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Production of metallothionein in copper- and cadmium-resistant strains of *Saccharomyces cerevisiae*

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SUMMARY

Certain mutants of the yeast *Saccharomyces cerevisiae* show copper or cadmium resistance. Both copper- and cadmium-resistant strains produce the same metallothionein with 53 amino acid residues which causes metal detoxification by chelating copper or cadmium. The metal detoxification role is the only known function of the metallothionein in yeast. The MT is encoded by the *CUP1* gene on chromosome VIII which is expressed by induction with metals. The *CUP1* is amplified to 3–14 copies with 2 kb-tandem-repeat units in the metal-resistant strains, whereas the wild-type strain contains only a single copy of the *CUP1*. Although transcription of *CUP1* is inducible by metals, the ACE1 protein serves a dual function as a sensor for copper and an inducer for *CUP1* transcription in the copper-resistant strain. In the cadmium-resistant strain, the heat-shock factor having a point mutation may be the regulator for *CUP1* transcription. Therefore, it has been clarified that production of MT in yeast is controlled by two systems, the amplification of *CUP1* and its transcriptional regulation.

METALLOTHIONEIN

Metallothionein (MT) was first isolated from horse kidney cortex as a zinc- or cadmium-binding protein. The protein is now known to be present in many organs of all vertebrates and has also been found in higher plants, eukaryotic microorganisms and some prokaryotes. MT is a ubiquitous family of cysteine-rich proteins which have a low-molecular weight of under 10 000 Da and which bind heavy metals such as zinc, copper, cadmium or silver [25].

Mammalian MTs are composed of approximately 61 amino acid residues with a molecular weight of 6000–7000 Da. They contain 20 cysteine residues in a configuration of Cys-X-Cys or Cys-Cys and no aromatic amino acids or histidines. Metals are associated with the MT exclusively through thiolate bonds to all cysteine residues. The MT consists of two domains which constitute the β-domain of amino acids residue 1–30 and the α-domain of the residue 31–61. The α-domain contains 11 cysteines, binding four zinc or cadmium ions or five to six copper ions, and the β-domain contains nine cysteines, binding three zinc or cadmium ions or six copper ions. The metals can be removed by exposure to low pH and the resulting apoprotein can also rebind to the above metals.

In mammalian cells, the synthesis of MT is induced by numerous substances including metals such as zinc, cadmium or copper, or steroid hormones, interferon, interleukin, cyclic AMP, lipopolysaccharides, protein kinase, etc. Regulation of MT synthesis occurs at the transcriptional level. Many transcriptional regulation proteins as a *trans*-acting factor are found which mediate through a specific DNA sequence in the promoter region of the MT genes.

Although MT was discovered 35 years ago, the biological functions specific to these proteins still remain a topic of discussion. The function of MT has been proposed to involve roles of metal metabolism such as transport, storage and especially detoxification of heavy metals ability in cells. Recently, many biological functions of MT have been suggested in animals. For example, the scavenging ability for active oxygen species, radicals and alkylating reagents has been an anticipated role for MT in protection of the body, and this property has been related to resistance to anticancer agents and has been utilized to prevent adverse effects in cancer chemotherapy [42]. A growth inhibitory factor has been isolated from human brain tissue and recently identified to be a new MT; this MT is down-regulated in Alzheimer's disease [41].

However, these new findings do not preclude the function of detoxification of heavy metals by MT. For example, some of the metal-resistant strains of yeast, such as *Saccharomyces cerevisiae* or *Candida glabrata*, produce MT when induced by metals [21,22,28,29]. The metal-detoxification role is known as only one physiological function of MT, and other functions of MT in yeast cells are not yet known [17]. The objectives of this review are to present recent information on MT and the amplification and expression of its structural gene, *CUP1*, in copper- and cadmium-resistant strains of *S. cerevisiae*.

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DETOXIFICATION OF METALS BY MT

Metal-resistant strains of S. cerevisiae

Heavy metals can be classified into two categories according to their biological function in microorganisms. Certain heavy metals such as copper and zinc, which participate in a variety of enzymatic reactions, in oxidation and in electron transfer, are essential trace elements for normal physiological functions of microorganisms. Even these essential metals inhibit normal metabolism if their intracellular concentrations rise above physiologically required levels. In contrast, other metals such as cadmium and mercury are nonessential for biological functions and serve as strong potent inhibitors of microorganisms even at low concentrations in the cells. In yeast cells, growth is hindered at the metal concentration level which inhibits normal metabolism, and cells die at concentrations higher than this. However, at such metal concentrations, certain strains survive through some metal-detoxification mechanisms. The survival of cells depends on their ability to limit the intracellular concentration of the heavy metals. The detoxification mechanism for the metal is achieved by regulating uptake and/or efflux, or by intracellular compartmentation, or sequestration [24,27,31,40]. One of the major molecules involved in intracellular sequestration of metals in *S. cerevisiae* is MT.

