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Biochemistry

Primary structure and transcription of an amplified genetic locus: The *CUP1* locus of yeast

(gene expression/gene amplification/copper chelatin/metallothionein)

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ABSTRACT **Copper resistance in yeast is controlled by the *CUP1* locus. The level of resistance is proportional to the copy number of this locus, which can be found in up to 15 tandemly iterated copies. To elucidate the molecular mechanisms controlling the amplification and expression of the *CUP1* locus, we determined its full nucleotide sequence. We have also identified and mapped two transcription units within the basic amplification unit of *CUP1* in laboratory yeast strains. One of those transcription units is inducible by copper and encodes a low molecular weight copper binding protein—copper chelatin. The increased production of chelatin, due to both gene amplification and induction of transcription, leads to increased resistance of yeast cells to copper ions.**

Copper resistance in the yeast *Saccharomyces cerevisiae* is controlled by the *CUP1* locus (1), which is located on chromosome VIII, 42 centimorgans distal to the centromere (2). Yeast of the *cup1*^{s} genotype are unable to grow in the presence of 0.3 mM $CuSO_4$ on complete plates, whereas *CUP1*^{r} strains can grow in the presence of up to 1.75 mM $CuSO_4$. Recently, we cloned the *CUP1*^{r} locus by complementation of the *cup1*^{s} allele (3). Restriction enzyme analysis of isolated *CUP1*-bearing plasmids and of genomic DNA from both *CUP1*^{r} and *cup1*^{s} strains revealed that the sensitive strain contains a single copy of the *CUP1* locus, whereas naturally occurring resistant strains contain 10 or more tandemly iterated copies of *CUP1* (3, 4). By selection to even higher levels of resistance, the copy number of the *CUP1* locus can be increased even further (4).

The genetic organization of the *CUP1* locus resembles the organization of the rRNA genes in yeast, *Drosophila*, *Xenopus*, silkworm, and sea urchin, which are arranged in clusters of tandemly arrayed repeating units (5–12). Histone genes of sea urchin, *Drosophila*, and chicken also show a similar arrangement in tandem arrays (13–16). The repeat units of any of those clusters, within a single species, show considerable homogeneity. Several mechanisms were proposed for the evolution of tandemly arrayed gene clusters and maintenance of homogeneity within clusters—e.g., master–slave correction (17), saltatory replication (18), gene conversion (19), and unequal crossing-over (20). None of those proposed mechanisms has been proven unequivocally as responsible for development of tandem gene arrays.

Because variants containing different copy numbers at the *CUP1* locus can be readily selected after growth in the presence of elevated copper levels, we suggest that this system would prove useful in elucidating the molecular mechanisms leading to gene amplification, generation of tandemly iterated gene clusters and multigene families, and maintenance of homogeneity within those structures. Yeast is an organism particularly adapted for such studies because it is possible to transfer cloned genes into its genome by DNA transformation and homologous recombination (21–23).

Recently, we demonstrated direct correlation between the copy number of the *CUP1* locus and the level of a small-sized copper-inducible mRNA in both laboratory strains (4) and natural yeast isolates (24). The locus copy number was also positively correlated with the copper resistance level of a given strain and with the production level of a low molecular weight copper binding protein. We determined the full nucleotide sequence of the *CUP1* locus and established that the basic amplification unit contains a copper-regulated transcription unit encoding a low molecular weight, cysteine-rich protein—copper chelatin (25–27). The sequence of this protein bears some resemblance to the more familiar heavy metal binding protein—metallothionein (28)—which is responsible for protecting mammalian cells against the toxic effects of cadmium ions (29, 30).

METHODS

Standard laboratory yeast strains X2180-1A(*CUP1*^{r}) and JW251-1A(*cup1*^{s}) were used for resistance and nucleic acid hybridization comparisons. The strains were backcrossed to each other eight times to approach isogenicity. For DNA transformations we have used the haploid strain BZ31-1-7Ba, carrying the following markers: *trp1-289*, *ura3-52*, *ade8-18*, *arg4-16*, *cup1*^{s}. P5A is a highly copper-resistant derivative of X2180-1A, obtained by step-wise selection (4).

