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Effect of some heavy metal ions on copper-induced metallothionein synthesis in the yeast *Saccharomyces cerevisiae*

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Abstract

Copper-induced metallothionein (MT) synthesis in *Saccharomyces cerevisiae* was investigated in order to associate this exclusively with Cu^{2+} *in vivo*, when cultured in nutrient medium containing other heavy metal ions. Expression of the *CUP1* promoter/*lacZ* fusion gene was inhibited by all heavy metal ions tested, especially Cd^{2+} and Mn^{2+}. By adding Cd^{2+} and Mn^{2+} at 10 μM concentration, the β-galactosidase activity decreased by about 80% and 50% of the maximum induction observed with 1 mM $CuSO_4$, respectively. Furthermore, cell growth was markedly inhibited by combinations of 1 mM-Cu^{2+} and 1 μM-Cd^{2+}. Therefore, the yeast *S. cerevisiae* could not rely on MT synthesis as one of the copper-resistance mechanisms, when grown in a Cd^{2+} environment. In contrast, the presence of Mn^{2+} in the nutrient medium showed alleviation rather than growth inhibition by high concentrations of Cu^{2+}. The recovery from growth inhibition by Mn^{2+} was due to decreased Cu^{2+} accumulation. Inhibitory concentrations of Co^{2+}, Ni^{2+} and Zn^{2+} on expression of the *CUP1*p/*lacZ* fusion gene were at least one order of magnitude higher than that of Cd^{2+} and Mn^{2+}. These results are discussed in relation to Cu^{2+} transport and Cu-induced MT synthesis in the copper-resistance mechanism of the yeast *S. cerevisiae*.

Introduction

Organisms are constantly exposed to changes in environmental factors such as nutrition, osmotic pressure, metal ions and so on. Under these conditions, cells have evolved homeostatic control mechanisms to grow and develop optimally. Copper is a vital divalent cation in living organisms, functioning as a cofactor of numerous enzymes such as Cu/Zn superoxide dismutase, cytochrome c oxidase, and laccase (Ochiai 1983; Collins & Dobson 1997), but it is also toxic in excess. The yeast *Saccharomyces cerevisiae* has developed a number of homeostatic regulations to overcome such changes in its external environment. With the increasing of Cu^{2+} concentrations in the external environment, the Cu^{2+} uptake system in the yeast *S. cerevisiae* is repressed by a transcription factor (Mac1p) (Graden & Winge 1997; Yamaguchi-Iwai *et al*. 1997; Jensen & Winge 1998), which functions as a repressor of the *CTR1* gene encoding a high affinity Cu^{2+} transporter (Dancis *et al*. 1994a, b). At the same time, the copper-dependent degradation of a Cu^{2+} transporter (Ctr1p) occurs (Ooi *et al.* 1996). Cu^{2+} entry into the yeast cells is, thereby, decreased. However, when Cu^{2+} ions further increase in the environment, these ions are accumulated in excess through a low affinity transport system (Yu *et al.* 1996).

In the yeast *S. cerevisiae*, resistance to Cu^{2+} is associated with the production of a metal-binding protein (metallothionein) (Welch *et al*. 1983; Butt *et al.* 1984; Ecker *et al.* 1986), mineralization (Ashida *et al.* 1963; Kikuchi 1965; Yu *et al.* 1996) and sequestration to the vacuoles (Eide *et al.* 1993; Ramsay & Gadd

1997; Szczypka *et al.* 1997), leading to a reduction in the cytoplasmic concentration of free copper ion. One of the major copper detoxification mechanisms in yeast *S. cerevisiae* was the Cu-induced synthesis of metallothionein (MT) that led to decreased binding of excess free-copper ions in the cytosol (Welch *et al.* 1983; Butt *et al.* 1984). This small Cu-MT was well characterized as a unique cysteine-rich protein having about 30% total amino acids and binding to metals such as Cd^{2+}, Zn^{2+}, Co^{2+} other than Cu^{2+}, *in vitro* (Hartman & Weser 1985; Winge *et al.* 1985; Inouhe *et al.* 1989). Furthermore, expression of the metallothionein gene (*CUP1*) in response to $CuSO_4$ or $AgNO_3$ was mediated by a trans-acting regulatory protein (Ace1p) (Furst & Hamer 1988; Thiele 1988; Welch *et al.* 1989; Butler & Thiele 1991), which was also a small MT-like protein. Therefore, many metals readily form a complex with Ace1p *in vitro* as well as with MT because of being a cysteine-rich protein (Furst *et al.* 1988; Szcypka & Thiele 1989; Dameron *et al.* 1993).