Copper- and cadmium-resistant strains of *S. cerevisiae* were isolated from wild-type (metal-sensitive) strains which could produce a large amount of MT under induction by the metals. The MT of *S. cerevisiae* was first isolated from a copper-resistant strain. The copper-resistant strain also showed resistance to silver but not to cadmium, nickel, cobalt, or mercury [11]. The cadmium-resistant strain [38], which was isolated as a strain growing in a medium containing 0.5 mM cadmium sulfate, also shows resistance to copper or silver but not to the other metals. The production of MT in *S. cerevisiae* has been detected only in copper- or cadmium-resistant strains. It is concluded that the metal-chelating ability of MT is the detoxification mechanism conferring metal resistance in these strains [28].

MT of yeast

The apoprotein of copper MT isolated from the copper-resistant strain is a 53-residue polypeptide of 5655 Da containing 12 cysteine residues and no aromatic amino acids [47]:

Gln-Asn-Glu-Gly-His-Glu-Cys-Gln-Cys-Gln-Cys-Gly-Ser-
Cys-Lys-Asn-Asn-Glu-Gln-Cys-Gln-Lys-Ser-Cys-Ser-Cys-
Pro-Thr-Gly-Cys-Asn-Ser-Asp-Asp-Lys-Cys-Pro-Cys-Gly-
Asn-Lys-Ser-Glu-Glu-Thr-Lys-Lys-Ser-Cys-Cys-Ser-Gly-Lys

Yeast MT has little primary sequence homology with mammalian MT except for a highly conserved region of Lys-Lys-Ser-Cys-Cys-Ser, but it is interesting that it has the sequences of Cys-X-Cys and Cys-X--X-Cys for metal ligands. Although Cu(II) ions are reduced to Cu(I) ions in the cells, this yeast MT molecule binds eight atoms of Cu(I) whereas mammalian MT can bind 12 atoms of Cu(I) [18]. The apoprotein molecule which removed copper can rebind eight atoms of Ag(I), or four atoms of Cd(II) or Zn(II) in vitro [6]. The yeast MT forms only one metal cluster, though the mammalian MT forms two metal clusters in each α- and β-domain [50].

On the other hand, the apoprotein of cadmium MT, which was isolated from the cadmium-resistant strain, is the same protein as copper MT from the copper-resistant strain, and can bind four atoms of Cd(II) [21]. Hence, the MTs which are produced by copper- and cadmium-resistant strains are the same protein, although the metal binding ability is specific to each resistant strain. As described previously, the copper-resistant strain shows resistance to copper and silver, and the cadmium-resistant strain shows resistance to cadmium, copper and silver. Based on these results, the resistance is concluded to be due to the ability to produce a large amount of MT depending on the metal concentration in the cells; the MT produced detoxifies the metal by chelation in the copper- and cadmium-resistant strains.

Does MT then not have any function other than metal detoxification in *S. cerevisiae*? A mutation resulting in a complete deletion of the MT gene showed little effect on vegetative growth and the cell cycle in a medium without metals, although the mutant cannot produce detectable MT and does not show any metal resistance [17]. That is to say, the metal-detoxification function is the only known function in yeast, whereas many biological functions of MT are suggested in animals [48].

AMPLIFICATION OF THE MT GENE

Structural gene of MT

Many animal MTs had first been studied with regard to protein structure; subsequent research interests have advanced to the molecular genetics of the MT gene, such as cloning and expression mechanisms. In *S. cerevisiae*, a genetic study of the copper-resistant strain was first reported nearly forty years ago [2]. Extensive genetic analysis of tetrad spores from the diploid of crosses between resistant and sensitive strains demonstrated unequivocally that copper resistance was mediated by a single gene designated *CUP1*. This occurred before the first isolation of MT from horse kidney. *CUP1* was mapped at a 28-cm distance from the marker *thr1* on chromosome VIII. Some time later it was suggested that *CUP1* is the structural gene of MT which determines the copper resistance of *S. cerevisiae*. Subsequently, the DNA fragment encompassing *CUP1*, which can transform the wild-type strain to copper resistance, was cloned by two independent research groups [4,5,26]. Base sequence analysis indicated that the *CUP1* encoded a 61-amino acid polypeptide which binds the octapeptide of Met-Phe-Ser-Glu-Leu-Ile-Asn-Phe to the N-terminal of the sequence of copper MT described previously. Therefore, this 8-amino acid residue may be removed by processing after translation, and the mature MT of 53 amino acid residues is formed in vivo. However, the mechanism or biological meaning of this process to form mature MT is not yet known.