Preparation of nucleic acids from yeast was as described (4, 24). Procedures for RNA analysis either by RNA transfer blot analysis (31) or by S1 nuclease mapping (32) have been described (4, 24, 30, 33). Nucleotide sequence analysis was by the dideoxy method (34) after cloning the appropriate restriction fragments into M13 vectors (35). Yeast cell transformation (21, 22) and analysis of copper-resistant colonies was as described (3). All recombinant DNA procedures were reported previously (30).

RESULTS

Mapping the Transcripts of the *CUP1* Locus. The structure of the *CUP1* locus in the standard wild-type resistant strain (*CUP1*^{r}) X2180 is shown in Fig. 1*a*. This map was derived by restriction enzyme analysis of plasmids bearing *CUP1* fragments (3) and by Southern blot analysis (36) of X2180 genomic DNA (4). We have used different portions of the basic repeat unit as hybridization probes to detect transcripts derived from the *CUP1* locus (see Fig. 1*a*). RNA prepared from control and copper-induced X2180 cells was separated by agarose gel electrophoresis and transferred onto nitrocellulose filters (31). After hybridization with probe A, two *CUP1*-derived RNA species are detected: an abundant transcript of about 500 nucleotides in length and a less abundant

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Abbreviation: kb, kilobase(s).

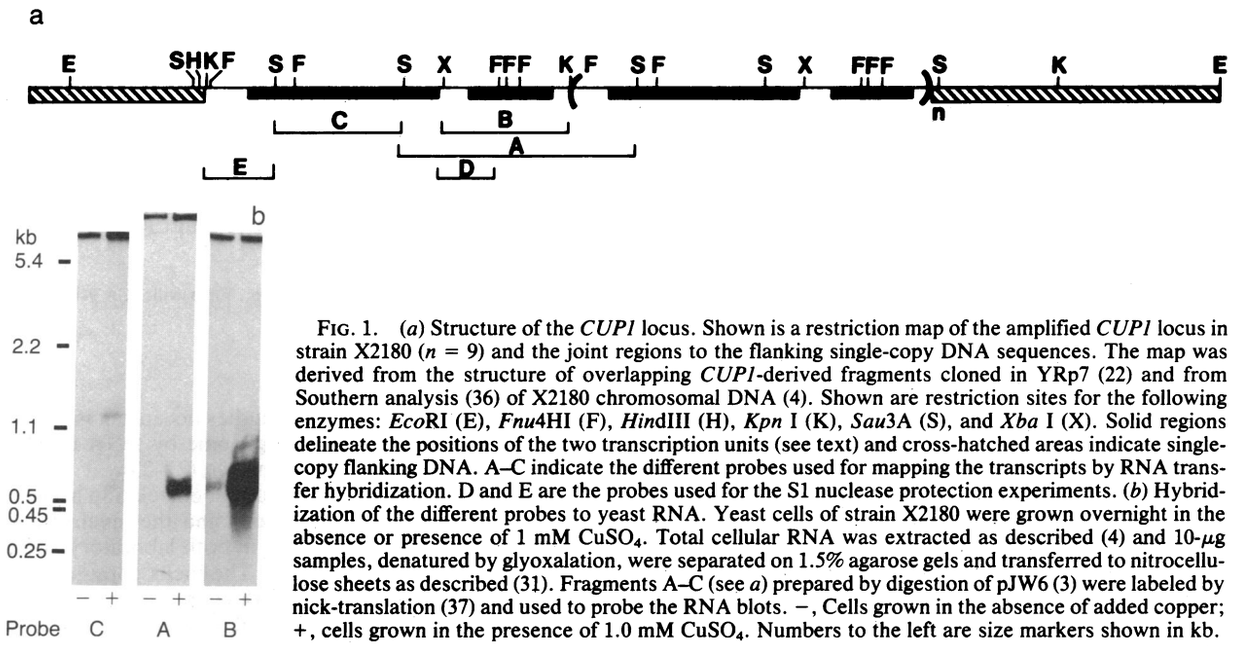

FIG. 1. (*a*) Structure of the *CUP1* locus. Shown is a restriction map of the amplified *CUP1* locus in strain X2180 ($n = 9$) and the joint regions to the flanking single-copy DNA sequences. The map was derived from the structure of overlapping *CUP1*-derived fragments cloned in YRp7 (22) and from Southern analysis (36) of X2180 chromosomal DNA (4). Shown are restriction sites for the following enzymes: *Eco*RI (E), *Fnu*4HI (F), *Hin*dIII (H), *Kpn* I (K), *Sau*3A (S), and *Xba* I (X). Solid regions delineate the positions of the two transcription units (see text) and cross-hatched areas indicate single-copy flanking DNA. A–C indicate the different probes used for mapping the transcripts by RNA transfer hybridization. D and E are the probes used for the S1 nuclease protection experiments. (*b*) Hybridization of the different probes to yeast RNA. Yeast cells of strain X2180 were grown overnight in the absence or presence of 1 mM $CuSO_4$. Total cellular RNA was extracted as described (4) and 10-μg samples, denatured by glyoxalation, were separated on 1.5% agarose gels and transferred to nitrocellulose sheets as described (31). Fragments A–C (see *a*) prepared by digestion of pJW6 (3) were labeled by nick-translation (37) and used to probe the RNA blots. −, Cells grown in the absence of added copper; +, cells grown in the presence of 1.0 mM $CuSO_4$. Numbers to the left are size markers shown in kb.

