains probe with no added extract.

Integration of CUP2 restores protein−DNA interactions

The results presented above suggest that mutant *cup2* is defective in one of the DNA-binding activities which recognize the *CUP1* promoter. Transformation of a recipient *cup2* strain with a plasmid bearing a copy of the *CUP2* gene and its subsequent integration into the genome to yield strain pJint restored expression of the *CUP1* gene (see Figure 3). We used this strain carrying both the *CUP2* and the *cup2* alleles to examine whether the restoration of *CUP1* expression correlated with the appearance of the DNA-binding activity which interacts with the *CUP1* promoter. As shown in Figure 5A, incubation of the −183 to −83 *CUP1* DNA probe with nuclear extracts from pJint cells led to formation of several distinct protein−DNA complexes: B1−B4. These are the same complexes formed upon incubation of this probe with the X2180 extract. Again, the B1 complex did not form upon incubation with the *cup2* extract. Although the intensity of the B1 protein−DNA complex observed in this experiment with the pJint extract is reduced in comparison to the X2180 extract, it is clear that transformation of *cup2* cells with the cloned *CUP2* gene leads to its reappearance.

Incubation of the pJint extract with the −163 to +7 *CUP1* probe generated at least three distinct protein−DNA complexes (B1−B3), only two of which (B2 and B3) were formed when this probe was incubated with the *cup2* extract (Figure 5B). Competition experiments indicated that two of these complexes, B1 and B2, were specific (Figure 5C) and the third, B3, which in this experiment was not resolved very well from the free DNA (F), was non-specific. A 100-fold excess of pCCPTaq, a plasmid containing the −163 to +7 region of the *CUP1*^{r} promoter competed successfully for formation of the B1 and B2 complexes while pBR322 DNA had no effect. Note that although different probes were used in Figures 4 and 5A versus Figures 5B and C, the complex which was specific to the *CUP1* expression strains, B1, was always the slowest migrating one.

Discussion

The experiments described above show that in *cup2* cells the *CUP1* gene is expressed at a low basal level and is refractory to copper induction. This defect appears to correlate well with the absence of a DNA-binding activity which recognizes the *CUP1* promoter, whereas, other DNA-binding factors binding to this promoter are common to both wild-type and *cup2* cells. While the exact sequences recognized by these factors are not yet known, we suggest that the activities which are present both in wild-type and mutant cells could respond to general transcription factors, for example a TATA-binding factor, which recognizes many promoters. On the other hand, the activity which appears to be unique to wild-type cells wherein the *CUP1* gene is active, is likely to correspond to the factor responsible for this gene's activation by Cu^{2+} ions. This activity is absent in *cup2* cells, but reappears after transformation of these cells with the *CUP2* plasmid. Thus, it is likely that this factor

is either a product of the *CUP2* gene itself or the product of yet another regulatory gene whose synthesis or activity is controlled by the *CUP2* gene. Since the genetic and biochemical analyses indicate that *CUP2* is a regulatory gene, responsible for both the basal level of *CUP1* expression and its induction by Cu^{2+} ions, the product of *CUP2* is likely to represent a factor which is either directly or indirectly involved in both basal and Cu^{2+} inducible *CUP1* transcription. The presence of other more general DNA-binding activities in similar levels in the *cup2* and wild-type extracts argues against a general susceptibility to proteolysis or refractoriness to extraction as being responsible for the observed differences in the CUP1 promoter recognizing activity between wild-type and *CUP2* extracts.

Previous analysis of the *CUP1* promoter region had indicated that the two copper response elements are likely to be centered around positions −123 (UAS_P) and −163 (UAS_D), while a third element, probably functioning as the TATA box at position −77, is involved in basal expression (Thiele and Hamer, 1986). It remains to be seen whether the factor, which is present in X2180 (WT) and pJint extracts but absent from *cup2* extracts, recognizes exactly the same DNA sequences that were proposed earlier by Thiele and Hamer (1986) to mediate copper induction. Likewise, one of the factors which are present in all three strains may be involved in controlling the basal expression of the *CUP1* promoter by interacting with its basal elements.