Amplification of CUP1

Genomic DNA from the wild-type (metal-sensitive) and copper-resistant strains of *S. cerevisiae* was digested with

restriction enzymes *Eco*RI or *Kpn*I, separated by electrophoresis and analyzed by blot hybridization to a *CUP1* specific probe [12]. DNA from the wild-type strain generated a band of 2.7 kb on digestion with *Kpn*I and a band of 5.2 kb on digestion with *Eco*RI. On the other hand, DNA from copper-resistant strains generated a band of 2.7 kb and a more intense band migrating at 2.0 kb on digestion with *Kpn*I, and a band of 23 kb, 25 kb or 31 kb on digestion with *Eco*RI. Based on these results and restriction maps of the DNA fragment encompassing *CUP1*, it became clear that the *CUP1* exists as 10–14 tandem repeated copies on chromosome VIII in copper-resistant strains, although the wild-type *S. cerevisiae* strain contains only a single copy of *CUP1* [44]. As indicated in Fig. 1, the 2.0-kb (the true figure is 1998 bp) fragment, including *CUP1*, is identical in size to the basic repeat units, and there is a single site for the *Kpn*I and no site for *Eco*RI in the basic repeat unit. Therefore, the number of tandem-amplified repeating units in the copper-resistant strain is determined from the following expression [12]:

Copy numbers $= (A - 3.2 \text{ kb}) \div 2.0 \text{ kb}$
A: Length of *Eco*RI fragment
3.2 kb: Length of flanking region (0.8 kb + 2.4 kb)
2.0 kb: Length of repeat unit (length of *Kpn*I fragment)

Subsequently, it became clear that the repeating units of 2.0 kb further contain one gene with 750 bases for a protein besides *CUP1* [4]. Although the gene product has not been isolated yet, it is expected that the protein has some function for metal resistance because of simultaneous amplification of the gene with *CUP1*.

The numbers of tandem repeats in the copper-resistant strains of *S. cerevisiae* are not constant, and the copy numbers of *CUP1* are variable from 3–14 between the resistant strains [9]. Although amplification of the MT gene has been reported in animals, amplification of *CUP1* in yeast shows some other interesting features [13,23]. In the resistant strain, resistance to copper increases as copy numbers of *CUP1* increase. Therefore, copper resistance is directly proportional to the copy numbers of *CUP1* on chromosome VIII in yeast. It was also confirmed that the transcription level of *CUP1* (level of mRNA of *CUP1*) also varied proportionally with the copy numbers of *CUP1* [16]. This result also supports the contention that the function of MT is a metal-detoxification role. On the other hand, the cadmium-resistant strain also has an amplified *CUP1* gene with 2.0 kb-repeating units as does the copper-resistant strain [39]. It may be concluded that *CUP1* encoding MT is amplified with a tandem repeat on the chromosome; a large amount of MT is then produced by expression of the *CUP1*s, and detoxifies the metal by chelation in both copper- and cadmium-resistant strains.

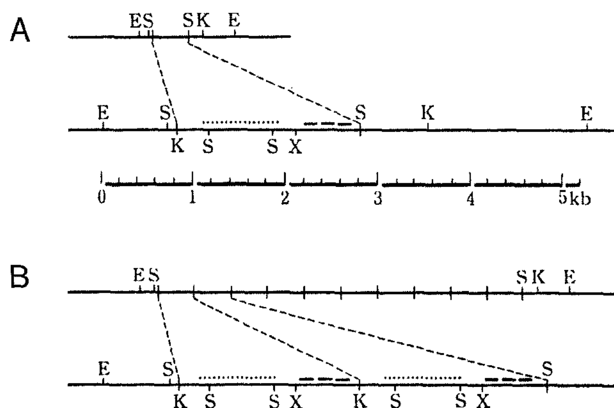

Fig. 1. Tandem amplification of *CUP1* with 2-kb repeating units [12]. (A) wild-type strain of *S. cerevisiae* with a single copy of *CUP1*; (B) copper-resistant strain with 10-tandem repeating units. Also shown are two expanded repeating units. The size marker in A and B shows the size of the expanded-repeating units. ---: *CUP1*; - - - - -: encoding region for the unknown protein. E, S, X or K shows the digestion site of *Eco*RI, *Sau*3AI, *Xba*I or *Kpn*I, respectively.