transcript of about 1,100 nucleotides in length (Fig. 1*b*). Only the shorter abundant species is induced by copper. The induction ratio is about 20-fold (as determined by densitometry). Probe B hybridizes only to the shorter copper-inducible transcript, and probe C hybridizes predominantly to the longer, less abundant transcript. (The low hybridization level of probe C to the short inducible transcript reflects cross-contamination of the probe with other fragments and the large excess of the short transcript.)

We conclude that the basic amplified *CUP1* unit contains two distinct transcription units. The expression of one unit is regulated by copper. Therefore, it probably codes for the inducible copper binding protein, chelatin (25–27), whose levels are increased in copper-resistant cells (4). Also, its size, 500 base pairs [0.5 kilobase (kb)] is consistent with the coding potential for a protein of 60 amino acids.

A modified S1 nuclease mapping procedure (32) was used to identify the direction and the start sites of each transcription unit. The two genes are transcribed in the same direction, from left to right, as indicated on the map in Fig. 1*a*. This conclusion is based on the fact that only end-labeled probes derived from the lower DNA strand (not indicated on the map) gave rise to stable hybrids with RNA derived from the *CUP1* locus. The copper-inducible transcription unit appears to have three different start sites, as three nuclease-resistant fragments can be detected (Fig. 2*a*). All of them are copper inducible. The transcripts map 118, 121, and 127 nucleotides upstream to the third *Fnu*4HI site on the map in Fig. 1*a*. The longer transcription unit appears to have two start sites, mapping 137 and 145 nucleotides upstream to the first *Sau*3A site (Fig. 2*b*). Therefore, the larger transcription unit lies between the *Kpn* I and the *Xba* I sites (5′ to 3′) and the smaller copper-inducible transcription unit is located between the *Xba* I and the *Kpn* I sites (see Fig. 1*a*).

Primary Structure of the *CUP1* Locus. The entire nucleotide sequence of the *CUP1* repeat unit has been determined (Fig. 3). The first 5′ nucleotide of the amplified unit (determined from the sequence of the junction with the flanking single-copy DNA, data not shown) is numbered +1. Computer analysis of the sequence predicts two large open reading frames whose map locations match the locations of the two transcription units. The potential open reading frame for the larger transcription unit is longer than the one that is probably used, because the first AUG codon is located upstream to the transcription start site. Starting from the first AUG, within the transcribed region, a reading frame for a protein, 246 amino acids in length, is found. To date, this protein's function has not been determined; therefore, we refer to it as protein X.