Insertion of synthetic copper-responsive elements upstream of a truncated *CYC1* promoter has indicated that these elements possess basal UAS activity even in the absence of copper. Incubation of cells with copper increased their activity 3.3-fold (Thiele and Hamer, 1986). In agreement with these observations we found that the *cup2* mutation not only prevented copper induction of the *CUP1* gene but also interfered with its basal expression. In this respect, the *cup2* phenotype is different from that of another regulatory mutant *ace1-1* which affects *CUP1* expression (Thiele, 1988). It was found that the *ace1-1* mutation interfered with the copper induction of *CUP1* but not with its basal expression. The *ace1-1* mutation maps at the same region as *CUP2* and the restriction map of the cloned *ACE1* gene, which complements the *ace1-1* mutation (Thiele, 1988) was identical to that of *CUP2*. These findings indicate that *ace1-1* is allelic to *cup2*. Thus, it will be interesting to determine the basis for their different effects on *CUP1* expression.

The identification and isolation of a regulatory gene involved in yeast gene activation by Cu^{2+} ions, and the identification of a DNA-binding activity which seems to be involved in this process, are expected to facilitate the elucidation of the molecular mechanism responsible for gene activation by metal ions not only in yeast but in higher eukaryotes, including mammals. For example, it may be possible to use antibodies directed against the yeast *CUP2* gene product to detect cross-reacting proteins in mammalian cells. Such proteins could play an important role in the regulation of the mammalian MT genes (Karin, 1985; Hamer, 1986). While all of the *trans*-acting factors involved in basal expression of the human $MTII_A$ gene and its induction in response to polypeptide and steroid hormones were identified and purified (Karin *et al.*, 1987), the *trans*-acting factors that mediate its induction in response to heavy metal ions have remained elusive. Thus, we anticipate that

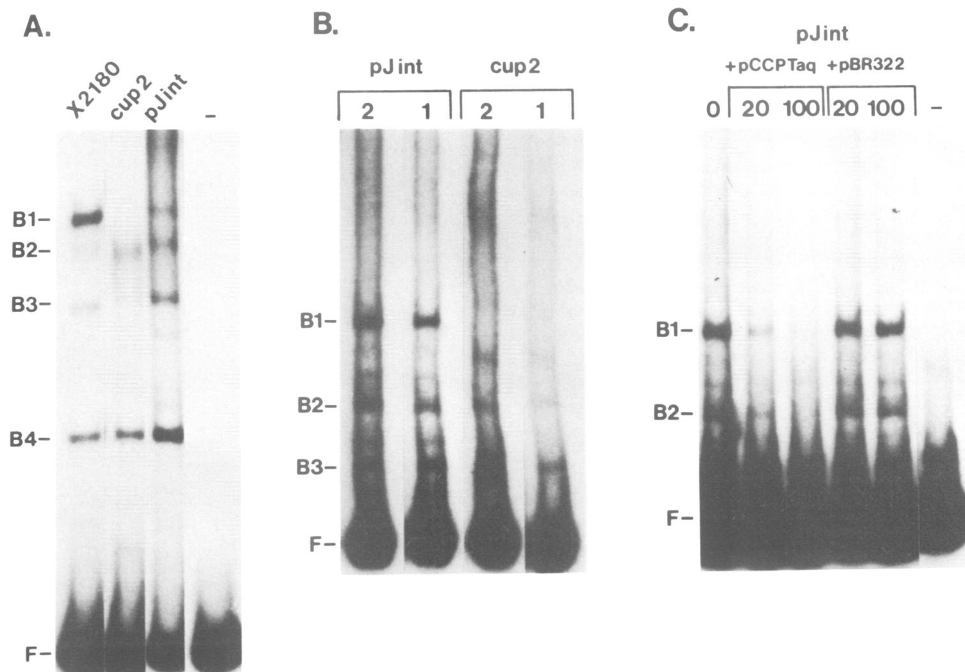

Fig. 5. Analysis of *CUP1* DNA-binding activities of extracts from *cup2* and pJint (*CUP2*) cells. **A**. Nuclear extracts X2180 (WT), *cup2* or pJint cells (1.0 μg protein), were incubated with 0.3 ng of the *CUP1* probe (−183 to −83) and analyzed by gel retardation. In addition to the free probe (F), at least four protein–DNA complexes (B1–B4) could be detected upon incubation with the X2180 (WT) and pJint extracts, while only three of them (B2–B4) formed with the *cup2* extract. **B**. pJint and *cup2* nuclear extracts (1–2 μg protein) were incubated with a *CUP1* probe (−163 to +7) and analyzed by gel retardation. Three protein–DNA complexes were observed: B1, B2 and B3, of which B1 was unique to pJint cells. **C**. The B1 and B2 complexes with *CUP1* probe (−163 to +7) were specifically competed by the plasmid bearing the −163 to +7 region of the *CUP1* promoter (pCCPTaq) used at either 20- or 100-fold molar excess, but not by pBR322 DNA. In this experiment the B3 complex was not resolved from the free probe (F). '0' lane contains no competing plasmids. '−', no added protein.

the identification and characterization of the *CUP2* gene and its product will facilitate the identification of the analogous mammalian factor. It will also be of interest to determine whether the *CUP2* product binds directly to the *CUP1* promoter and whether it is the sensor that responds to the changes in the intracellular level of Cu^{2+} ions.

