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Genetics

# Tandem gene amplification mediates copper resistance in yeast

(tandem iteration/repeats/heavy metal)

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**ABSTRACT** **Resistance to copper's toxicity in yeast is controlled by the *CUP1*$^r$ locus. This gene was cloned by transforming sensitive recipients (*cup1*$^s$) with a collection of hybrid DNA molecules, consisting of random yeast DNA fragments inserted into the vector YRp7. Four resistant transformants were studied in detail. Autonomously replicating or integrated by homologous recombination into chromosomal sites, the corresponding plasmids and several subclones confer resistance on sensitive recipients carrying the natural variant allele, *cup1*$^s$. Tetrad analysis and genetic mapping established that integration occurs typically at the *cup1*$^s$ site located 28 centimorgans distal to *thr1*, a chromosome VIII marker. Restriction endonuclease cleavage and electrophoretic mobility studies revealed that the *CUP1*$^r$ locus consists of a tandem array of repetitive units. Each unit is 1.95 kilobases in length and contains single sites for *Kpn* I and *Xba* I and two *Sau*3A sites. The sensitive allele represents one repeat and the resistant allele embraces 15 tandemly arrayed repeat units. Progressive selections in higher copper concentrations establish strains with markedly enhanced resistance. Resistance, we propose, is mediated by a gene amplification mechanism based on unequal sister chromatid exchange.**

Resistance to the toxic effects of heavy metal ions in *Saccharomyces cerevisiae* is mediated by the inducible synthesis of a low molecular weight, cysteine-rich, metal-binding protein—metallothionein. Previous investigations of heavy metal metabolism have centered on the role of metal-binding proteins in the cellular economy, their interactions with metal ions such as cadmium, zinc, mercury, and copper, and their possible function as detoxification agents. A comprehensive review concerning metalloproteins as related to medicine, nutrition, toxicology, and physical biochemistry is available (1). Metallothioneins display strikingly similar amino acid sequences in phylogenetically diverse organisms such as man, horse, crab, and *Neurospora* (1–3). Nearly one-third of the approximately 60 amino acids in the metallothionein molecule are cysteines, and the locations of these residues are virtually constant within the protein sequence. The metallothionein molecule exhibits a remarkable conservatism in evolution.

Naturally occurring copper-sensitive (*cup1*$^s$) and copper-resistant (*CUP1*$^r$) variant yeast strains were described in 1955 (4). Genetic analysis of tetrads from sporulated cultures of crosses between *CUP1*$^r$ and *cup1*$^s$ strains demonstrated that copper resistance is mediated by a single gene. Subsequently, these polymorphisms served extensively as markers in several fundamental studies on gene conversion (5–7). The *cup1*$^s$ allele renders yeast cells sensitive to 0.3 mM $CuSO_4$ on synthetic complete plates. Isogeneic resistant cells grow confluently on agar plates containing copper at concentrations up to 1.75 mM. The *CUP1*$^r$ gene is semidominant and the *CUP1/cup1* heterozygote displays a resistance level (1.0 mM) intermediate between that of the two homozygotes. Located on chromosome VIII, 42 centimorgans (cM) distal to the centromere, the *CUP1* locus delineates a region of high positive chiasma interference indicated by a second division segregation frequency of 76% (8).

When *cup1*$^s$ cells become disomic for chromosome VIII, they manifest enhanced copper resistance and grow confluently on plates containing 0.3 mM copper, a concentration inhibitory to the growth of the parental *cup1*$^s$ haploid cells. Disomic derivatives arise spontaneously as papillae on a nongrowing *cup1*$^s$ background. *CUP1*$^r$ cells show a similar dosage effect. Strains disomic for chromosome VIII but carrying a *CUP1*$^r$ locus grow on agar plates containing copper at 2.0 mM, a concentration inhibitory to the growth of the parental haploid.

Selection for resistance to progressively higher copper concentrations yields a family of isolates with graded resistance levels up to 12 mM $CuSO_4$, approximately a seven-fold increase. Although the copper resistance levels of some isolates are unstable, most retain their resistance capabilities even under nonselective conditions and after numerous vegetative transfers. We propose that the enhanced resistance levels reflect gene amplification that occurs as a consequence of sister chromatid exchange. The observed effect is reminiscent of the amplification reported by Schimke and co-workers for methotrexate resistance in mammalian cells (9, 10). This effect may be diagnostic for shifts in chromosomal aneuploidy as well as increases in the extent of tandem iterations at a specific genetic locus.