The smaller transcription unit contains an open reading frame for a cysteine-rich protein, 61 amino acids in length. Its predicted amino acid composition was compared to the amino acid composition of yeast copper chelatin (25) and to

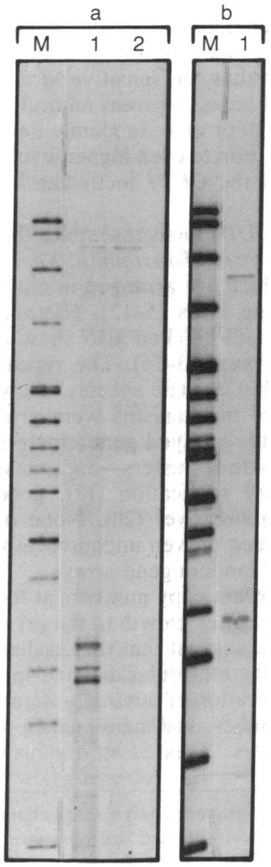

FIG. 2. Mapping the start sites of the two *CUP1* transcription units. (*a*) Mapping the start site of the short transcription unit. We have used a modification of the nuclease mapping technique of Weaver and Weissman (32). Briefly, 50 μg of RNA from cultures of strain X2180 grown in the presence of 1.0 mM Cu^{2+} (lane 1) or in its absence (lane 2) was hybridized to about 10^5 cpm of probe D (see Fig. 1*a*), end labeled at the *Fnu*4HI site, at 40°C for 12 hr. Digestion with mung bean nuclease was for 90 min at 30°C. Lane M, end-labeled *Hpa* II fragments of pBR322 were used as size markers. (*b*) Mapping the start site of the long transcription unit. Conditions were as described above, except that 10^5 cpm of probe E, end labeled at the *Sau*3A site, was used.