In the natural environment, organisms face a variety of conditions containing several ions rather than a single metal. Cu^{2+} has been reported to interact with other metals at proteins (Winge *et al.* 1985; Dameron *et al.* 1993), but these experiments were performed *in vitro* with purified components, and the interactions have not been convincingly linked to a toxic effect. The detoxification of Cu^{2+} mediated by MT in the yeast *S. cerevisiae* in coexistence with other metal ions also has not been elucidated *in vivo*. The purpose of this study was to ascertain in a combination of Cu^{2+} and other metals whether the Cu-induced MT synthesis in the yeast *S. cerevisiae* is associated exclusively with the copper ion *in vivo* and whether the cells are able to grow in a combination of large amounts of Cu^{2+} and other heavy metal ions.

Materials and methods

Culture

Saccharomyces cerevisiae strain XTY-14 (*MATa, ura3-52, his6, LEU2::YipCL, CAD2, CUP1^r*) involving an integrated *CUP1* promoter/*lacZ* fusion gene and strain X2180-1B (*MATa, SUC2, mal, mel, gal2, CUP1^r*) were used. Strain XTY-14 was isolated from segregants of tetrad spores of a cross between XTT-1d (*MATa, CAD2*) described previously (Tohoyama *et al.* 1990) and DTY-22 (*MATa, ura3-52, his6, LEU2::YipCL, CUP1^r*). Strain DTY-22 was donated from Dr. D.H. Hamer. Cells were cultured at 30° in a nutrient medium of the following composition (g l^{-1}): glucose 20, yeast extract (Difco) 4.0, polypeptone (Wako Pure Chem.) 5.0, KH_2PO_4 5.0, $MgSO_4 \cdot 7H_2O$ 2.0. The heavy metals tested were all of the sulfate form. These metal solutions were sterilized separately and added to the culture medium before cell inoculation.

Measurement of growth

Following preculture at 30° for 48 h, cells were inoculated into the nutrient medium (20 ml, in 100-ml Erlenmeyer flasks) and incubated at 30° on a reciprocating shaker. Growth was measured using spectrophotometric measurement at $OD_{600\ nm}$. Culture samples were also centrifuged for 5 min (3000 rev/min), washed twice with distilled water and dried at 95° for 2 days to determine the dry weight.

β-Galactosidase assay

One ml of yeast cell cultures was grown in nutrient medium and harvested when the cell densities reached about 1.0 at $OD_{600\ nm}$. The cells were washed in Z buffer (Miller 1972), resuspended in 1 ml of Z buffer, and permeabilized with 2 drops of chloroform and 1 drop of 0.1% SDS. ß-galactosidase assays were done as described in Miller (1972) using 100 μl of the permeabilized cells. β-galactosidase activity was expressed as units where one unit $= 1000 \times OD_{420}/CVt$ with OD_{420} being the absorbance at 420 nm, C; the density of the cell suspension at 600 nm, V; the volume of the cell suspension (ml) and t; the total incubation time of the assay. All assays were carried out triplicate.

Measurement of metal content

Washed cells (about 20 mg dry weight of cells) were digested by adding 0.5 ml 6N HNO_3 in boiling water for 20 min. After this acid extraction, the samples were diluted to 5 ml with distilled water, then mixed and centrifuged to remove any undigested material. The copper content in these extracts was measured with an atomic absorption spectrophotometer (Hitachi 207, Japan).