Our recent knowledge concerning gene amplification—i.e., increase in the copy number of a specific DNA sequence per cell—has been enlarged by the analytical capabilities inherent in recombinant DNA technology. Four major classes of amplified DNA sequences are commonly encountered: Highly repetitive and widely dispersed repeats of unknown function have been found in human (11, 12) and sea urchin DNA (13). Second, changes in gene dosage may reflect differential DNA replication at specific loci as in egg chorion proteins (14). Gene dosage shifts may also be occasioned by autotetraploidy accompanied by segmental aneuploidy as in cadmium resistant mouse cell lines (15). Finally, in contrast with these organizational modes, there are gene arrays coding for rRNA in yeast and *Drosophila* (16, 17), yeast $\alpha$ mating type factor (J. Kurjan and I. Hershkowitz, personal communication), the amplified dihydrofolate reductase (DHFR) gene in mice (9, 10), and the *CAD* gene of hamster cells that provides resistance to an inhibitor of aspartate transcarbamoylase, *N*-(phosphonacetyl)-L-aspartate (18). These sequences are amplified by means of iterated tandem arrays within a single localized chromosome region.

The present study concerns the molecular mechanism of resistance to copper in *S. cerevisiae*. We report here the isolation, purification, and characterization of cloned yeast DNA fragments that confer copper resistance on otherwise genetically

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Abbreviations: cM, centimorgans; kb, kilobase(s); bp, base pair(s).

sensitive cells. These cloned DNA fragments contain one or more tandemly repeated DNA sequences with a unit length of 1.95 kilobases (kb). The repeat units are tandemly arrayed within the yeast chromosomal *CUP1*$^r$ locus and copper-resistant yeast strains such as X2180-1A contain as many as 15 iterated repeats.

## MATERIALS AND METHODS

**Strains and DNAs.** DNA transformations were performed with the haploid *S. cerevisiae* strain BZ31-1-7Ba, which carries *cup1*$^s$ and the following auxotrophic markers: *trp1-289*, *ura3-52*, *ade8-18*, and *arg4-16*. Copper resistance (*CUP1*$^r$) genes were selected from a pool of hybrid DNA prepared by Nasmyth and Reed (19), and provided by Terrance Cooper. This pool was prepared from a *Sau*3A partial digest of AB320 yeast DNA inserted into the *Bam*HI site of the plasmid YRp7 and cloned. The genomic DNA used in the construction of this hybrid pool was prepared from strain AB320 (*HO*, *ade2-1*, *lys2-1*, *trp5-2*, *leu2-1*, *can1-100*, *ura3-1*, *met4-1*) by Maynard Olsen. This strain is a segregant from hybrid W87 (20) and it probably is closely related to haploid X2180. *Escherichia coli* strain HB101 was used for bacterial transformations.

The YRp7 vector is a pBR322 derivative that carries a 1.43-kb *Eco*RI segment containing the *TRP1*$^+$ yeast gene and sequences capable of conferring autonomous replication in yeast (*ars1*) (21). The YRp17 vector contains the *URA3*$^+$ yeast gene at the *Ava* I site of pBR322, as well as the 1.43-kb *TRP1*$^+$ *Eco*RI fragment. This vector was a gift from Ronald Davis.

**Cultivation of Microorganisms.** *E. coli* was grown in LB medium (22). Ampicillin was added (50 μg/ml) when plasmid-containing cells were grown. Yeast culture conditions and handling followed published procedures (23). Copper medium (0.3 mM $CuSO_4$) is a synthetic complete medium solidified with 1.5% Phytagar (GIBCO). Synthetic complete medium is a comparatively rich, chemically defined medium that permits confluent growth for most auxotrophic mutants.