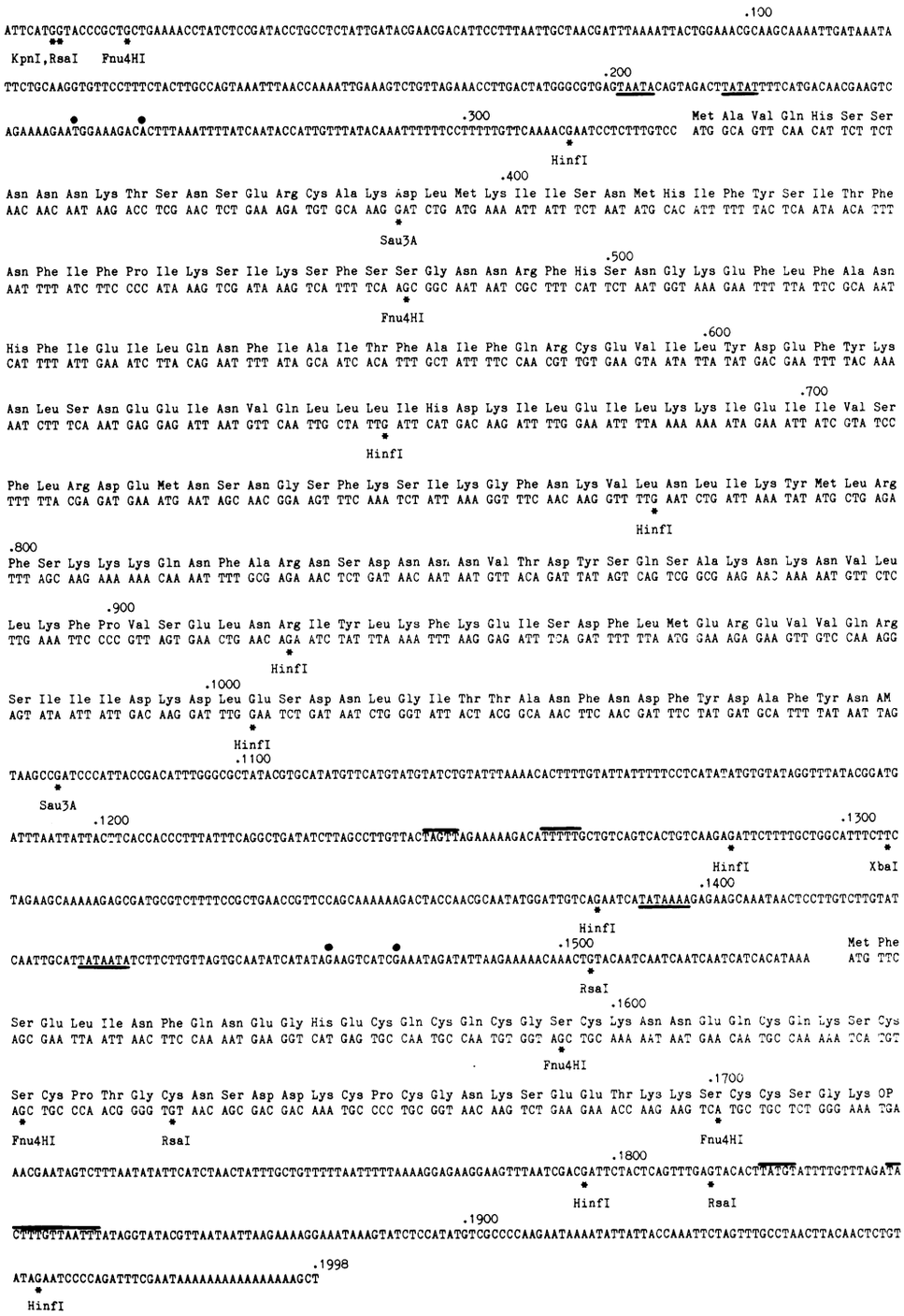

FIG. 3. Primary structure of the *CUP1* locus. Shown is the nucleotide sequence of the basic repeat unit of the *CUP1* cluster (Fig. 1*a*). The numbering starts from the first nucleotide of the amplified unit. ●, Major transcription start sites. Minor start site consensus sequences involved in transcription initiation are underlined, and those involved in transcription termination are overlined. Recognition sites for restriction endonucleases used in nucleotide sequence analysis and transcriptional mapping are also indicated. The repeat unit was subjected to sequence analysis by the dideoxy method (34) after subcloning all of the *Hin*fI and *Rsa* I fragments and the 0.7-kb *Xba* I–*Kpn* I fragment into M13 vectors (35). We have also sequenced from the internal *Sau*3A sites of those fragments. Sequence analysis reactions were done in both directions, at least twice. The predicted amino acid composition of copper chelatin according to that sequence is (expressed as mol %): Cys = 20, Lys = 12, Ser = 13, Met = 2, Phe = 3, Glu = 10, Gln = 8, Gly = 8, Leu = 2, Ile = 2, Asn = 10, His = 2, Pro = 3, Thr = 3, and Asp = 3. The amino acid composition of copper-thionein (26) is: Cys = 20, Lys = 12, Ser = 10, Met = 1, Phe = 0, Glx = 16, Gly = 10, Leu = 1, Ile = 1, Asx = 14, His = 2, Arg = 1, Val = 2, Pro = 5, Thr = 5, and Ala = 2. The published amino acid composition of chelatin (25) is: Cys = 13, Lys = 9, Ser = 5, Met = 1, Phe = 2, Glx = 16, Gly = 12, Leu = 3, Ile = 2, Asx = 11, His = 2, Arg = 4, Asx = 11, His = 2, Arg = 4, Val = 4, Pro = 4, Thr = 4, and Ala = 6.

the protein copperthionein (26). For most residues, there is close agreement between the amino acid compositions predicted by the nucleotide sequence and the ones published by the two independent groups (see legend to Fig. 3). These groups used similar purification schemes and isolated what may be an identical protein, though the derived amino acid compositions are slightly different. We believe the deviations between our predicted sequence and the previously published amino acid compositions of the yeast copper binding protein arise both from experimental difficulties in accurately determining various residues and from impurities present in the preparations. Actually, our predicted amino acid composition agrees best with the average of the two previously determined compositions.