The copy numbers of *CUP1* in *S. cerevisiae* are not constant but vary as described previously. when a strain with a low copy number of *CUP1* is cultured on an agar medium containing a high copper concentration, some colonies increase the copy numbers of *CUP1* with a 2.0-kb repeating unit. In contrast, when a subculture of the copper-resistant strain in a medium with no metal addition is repeated ten times, some colonies decrease the copy number of the *CUP1* with a 2.0-kb repeating unit. What is the mechanism of amplification or decrease in 2.0-kb units of the DNA fragments containing *CUP1*? It is presumed that recombination of DNA takes part in the mechanism though this question cannot be answered yet [30,45,46]. It is uncertain whether amplification of *CUP1* is induced by metals, or the amplification is caused constitutively without metals and the amplified strain is selected by the metals. However, it is known that the amplification of *CUP1* occurs on treatment with carcinogens such as *N*-methyl-*N′*-nitro-nitrosoguanidine (MNNG) or 4-nitroquinolin-*N*-oxide (4NQO) as well as metals [1]. The elucidation of the mechanism for this phenomenon is expected to be a model of specific gene amplification on the chromosome in the eukaryote.

INDUCTION FOR TRANSCRIPTION OF *CUP1*

Metal-specificity for induction of MT synthesis

A remarkable feature of MT synthesis in animals is its inducibility at the level of gene transcription. In copper-resistant *S. cerevisiae* as well as in animals, MT is not detected in cells grown in a medium with no metal addition, and synthesis is induced by copper or silver but not by other metals [49,53]. It is suggested that copper or silver can specifically induce the expression of the *CUP1*. Actually, it was determined that *CUP1* transcription is controlled by its promoter based on the results of Northern analysis and experiments with fused plasmids. For instance, the strain with a fused *CUP1* gene, the promoter of which is substituted by the promoter of the *TDH3* gene having constitutive expression ability, can transcribe *CUP1* and produce MT in a medium without metal addition. The strain with this fused gene also shows resistance to cadmium in addition to copper or silver. In contrast, the transformant with a fused *lacZ* gene, the promoter of which is substituted by the promoter of *CUP1*, increases β-galactosidase activity according to the level of additional copper or silver, although the activity cannot be detected when the metal is not

added. It is thought that copper and silver, but not cadmium, induce the production of MT by the action of the *CUP1* promoter in the coppper-resistant strains. However, the MT produced can also bind cadmium in addition to copper or silver.

Transcription factor as a copper sensor

What is the mechanism of metal-specific inducible *CUP1* transcription by copper or silver? It could not have been considered that the metal itself regulates the ability of the *CUP1* promoter directly. The transcription factor, which senses the intracellular metal level and acts as the *CUP1* promoter, was sought as the intermediate substance between the metal and the promoter. A mutant which could not induce *CUP1* transcription by copper was isolated from the copper-resistant strain [35]. The recessive *ace1* (*a*ctivation of *CUP1* *e*xpression) gene which controls *CUP1* transcription was mapped at 9 cm distance from the centromere on the left arm of chromosome VIII. A DNA fragment containing *ACE1* was isolated from the wild-type strain, and it was confirmed that the *ace1* strain transformed with the *ACE1* fragment to recover the *CUP1* inducible ability by copper. Although the isolated *ACE1* fragment encoded three kinds of proteins, one of these products was demonstrated to be the transcription factor for *CUP1* and it was designated as an ACE1 protein [34]. Although another research group cloned the same gene independently and designated it as *CUP2*, we use the *ACE1* terminology in this review [3,43].

The ACE1 protein is produced in both the copper-resistant and wild-type strains of *S. cerevisiae* in the presence or absence of the metals. The molecular weight of the ACE1 protein is 24 000 Da, and the protein consists of 225 amino acid residues. The N-terminal moiety is rich in basic amino acids and contains 12 cysteine residues in five configurations of Cys-X-Cys or Cys-X-X-Cys, though the C-terminal moiety is rich in acidic amino acids. It was observed that the ACE1 protein from cells grown in a medium containing copper bound six or seven atoms of Cu(I). Although this protein is not classified in the category of MT, the Cys-X-Cys and Cys-X-X-Cys can chelate copper or silver as can the MT [7,8]. Furthermore, it is confirmed that the copper-chelating ACE1 protein can bind the promoter of *CUP1*, though the ACE1 protein without the metal cannot act as the promoter [14,15]. As a result of gene-deletion and gel-shift experiments, it became clear that the N-terminal moiety of the 1–101 amino acid residue of the ACE1 protein is the binding-domain for copper and the promoter. Therefore, the ACE1 protein serves a dual function as a sensor for copper and as an inducer for *CUP1* transcription according to the copper-concentration in the cell.