**DNA Transformations and Preparations.** Yeast transformations were according to Hinnen *et al.* (24) with the following exception: after resuspension in 1 M sorbitol and 50 mM $NaPO_4$, pH 7.5, cells were treated at 30°C for 1 hr with 0.1% 2-mercaptoethanol and lyticase (gift from R. Schekman) at 40 units/ml. *E. coli* transformations were performed as described (22, 25). Rapid DNA preparations were according to Struhl *et al.* (21). DNA subcloning procedures were according to Davis *et al.* (22). Agarose gels (0.7%) were prepared in 40 mM Tris/20 mM acetic acid/2 mM $Na_2EDTA$, pH 8.2, containing ethidium bromide at 0.5 μg/ml, and the electrophoresis was run in a horizontal gel apparatus at 1–1.5 V/cm (22, 26). DNA bands were retrieved from gels by electrophoretic collection on Whatman DE 81 DEAE-cellulose filters (27). Elution in 1 M NaCl was followed by ethanol precipitation. Genomic DNAs for DNA·DNA hybridization were prepared according to Struhl *et al.* (21), separated on 0.5% agarose gels by electrophoresis, and transferred to cellulose nitrate as in Southern's procedure (28). These were hybridized with a nick-translated $^{32}P$-labeled probe prepared from the 1.25-kb *Sau*3A DNA fragment (22, 29); see Fig. 1.

**Notation.** YJW6, -9, -10, and -11 denote yeast strains containing plasmids (pJW6, -9, -10, and -11). The cloned DNA sequences are designated JW6, -9, -10, and -11 corresponding to the inserts contained within the plasmids.

## RESULTS

The copper-sensitive (*cup1*$^s$) recipient haploid yeast strain BZ31-1-7Ba was transformed to *TRP1*$^+$ with a collection of hybrid DNA molecules, consisting of random yeast DNA fragments inserted into the YRp7 vector. This pool is described in *Materials and Methods* and ref. 19. Of nearly 500 *TRP1*$^+$ transformant colonies, 12 grew confluently when transferred to 0.3 mM copper plates, a concentration completely inhibitory to the growth of the recipient yeast strain BZ31-1-7Ba. Insert-bearing plasmids, autonomously replicating in yeast, were detected in four isolates: YJW6, YJW9, YJW10, and YJW11, and these were subsequently used to transform *E. coli*. The resultant ampicillin-resistant tetracycline-sensitive bacteria were grown for large scale plasmid preparations. Plasmid DNAs were analyzed by

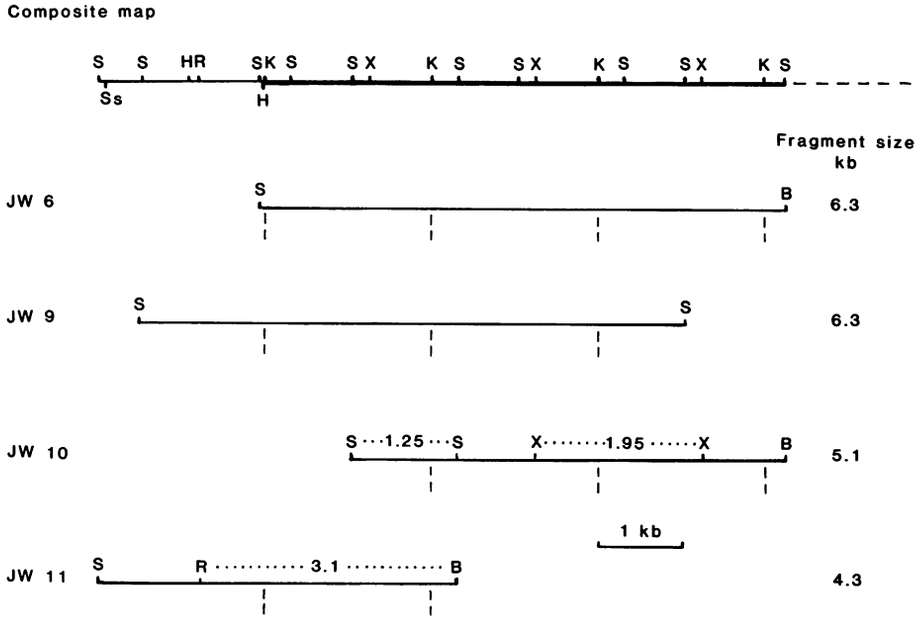


FIG. 1. Restriction maps of JW6, JW9, JW10, and JW11, which carry, respectively, three, two, two, and one *Kpn* I repeat units, indicated on the fragments by vertical broken lines. The known extent of the repetitious sequences is indicated by the thick black line. Further extension of the repetitive DNA is indicated by the horizontal broken line. The subcloned fragments and their lengths in kb are shown (dotted lines). Restriction endonuclease sites: B, *Bam*HI; H, *Hin*dIII; K, *Kpn* I; R, *Eco*RI; S, *Sau*3A; Ss, *Sst* I; X, *Xba* I.