Purification of metallothionein

The yeast Cu-MT was purified according to a modification of the method described previously (Inouhe

et al. 1989). The copper-resistant yeast *S. cerevisiae* strain X2180-1B was grown in 1.0 mM Cu-containing medium at 30° for 48 h. The cells were harvested, washed with distilled water by centrifugation and then resuspended in 20 mM Tris/HCl (pH 8.0). After cell disruption by a Braun's homogenizer at 0°, the homogenates were centrifuged at 8000 *g* for 20 min. The supernatant was treated at 75° for 5 min and then placed immediately on ice for 30 min. The heat-denatured materials were removed by centrifugation at 15,000 *g* for 30 min. Subsequently, the sample was applied to a Sephadex G-50 column (25 × 900 mm) and eluted with 750 ml of 20 mM Tris/HCl (pH 8.0) at flow rate 0.5 ml/min. The copper-inclusive fraction was collected, loaded on a DEAE-Sephadex A-50 column (13 × 130 mm) and eluted with 500 ml of 20 mM Tris/HCl (pH 8.0) containing a linear gradient of 20–500 mM NaCl. Following dialysis of the copper-fraction, the sample was lyophilized. The crude MT was further purified by rechromatography using a Sephadex G-50 column. The purified MT sample was detected as a single protein band at 15% SDS-PAGE.

Preparation of antibody

To obtain the antibody for Cu-MT, rabbits were primed intradermally with purified yeast MT (about 1 mg) emulsified in Freund's incomplete adjuvant. After three weeks, rabbits were boosted with subcutaneous injections of the same immunogen mixed with Freund's incomplete adjuvant. After rabbits were bled, sera involved in MT-antibody were harvested by centrifugation and stored at −80°.

Measurement of metallotionein

Cells were grown in 20 ml nutrient medium with Cu^{2+} ± Cd^{2+} (or Mn^{2+}) for 16 h at 30°. Metallothionein was extracted from yeast cells as described previously (Tohoyama *et al.* 1990). Washed cells were resuspended in 20 mM Tris/HCl (pH 7.5) buffer at a cell density at $OD_{660\ nm}$ = 10–20. One ml of cell suspension was treated in a small sealed test tube at 75° for 20 min. Following the hot-buffer extraction, the samples were rapidly cooled in ice cold water and then centrifuged by 10,000 *g* for 1 min. Metallothionein in hot-buffer extractable fraction was measured by ELISA.

Results

Effects of some heavy metal ions on expression of yeast CUP1 promoter/lacZ fusion gene

In a 1-h induction with $CuSO_4$, expression of β-galactosidase from the yeast *CUP1*p/*lacZ* fusion gene in strain XTY-14 is shown in Figure 1a. An increase in β-galactosidase activity was related to the concentrations of Cu^{2+} and was maximal at 1 mM $CuSO_4$. To examine whether any transition metal cations interfere with the Cu-induced expression of the *CUP1*p/*lacZ* fusion gene in the yeast *S. cerevisae*, cells were incubated in 1.0 mM Cu-containing medium with another cation for 1 h. The β-galactosidase activity was drastically decreased in the proportion to the increased concentrations of Cd^{2+}. The addition of 10 μM Cd^{2+} inhibited about 80% of the induction observed with 1 mM $CuSO_4$ (Figure 1b). Furthermore, Mn^{2+} also affected the Cu-induced expression of the *CUP*1p/*lacZ* fusion gene, although this was not greater than that observed in the presence of Cd^{2+} (Figure 1c). In the presence of Mn^{2+} at 10 μM, the β-galactosidase activity was approximately 50% of that observed with no added Mn^{2+}. Compared to Cd^{2+} or Mn^{2+}, the inhibitory effect of Zn^{2+}, Co^{2+} and Ni^{2+} ions on Cu-induced expression of *CUP1*p/*lacZ* gene is relatively low. The β-galactosidase activity was decreased approximately 60% by Co^{2+} (Figure 1d) and Zn^{2+} (Figure 1e) at 100 μM and by Ni^{2+} (Figure 1f) at 1 μM, respectively. The reduced β-galactosidase activity in combinations of Cu^{2+} and these three kinds of metal ions appears to decrease the entry of copper into the cells (Lin *et al.* 1993) or lower the interaction of metals in the Cu-induced expression processes of the *CUP1*p/*lacZ* gene by at least one order of magnitude higher concentrations than the Cd^{2+} or Mn^{2+} does.