There are two major differences between the yeast copper binding protein and the family of mammalian heavy metal binding proteins, metallothioneins (28). Mammalian proteins contain 30% cysteine, and the yeast protein contains only 20%. There is a complete absence of aromatic amino acid residues in mammalian metallothioneins. In contrast, the yeast protein contains two phenylalanine residues. In view of such differences and the absence of extensive sequence homology to metallothioneins, we prefer to use the name copper chelatin to describe the yeast protein as suggested by Premakumar *et al.* (25), instead of copperthionein (26). Nevertheless, some partial homology exists between copper chelatin and metallothioneins. For example, the sequence Lys-Lys-Ser-Cys-Cys-Ser is common to all proteins. Also there is an abundance of Ser-Cys pairs, and the three most abundant amino acids in these proteins are cysteine, serine, and lysine. We believe those similarities reflect the common function served by these proteins.

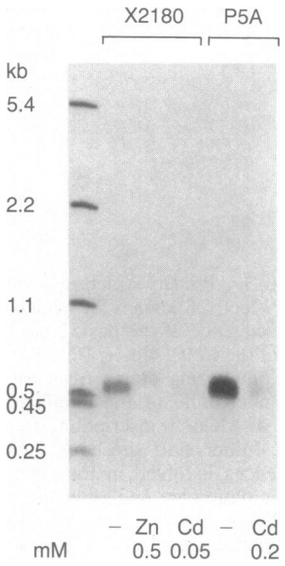

FIG. 4. Effects of Zn^{2+} and Cd^{2+} on chelatin mRNA levels. Yeast cells were incubated with 0.5 mM Zn^{2+}, 0.05 mM Cd^{2+} (strain X2180), or 0.2 mM Cd^{2+} (strain P5A) or in the absence (–) of any added metal. Total RNA was extracted and analyzed by blot hybridization as described in the legend to Fig. 1. The probe used was nick-translated (37) fragment B (see Fig. 1*a*). Numbers to the left are size markers shown in kb.

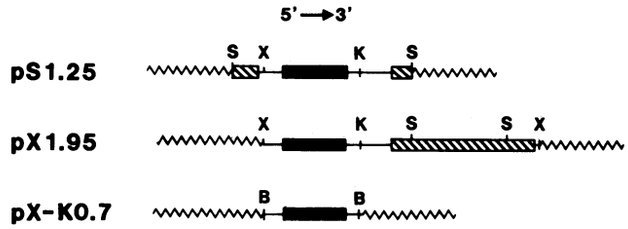

FIG. 5. Structure of copper chelatin plasmids used for transformation. Solid boxes indicate the location of the copper chelatin gene and cross-hatched boxes indicate the location of the larger transcription unit of *CUP1*. Wavy lines indicate plasmid sequences. The following restriction enzyme sites are indicated: *Bam*HI (B), *Kpn* I (K), *Sau*3A (S), and *Xba* I (X). pS1.25 and pX1.95 were constructed by subcloning the 1.25-kb *Sau*3A and the 1.95-kb *Xba* I fragments of JW6 (3) into the *Bam*HI and *Xba* I sites, respectively, of the yeast vector YRP17 (22). pX-K0.7 was constructed the following way: The 0.7-kb *Xba* I–*Kpn* I fragment from JW6 was isolated by electrophoresis on a 5% polyacrylamide gel and electroelution. The isolated fragment was blunt ended by using mung bean nuclease (P-L Biochemicals), ligated to *Bam*HI linkers (Collaborative Research, Waltham, MA), and cloned into the *Bam*HI site of YRP17. Bacterial colonies containing the desired plasmid were identified by a colony screen (41) with the 0.7-kb *Xba* I–*Kpn* I fragment as a probe.