restriction endonuclease digestion.

One inserted fragment (JW6) was cleaved by three enzymes: *Hin*dIII, *Xba* I, and *Kpn* I. Located approximately 50 base pairs (bp) away from the left-most *Kpn* I site, the unique *Hin*dIII restriction site defines one terminus of the iterated segment within the cloned DNA fragment (see Fig. 1). The pJW6 digests prepared with *Xba* I and *Kpn* I displayed two bands on agarose gels that sum to less than the lengths of the insert and the YRp7 vector. In addition, the two bands produced with pJW9, -10, and -11 do not have equal intensities. Although a linear form of pJW6 is 12.1 kb in length—i.e., 5.8 kb (vector) + 6.3 kb (insert)—in both *Xba* I and *Kpn* I digests, the observed bands sum to only 8.15 kb (6.2 kb and 1.95 kb). In addition, digestion with the restriction endonuclease *Sau*3A yields the result shown in Fig. 2. The 0.7-kb and the 1.25-kb restriction fragments are in molar excess compared to the vector DNA fragments. Similarly, electrophoresis of *Dde* I and *Dpn* I restriction enzyme digests also exhibit unique nonvector bands of increased fluorescence intensity.

To account for these collective observations, as well as the high frequency of copper-resistant colonies among transformants—i.e, about 2%—we postulate that the chromosomal region controlling the level of copper resistance is composed of tandemly iterated 1.95-kb DNA segments. Each repeat unit contains single *Kpn* I and *Xba* I sites along with two *Sau*3A/*Dpn* I sites containing methylated adenine residues.

The remaining three plasmids (pJW9, pJW10, and pJW11) include DNA inserts that also confer resistance to copper. Their restriction fragment patterns are similar to the pattern displayed in Fig. 2 by pJW6. However, as shown in Fig. 1, plasmids pJW9 and pJW11 contain longer, nonrepetitious DNA segments that extend beyond the terminal *Hin*dIII site. From these clones alone, we cannot estimate the number of the repeated segments that make up the $CUP1^r$ locus in the resistant strain from which the DNA pool was prepared. Data from the other eight transformants may provide useful information on this issue. Within the repeat sequence there are no sites for the following restriction enzymes: *Ava* I, *Bam*HI, *Bgl* II, *Eco*RI, *Hae* III, *Hin*dII, *Hin*dIII, *Hpa* I, *Pst* I, *Pvu* I, *Sal* I, and *Sst* I. Taken collectively, the present data based on restriction enzyme mapping indicate that the cloned DNA fragments capable of conferring resistance to copper in *S. cerevisiae* embrace three and a fraction of a fourth repetitive 1.95-kb units.

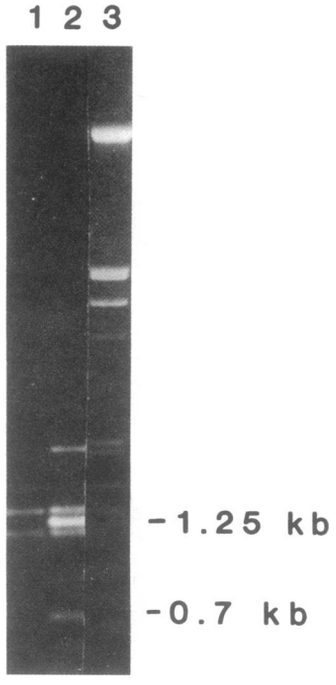


FIG. 2. Identification of *Sau*3A fragments. Lane 1 is vector YRp7; lane 2 is vector with insert JW6; lane 3 is λ DNA standard digested with *Hin*dIII/*Eco*RI. The bright bands in lane 2 are the two *Sau*3A fragments that make up the repeat unit 1.25 kb and 0.7 kb.