Cadmium inhibits growth and MT accumulation in the presence of copper

Whether cells are capable of growing in combination of Cu^{2+} and Cd^{2+} in culture medium was tested, involving a reduced Cu-induced expression of *CUP*1p/*lacZ* fusion gene. Figure 2 shows the growth yield in Cu-containing medium with or without cadmium. Growth was inhibited only 10% by Cd^{2+} and Cu^{2+} at 1.0 μM and 1.0 mM, respectively. However, in combination with 0.1 μM Cd^{2+} and 1.0 mM Cu^{2+}, growth inhibition was 77%.

Figure 3 shows the relative MT content of cells which grew in combinations of Cu^{2+} and Cd^{2+} at var-

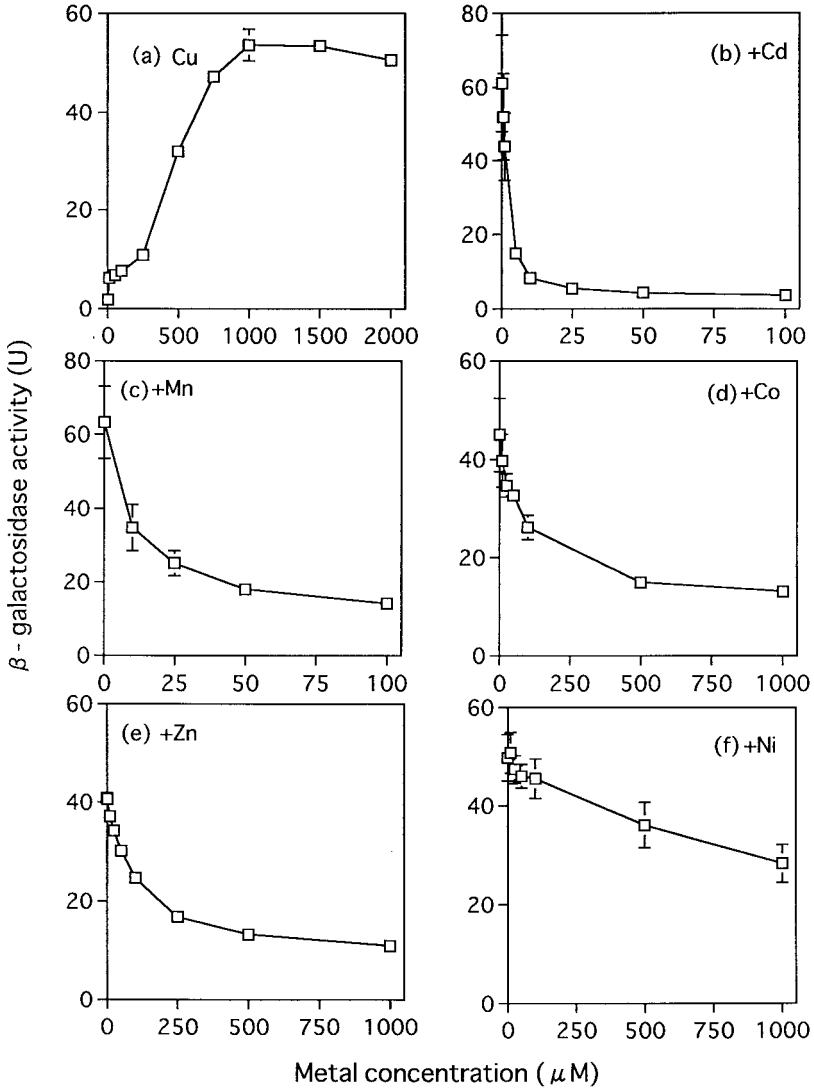

Fig. 1. Effect of metal ions on expression of *CUP1*p/*LacZ* fusion gene in *S. cerevisae* strain XTY-14. The cells were incubated for 1 h in nutrient medium with various amounts of $CuSO_4$ (a) or 1 mM Cu^{2+} plus other metal salts in sulfate form; + Cd^{2+} (b), + Mn^{2+} (c), + Co^{2+} (d), + Zn^{2+} (e) and + Ni^{2+} (f). The cells permeabilized with chloroform and SDS as described in Materials and methods were used for assay of β-galactosidase activity. Values were normalized to the cell density and the incubation time. Mean values from triplicate determinations with standard deviation are shown where these exceed the dimensions of the symbols.