The proposed induction-expression mechanism of *CUP1* by copper in the copper-resistant strain of *S. cerevisiae* is shown in Fig. 2. 1) Cu(II) is reduced to Cu(I) when it is transported into the cells; 2) some Cu(I) bind the inactive ACE1 protein in the cytoplasm and the active complex Cu(I)-ACE1 protein is generated; 3) the active Cu(I)-ACE1 protein binds the promoters of amplified *CUP1*s and induces the transcription of the genes in the nucleus; 4) large amounts of MT are produced which bind the copper ions in the cytoplasm.

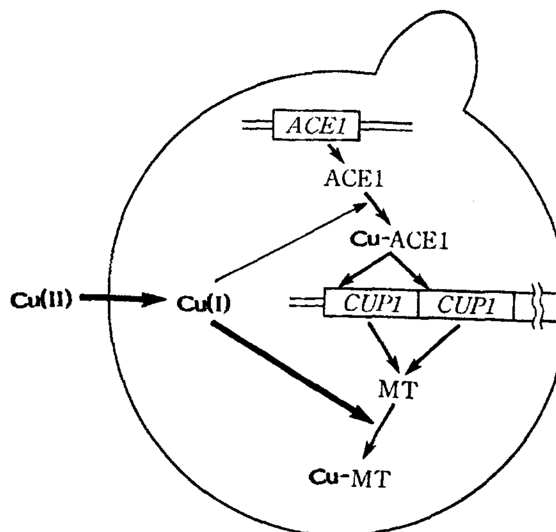

Fig. 2. Induction mechanism for expression of *CUP1* by copper in copper-resistant *S. cerevisiae*. *ACE1* or *CUP1* in the box shows each gene.

Actually, the Cu-ACE1 protein binds to the UAS (*u*pstream *a*ctivation *s*equence) upstream of the *CUP1* promoter on chromosome VIII [19,20]. The base sequence of the UAS has been determined [36], and the interaction of the Cu-ACE complex and the UAS of *CUP1* are being studied intensively. In addition to copper, silver (but not other metals) activates the ACE1 protein by binding and the active Ag-ACE1 complex can bind to UAS. Why is the ACE1 protein activated by binding with copper or silver? It may be concluded that the conformation of the ACE1 protein changes into an appropriate form for binding to the UAS of *CUP1* by binding with copper or silver. The ACE1 protein increases the stability to proteinase by binding with copper, and the cadmium-binding ACE1 protein cannot induce *CUP1* transcription. These results support the finding of copper- and silver-specific induction of *CUP1* by the ACE1 protein.

Transcription in cadmium-resistant S. cerevisiae

A transcription factor which, in addition to the ACE1 protein, can induce *CUP1* transcription has been discovered. A mutant involving a single-amino acid substitution in the DNA binding domain of the heat-shock-transcription factor (HSF1 protein encoded by the *HSF1* gene) dramatically enhanced *CUP1* transcription [32,33]. Therefore, the copper-resistant strain with the mutated *HSF1* can transcribe the *CUP1* with no metal addition, and the transcription level increases with the addition of cadmium [51]. This phenomenon was also confirmed in an experiment using the fused plasmid of the *CUP1* promoter and the *lacZ* gene. The mutated HSF1 protein bound strongly to the promoter of *CUP1*, whereas the wild-type-HSF1 protein interacted weakly with the same region. Therefore, the mechanism is suggested to be related to the binding ability of the HSF1 protein to the promoter sequence of *CUP1* [52]. However, it is not known yet why the transcription level of *CUP1* in the mutated *HSF1* strain is increased by cadmium.

The cadmium-resistant strain transcribes *CUP1* at the basal

level without metal addition, and the level is increased by addition of cadmium or copper [37]. It is known that the gene of the transcription factor for *CUP1* isolated from the cadmium-resistant strain is itself of the mutated *HSF1* according to the restriction map (T. Miyagawa, unpublished data). Therefore, it may be concluded that the cadmium-resistant strain involves two mutations which are the amplification of *CUP1* and the point mutation of *HSF1*. The similarity of the induction mechanism of proteins under environmental stresses such as metal and heat is very interesting.

A mutant which can produce detectable MT from *CUP1* has not been isolated other than these copper- and cadmium-resistant strains. The transcription factor for *CUP1* may be restricted to ACE1 and mutated HSF1 proteins based on this evidence. Further research on the RNA-polymerase-action mechanism by binding of these transcription factors to the *CUP1* promoter is expected [10].

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