With a view towards delineating the smallest DNA fragment that would impart resistance to increased copper concentrations, we subcloned several DNA fragments. Fig. 1 illustrates the various subcloned DNA fragments. The 1.25-kb *Sau*3A restriction fragment constituting less than one basic repeat unit and the 1.95-kb *Xba* I fragment, one repeat unit, were collected from pJW10 digests. When pJW11 is cleaved with *Eco*RI and *Bam*HI a 3.1-kb fragment of yeast DNA containing more than one repeat unit and 800 bp of nonrepetitive DNA sequences is generated. These three DNA fragments were inserted into the vector YRp17 ($URA3^+$, $TRP1^+$, and $ARS1^+$), subcloned, and used to transform a copper-sensitive ($cup1^s$) yeast strain (BZ31-1-7Ba) to $TRP1^+$ and $URA3^+$.

In each instance, the recipient cells, transformed to $TRP1^+$ and $URA3^+$, displayed resistance to 0.3 mM copper. The 0.7-kb *Sau*3A fragment remains to be evaluated in the same manner. When the transformed yeast cells were grown under nonselective conditions, they exhibited the phenotypic instability associated with autonomously replicating plasmids—i.e., high frequencies of $cup1^s$, $trp1^-$, $ura3^-$ cells were regenerated. Resistance to copper ions can be conferred by the presence of the 1.25-kb *Sau*3A fragment. The copper resistance phenotype is likely to be associated only with that fragment. If the 1.25-kb *Sau*3A DNA fragment contains sequences homologous to the $CUP1^r$ locus, it should confer upon the autonomously replicating plasmid the ability to recombine into that same chromosomal locus.

We isolated several clones in which copper-resistance DNA regions had been integrated into yeast chromosomal DNA in various forms. The first, a 3.1-kb (JW11, *Eco*RI/*Bam*HI) DNA fragment recloned in YRp17, integrated near the *ura3-52* site close to the chromosome V centromere via the *URA3* sequence present in the vector molecule. This integrant was crossed to a strain carrying a wild-type allele at *ura3*. Sixteen four-spored asci were recovered and subjected to tetrad analysis. All exhibited a $4^+:0^-$ segregation pattern for the uracil requirement. The second, pJW10, integrated at *trp1-289*. The integrant at *trp1-289* was also crossed to a wild-type strain and then subjected to meiotic analysis. Twenty-two complete tetrads yielded a $4^+:0^-$ segregation for the tryptophan requirement. The third, pJW9, recombined at the *cup1* site on chromosome VIII. This presumptive integrant was crossed to a strain genetically marked on chromosome VIII by the presence of *thr1*. The resultant diploid was sporulated and 61 unselected asci were subjected to tetrad analysis (30). The markers $cup1^s$ and *trp1* cosegregated $2^+:2^-$ in all tetrads. Complete linkage was observed for these markers and recombinants between them were not detected. This behavior is expected if a DNA fragment bearing the $CUP1^r$ locus, inserted into the YRp7 plasmid containing $TRP1^+$, integrated near the *cup1* site. Parental ditypes (PD), nonparental ditypes (NPD), and tetratypes (T), 30:0:31, were found for either *CUP1* and *THR1* or for *TRP1* and *THR1*. Five additional independent integrants of pJW10 were crossed, and a total of 84 unselected asci were analyzed. The PD:NPD:T ratio for these same markers was 37:0:47. The complete absence of nonparental ditypes in a combined total sample of 145 tetrads (67:0:78) provides a clear indication of gene–gene linkage and the calculated map distance between *CUP1* or *TRP1* and *THR1* is 27 cM, an estimate well within the sampling limits