ious concentrations. The MT content as well as the expression of *CUP1*p/*lacZ* fusion gene as shown in Figure 1b was related to the increasing concentrations of Cu^{2+}. In 0.5 mM and 1.0 mM Cu-containing media, the MT content was gradually decreased with increasing Cd^{2+} at various concentrations. When Cd^{2+} was added to the nutrient medium at 1.0 μM, the MT content of cells cultured in the presence of 0.5 mM and 1.0 mM Cu^{2+} was 60% and 40% of each control of Cd-free medium, respectively. No significant decrease in MT content by Cd^{2+} was observed in lower Cu-containing medium. The facts obtained from the results of Figures 1b and 3 suggested that the reduced growth in combinations of Cu^{2+} and Cd^{2+} was caused by decreased Cu-induced expression of the *CUP1* gene, leading to a decrease in the accumulation of MT.

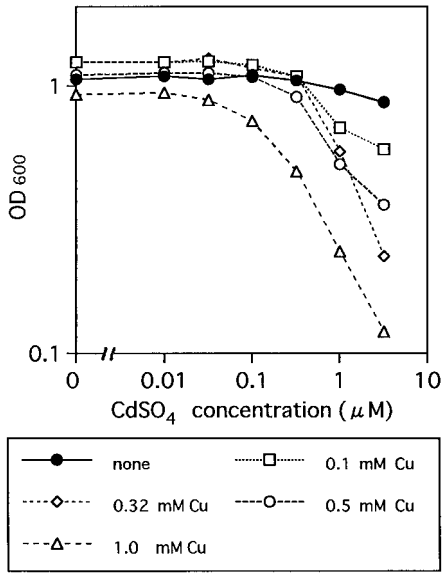

Fig. 2. Effect of combination of Cu^{2+} and Cd^{2+} on the growth of *S. cerevisiae* strain XTY-14. The cells were grown in nutrient medium with Cu^{2+} and/or Cd^{2+} for 16 h. Shown are the mean values of two experiments.

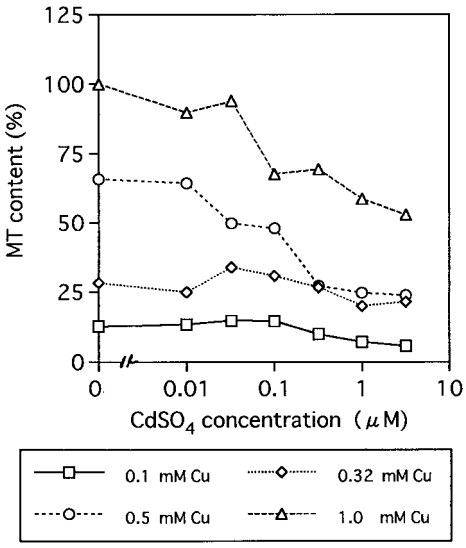

Fig. 3. Effect of combination of Cu^{2+} and Cd^{2+} on MT accumulation of *S. cerevisiae* strain XTY-14. The MT contents were measured in cells obtained from the experiment described in Figure 2. Data were expressed as % of the MT content obtained with 1 mM Cu-cultured cells and were the mean values of two different experiments.

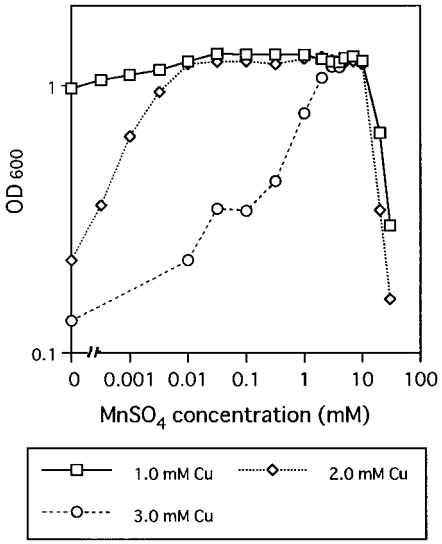

Fig. 4. Effect of combination of Cu^{2+} and Mn^{2+} on the growth of *S. cerevisiae* strain XTY-14. The cells were grown in nutrient medium with Cu^{2+} and/or Mn^{2+} for 16 h. Shown are the mean values of two experiments.