The sequences T-A-T-A-A-T-A and T-A-T-A-A-A are found 26 and 70 nucleotides upstream to the first start site of the chelatin gene (Fig. 3). A multiplicity of T-A-T-A-A-like sequences that are involved in the initiation of transcription (38) have been described earlier for other yeast genes (39). Two similar sequences are found 35 and 50 nucleotides upstream to the start site of the larger transcription unit. The sequence C-A-A-T is repeated four times within the 5′ untranslated region of the chelatin gene. It bears similarity to the RC-A-C-A-C-A (where R = purine) sequence implicated in translation initiation (39). Both transcription units contain close to their predicted ends the sequences T-A-G-T-T·····T-T-T or T-A-T-G-T·····T-T-T implicated in transcription termination (40).

Copper Chelatin Is Not Induced by Cd^{2+} or Zn^{2+}. In contrast to metallothionein, which can bind virtually any heavy metal ion assigned to groups Ib and IIb of the periodic table (28), yeast copper chelatin binds only copper, *in vivo* (26). An interesting property of metallothioneins is their induction by the same heavy metal ions to which they bind (28). Thus, it was of interest to determine whether the mRNA for copper chelatin, known to be highly inducible by copper ions (Figs. 1 and 2), is inducible to any extent by Cd^{2+} or Zn^{2+}. These ions do not bind to the protein *in vivo* (26) but are effective inducers of metallothionein (28). We tested the effect of incubation with either Zn^{2+} or Cd^{2+} on chelatin mRNA levels in two yeast strains, X2180 and P5A. The two strains differ in their *CUP1* locus copy numbers and in their levels of copper resistance (4). We find that unlike incubation with Cu^{2+} ions, which leads to induction of chelatin mRNA (Figs. 1 and 2), incubation with Zn^{2+} or Cd^{2+} does not lead to induction but actually causes a decrease in the basal level of chelatin mRNA in the yeast cell (Fig. 4).

The Chelatin Gene Alone Is Responsible for Copper Resistance. The *CUP1* region is a structural locus responsible for high levels of copper resistance in yeast. As we have demonstrated, this locus actually contains two different genes, one of which encodes copper chelatin, a copper binding protein. However, there is no direct proof that the chelatin gene is the one responsible for copper resistance. We have tested whether a *cup1*^{s} strain will be converted to a copper-resistant phenotype after transformation with plasmids carrying different fragments of the *CUP1* locus. All of the plasmids (Fig. 5) that contain an intact chelatin gene can transform *cup1*^{s} cells with high efficiency to a copper-resistant phenotype (as judged by growth in the presence of 0.3 mM $CuSO_4$). Because both pX1.95 and pX-K0.7 do not contain an intact transcription unit for protein X, this protein does not seem to play an essential role in expressing a copper-resistant phenotype. Transformation with the YRp17 (22) vector itself does not confer increased resistance to copper on the *cup1*^{s} host cells.

DISCUSSION

Yeast's *CUP1* locus is the primary determinant of copper resistance. At the DNA level it consists of a basic repeat unit, 2 kb in length. Sensitive (*cup1*^{s}) strains contain a single copy of this unit, whereas resistant (*CUP1*^{r}) strains contain multiple tandemly iterated units (4). The basic repeat contains two transcription units. The smaller one encodes copper chelatin, a low molecular weight, cysteine-rich, copper binding protein. The other transcription unit contains an open reading frame for a yet unidentified protein. The chelatin gene alone seems to be sufficient for development of copper resistance, when it is present in a sufficiently high copy number. Yet, all copper-resistant strains of *S. cerevisiae* we have examined contain multiple copies of the same basic repeat unit. Therefore, they carry multiple copies not only of the chelatin gene but also of gene X.