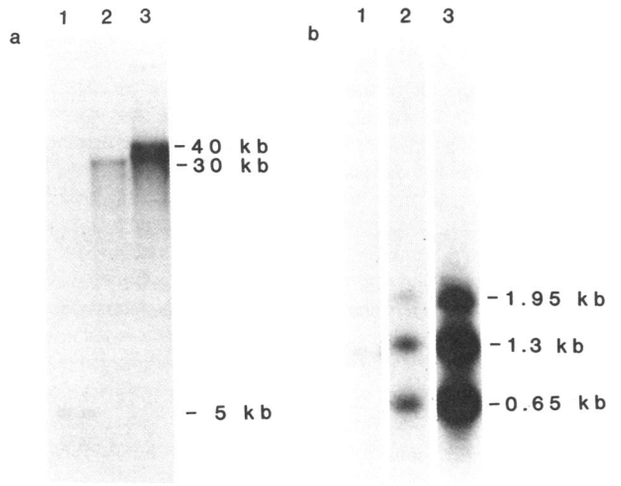


FIG. 3. DNA·DNA hybridization of $^{32}$P-labeled *Sau*3A 1.25-kb probe (see *Materials and Methods* and Fig. 1) involving *Eco*RI-digested (*a*) and *Xba* I/*Kpn* I double digested (*b*) genomic DNA from copper-sensitive cells (*cup1*$^s$) in lanes 1, copper-resistant cells (*CUP1*$^r$) in lanes 2, and hyper-resistant cells in lanes 3. In *b*, lane 1 contains only the 1.3-kb band, even under conditions of overexposure. The three lanes in *b* are all from the same Southern hybridization.

of the published value of 28 cM (31). Considered collectively, these data establish that the cloned DNA fragments confer a stable copper resistance phenotype on *cup1* sensitive recipient cells, and that the fragments contain functional segments of the *CUP1*$^r$ locus located distal to the *thr1* marker in chromosome VIII.

In an attempt to determine the upper limit of the gene copy number, we conducted an analysis by Southern's (28) DNA·DNA hybridization method using a nick-translated $^{32}$P-labeled probe prepared from the 1.25-kb *Sau*3A DNA fragment. Genomic DNA prepared from copper-sensitive and copper-resistant yeast cells was digested with *Eco*RI, a restriction enzyme for which there are no cleavage sites within the repeat unit. We find that our *cup1*$^s$ strain carries a hybridizable segment of 5 kb. This represents a single 1.95-kb repeat unit in addition to nonhomologous flanking sequences. This is compared to the approximately 30-kb segment composed of about 15 tandemly iterated repeat units in X2180-1A, a *CUP1*$^r$ strain, and the approximately 40-kb segment or about 20 repeat units in a hyperresistant strain, JWP5A. See Fig. 3*a*. *Xba* I or *Kpn* I digests of *CUP1*$^r$ genomic DNA contain 1.95-kb hybridizable fragments. These indicate repetitive restriction enzyme sites. Digested *cup1*$^s$ genomic DNA fails to exhibit a 1.95-kb band; thus, sensitive cells have one repeat unit with only single *Xba* I and *Kpn* I sites. However, double digestion with *Kpn* I and *Xba* I generates a 1.3-kb fragment from within the repeat unit. We expect one such fragment per *cup1*$^s$ genome, about 15 copies per *CUP1*$^r$ genome, and about 20 copies per hyperresistant genome. The 0.65-kb fragment spans the junction between repeat units. Fig. 3*b* depicts a semiquantitative *Kpn* I/*Xba* I double digest. The 1.95-kb band constitutes a partial digestion product.

## DISCUSSION

Ubiquitous, small, and cysteine-rich proteins, the metallothioneins, immobilize heavy metals such as zinc, cadmium, mercury, and copper. Composed of about 60 amino acid residues, these proteins play an important role in regulating heavy metal metabolism and in cellular detoxification (1). Beach and Palmiter (15) found that, with respect to the mouse *MT-1* (metallothionein) locus, cadmium-resistant cells have 14-fold more mRNA, a higher rate of gene transcription, and 6 times as many gene copies compared to sensitive cells. Resistant cells are virtually tetraploid and in addition contain three small chromosomes not encountered in ordinary sensitive diploid strains. Accordingly, the observed gene amplification occurs as a consequence of increase in both the number of whole chromosomes and the number of specific chromosome segments—i.e., via autotetraploidy and segmental aneuploidy.