Materials and methods

Details concerning media, culture and EMS mutagenesis have been published – see Fogel *et al.* (1981), also see Fogel and Welch (1982) for procedures in molecular cloning and physical studies on DNA.

Nuclear extract preparation

Yeast cultures were grown to stationary phase in YEPD, centrifuged, and resuspended in 1 M sorbitol, 50 mM Tris (pH 7.8), 10 mM $MgCl_2$ and 30 mM DTT at the ratio of 1 g yeast/ml buffer. The suspension was frozen as small pellets in liquid nitrogen and stored at −80°C. Nuclear extracts were prepared as previously described (Wiederrecht *et al.*, 1987) except that diisopropyl-fluorophosphate was omitted.

Band shift assays

Assay conditions were similar to those previously described (Fried and Crothers, 1981; Garner and Revzin, 1981; Carthew *et al.*, 1985). Binding reactions (15 µl) contained up to 16 µg of protein extract, 0.3 ng ^{32}P-end labelled probe (15 – 35 000 c.p.m.), 0.1 – 1.0 µg poly(dI – dC), 12% glycerol, 12 mM Hepes – NaOH (pH 7.9), 60 mM KCl, 5 mM $MgCl_2$, 4 mM Tris – HCl (pH 7.4), 0.6 mM EDTA, 0.6 mM DTT and competitor DNA were indicated. After a 30 min incubation at 30°C, reaction mixtures were diluted 10-fold with 10 mM Tris – HCl (pH 7.4), 1 mM EDTA, 10 µg/ml xylene cyanol, 10 µg/ml bromophenol blue and 5% glycerol. Aliquots (8 µl) were loaded on 5% polyacrylamide gels (0.15 × 16 cm; acrylamide:bisacrylamide ratio of 80:1). Gels were pre-electrophoresed for 2 h at 20 mA in a buffer consisting of 22.5 mM Tris-borate and 0.5 mM EDTA. Probes span 101 bp (−183 to −83 relative to the distal major transcription start site, Karin *et al.*, 1984) or 170 bp (−163 to +7). There were no differences in binding activities when the yeast cultures were grown in the presence of $CuSO_4$ prior to preparation of nuclear extracts (data not shown).

Northern hybridizations

Total RNA was prepared from stationary phase yeast cultures. French press lysates were resuspended by vortexing in 5 M guanidine thiocyanate, 50 mM Tris (pH 7.5), 10 mM EDTA and 0.7 M β-mercaptoethanol as a ratio of 10 ml buffer/100 ml yeast culture. LiCl was added to a final concentration of 3.5 M and the RNA was precipitated at 4°C for 20 h and centrifuged at 6500 r.p.m. for 1.5 h at 4°C. The RNA pellet was suspended in H_2O, vortexed and the solution adjusted to 10 mM Tris (pH 7.5), 1 mM EDTA, 0.1% SDS and centrifuged as before. The supernatant was saved and the pellet re-extracted. The combined supernatants were extracted with phenol and chloroform. The RNA was resuspended in 10 mM Tris (pH 7.5), 0.1% SDS and 1 mM EDTA. 20 µg of RNA were precipitated and resuspended in 1 × running buffer (10 mM $NaPO_4$, pH 7, 1 mM EDTA, 5 mM sodium acetate), 50% formamide, 2.2 M formaldehyde, and heated for 5 min at 68°C. Samples were electrophoresed on a 1.5% agarose, 2.2 M formaldehyde gel in 1 × running buffer. The RNA was transferred to nitrocellulose in 20 × SSC and the membranes were hybridized at 42°C in 5 × SSC, 3 × Denhardt's, 50 mM $NaPO_4$ (pH 7), 0.1% SDS and 50% formamide. Filters were washed in 0.1% SDS, 0.2 × SSC at 65°C and autoradiographed. The probes used were: 2 kb *Kpn*I fragments from pJW6 (Fogel and Welch, 1982) and a *Hin*dIII fragment from the glyceraldehyde 3-phosphate dehydrogenase gene (Musti *et al.*, 1983).

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