The nucleotide sequence of the left junction region between *CUP1* and the flanking single-copy DNA was determined (data not shown). This allowed us to map accurately both ends of the amplified unit. The breakpoints seem to occur in the nontranscribed spacer region, in such a way that functional integrity of both transcription units is likely to be maintained. It is not clear why gene X, which does not seem to be required for copper resistance, is coamplified along with the chelatin gene. Some possible explanations are (*i*) presence of a "hot spot" for recombination 5′ to gene X, which causes the first breakpoint to occur in that region, and (*ii*) amplification of gene X might lead to some selective advantage other than copper resistance. The evolutionary basis for the development of copper resistance is not clear. Copper-resistant strains maintain multiple copies of *CUP1*, even in the absence of selective pressure (24). However, selection for increased copper resistance does lead to further amplification of *CUP1* (4). Nonetheless, it is possible that growth in copper-brewing vats of the original *S. cerevisiae* strains, from which the current laboratory strains were derived (1), provided the pressure for selection of present-day copper-resistant strains.

The organization of the amplified *CUP1* locus resembles the organization of the rDNA locus of *S. cerevisiae* (5, 42),

especially with respect to the location of the junctions. In both gene clusters, the junctions are formed by fusion within the nontranscribed regions of the different genes. By such nonrandom assignment of breakpoints, the transcription units located within the distal repeat units are maintained in a functional state. Such arrangements of tandemly iterated genes might not be unique. Rather, it is probably operative in other tandem arrays, such as DNA encoding rRNA and histone genes (5–16). However, this type of arrangement does not seem to be the case for amplified genes observed under selective pressure in mammalian cells (43), indicating they probably arise by a different mechanism.

Maintenance of functional integrity of the more distal transcription units within a highly amplified tandem array is not critical because the proximal (internal) gene copies are functional. We believe that the organization of the *CUP1* locus is a relic of the first events leading to the generation of tandem repeats. During the early stages of amplification—e.g., when only two or three gene copies are present—the functional integrity of each single copy is highly significant. Otherwise, no selective advantage arises from maintaining such structures. Therefore, our results, and those of Zamb and Petes (42), suggest that tandem gene arrays originate from a primary gene duplication event. After the first duplication, by an undefined mechanism, the gene cluster can expand by unequal sister chromatid exchange. In agreement with this model, we failed to amplify a single copy of the *CUP1* locus. But starting from two copies (introduced by homologous recombination with a cloned *CUP1* present on a plasmid), highly copper-resistant strains were easily obtained. Such strains contain multiple copies of the *CUP1* locus (unpublished results).

Although chelatin's primary structure bears some homology to metallothionein (28), we tend to favor the view that this may reflect convergent evolution. The two proteins provide similar function—i.e., heavy metal binding. However, it is conceivable that the sequence common to the two proteins, Lys-Lys-Ser-Cys-Cys-Ser, is a relic of a "primordial" heavy metal ligand. Unlike metallothionein (28), yeast chelatin is a specific copper binding protein (26) and, accordingly, chelatin mRNA is induced by copper ions but not by zinc or cadmium (Fig. 4). Metallothioneins, on the other hand, are induced by all group Ib and IIb metal ions (28). Cadmium resistance in yeast is encoded by a genetic locus other than *CUP1* (unpublished results). Therefore, unlike mammalian cells, yeast seems to possess several distinct systems, wherein each confers protection against a different heavy metal ion. It is possible that the gene product responsible for cadmium resistance will exhibit greater homology to mammalian metallothioneins.

The ability of various *CUP1* locus fragments carried on the yeast vector YRp17 (22) to transform *cup1*^{s} cells to a *CUP1*^{r} phenotype was tested. We found that maintaining integrity of the large transcription unit is not necessary and that a vector, pX-K0.7, carrying only the short transcription unit encoding chelatin, can efficiently convert cells to a resistant phenotype. There still remains the possibility that the single copy of gene X present in the host may contribute somehow to copper resistance. We also found that the episomal chelatin gene still retains its inducibility by copper (unpublished results).

Although the natural mechanism for generation of heavy metal-resistant cells is by amplification of either the metallothionein (29) or chelatin (4, 24) genes, it is possible to achieve the same phenotype, by transforming the appropriate host with autonomously replicating plasmid vectors carrying either the metallothionein (30) or chelatin genes. Such vectors provide simple, economical, and semidominant selection strategies that can be used for introducing other genes of interest into either mammalian (30) or yeast cells.

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