The present study reports on the molecular cloning and isolation of a tandemly iterated segment of genomic DNA from *S. cerevisiae*. The fundamental repeat unit, 1.95 kb, confers a phenotype of copper resistance to otherwise sensitive yeast cells. This phenotypic alteration occurs when the plasmid is present either as an autonomously replicating molecule or as a unit stably integrated into a yeast chromosome. We have observed homologous integrations into three different chromosomal sites—*ura3-52*, *trp1-289*, and *cup1*$^s$. Approximately the same level of resistance enhancement is observed in each instance and no evidence for a position effect in the expression of copper resistance has been found. Three and part of a fourth tandemly arrayed units have been identified within the cloned DNA fragments. These DNA sequences were shown to be homologous to the *CUP1* locus of yeast chromosome VIII because the insert-bearing vector undergoes homologous recombination and integration at this genetically defined position.

Our accumulated data on cloning and analysis by restriction mapping provides only a minimal estimate for the copy number of the sequence responsible for copper resistance. For a more accurate copy number estimate, we analyzed *Eco*RI-digested genomic DNA from copper-sensitive and copper-resistant cells by Southern's (28) DNA·DNA hybridization method, using a nick-translated $^{32}$P labeled probe prepared from the 1.25-kb *Sau*3A DNA fragment. The probe hybridizes to a 5-kb segment from *cup1*$^s$ cells compared to about 30 kb or about 15 repeat units in the *CUP1*$^r$ strain. Thus, sensitive and resistant strains may differ from each other not with respect to the kind of DNA sequence at the locus, but rather in the number of iterated units, and consequently exhibit enhanced resistance levels directly proportional to the number of repeat units. These sequences span considerably more than the 200–300 bp required to code for a metallothionein of molecular weight about 7,000. Other functions may be encoded in the repeat unit sequence. We may expect to find segments that modulate the expression and regulation of the structural gene. Also, it is plausible that the repeat unit may contain several functional genes.

The genetic behavior of some tandemly repeated DNA sequences has been reported. Szostak and Wu (32) and Petes (16) demonstrated that tandemly arrayed clusters of some 100 genes for rRNA exhibited a high frequency of unequal sister chromatid exchange in both meiotic and mitotic cell division. In addition, for these repeated sequences, Petes (16) showed that meiotic crossing-over or reciprocal exchange is strongly suppressed during meiosis. Though 100–140 adjacent genes occupy this region, the recombination frequency within the cluster is only 0.05 per meiosis. Some 3.5–5 exchanges would be expected because the ribosomal DNA region corresponds to 5–10% of the genomic DNA with a total map length of 4,500 cM (31). Other studies on meiotic recombination within a tandem array of eight *LEU2* genes, by Petes and Klein (33), established that these iterated sequences display more sister-strand than homologous chromosome recombinational events compared to single-copy genes, and these events occur at frequencies proportional to the copy number of the relevant genetic element. Also, they hypothesized that sister-strand and chromosome exchanges occur competitively. These theoretical constructs and experimental findings impinge significantly on our notions concerning the

molecular and genetic basis for heavy metal resistance in yeast.

Variations in the number of repeat units that confer copper resistance could arise readily via unequal crossing-over between homologous chromosomes or sister chromatids. Also, in addition to classical gene conversion (30) and intrachromosomal gene conversion, reported for nontandem duplications (33–36), selection provides a reliable mechanism for maintaining genetic homogeneity among the repeated segments, especially in view of Fogel and Mortimer's (37) demonstration that gene conversion operates as an essentially conservative process marked by a high fidelity down to the level of a single nucleotide. Accordingly, unequal crossing-over between homologues or sister chromatids and intrachromosomal gene conversion are themselves sufficient to generate tandem linear arrays of variously amplified, homogeneous repeat units.

The *CUP1* locus is situated 40–50 cM from the centromere of chromosome VIII and it delimits a region of high chiasma interference. Also, it is flanked by the outside markers *thr1* and *cdc 12*, respectively 28 and 15 cM removed from *CUP1*. Accordingly, these circumstances should allow us to investigate the recombinational profile of a genetic sequence that is amplified by tandem iterations. We are especially concerned with the regularities of sister chromatid exchange, intrachromosomal gene conversion, postmeiotic segregation, and "unequal" crossing-over.

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