Manganese inhibits MT accumulation but does not inhibit growth in the presence of copper

The addition of small amounts of Mn^{2+} as well as Cd^{2+} repressed the expression of the *CUP1*p/*lacZ* fusion gene by Cu^{2+} (Figure 1b). We examined whether Mn^{2+} inhibited the growth through a mechanism involving the decreased Cu-induced expression of the *CUP1*p/*lacZ* fusion gene as well as Cd^{2+} did. Figure 4 shows the effect of $MnSO_4$ on growth in a Cu-containing medium. The growth yield was about 30, 80 and 90% with the addition of 1.0, 2.0 and 3.0 mM $CuSO_4$ in comparison with its yield in Cu-free medium as shown in Figure 2, although the yeast maintained 3 copies of the *CUP1* gene encoding MT. However, its growth inhibition by Cu^{2+} was almost alleviated by adding $MnSO_4$ to the medium. The minimal effective concentration of Mn^{2+} for complete recovery from growth inhibition was 0.01 and 3.0 mM in 2.0 and 3.0 mM Cu-containing medium, respectively.

Figure 5 shows the effect of Mn^{2+} on Cu-induced MT synthesis. The cellular MT content is related to the concentrations of Cu^{2+} in the culture medium and was maximal at 1 mM $CuSO_4$ based on the results shown in Figures 3 and 4. The MT content was 90 and 71% in 2.0 and 3.0 mM Cu-containing media obtained at 1.0 mM Cu^{2+}, respectively. The MT content decreased to 32% with the combination of 0.01 mM

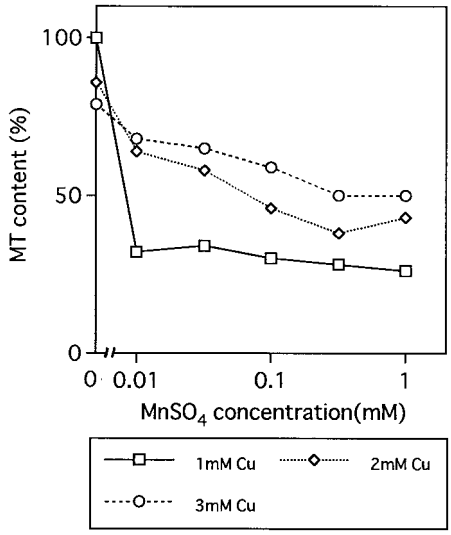

Fig. 5. Effect of combination of Cu^{2+} and Mn^{2+} on MT accumulation of *S. cerevisiae* strain XTY-14. The MT contents were measured in cells obtained from the experiment described in Figure 4. Data are expressed as% of the MT content obtained with 1 mM Cu-cultured cells and are the mean values of two different experiments.

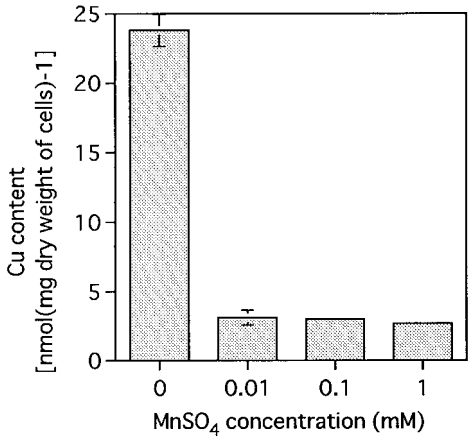

Fig. 6. Effect of Mn^{2+} on copper accumulation of *S. cerevisiae* strain XTY-14. The cells were grown in 1.0 mM Cu-medium with or without Mn^{2+} for 24 h. Values are means of three different experiments with standard deviations shown in the figure.

Mn^{2+} and 1.0 mM Cu^{2+}. A further increase in Mn^{2+} in the incubation medium gradually led to a decrease in Cu-induced MT synthesis. The degree of decreased MT content due to Mn^{2+}, however, was lower in a 3.0 mM Cu-containing medium than in a 1.0 mM Cu-containing medium. The result may indicate non-specific increased penetration of copper into the cells according to the increased Cu^{2+} concentrations in the medium.

Effect of manganese on copper accumulation

By adding Mn^{2+}, the Cu-induced MT synthesis by the *S. cerevisiae* strain XTY-14 was decreased but growth inhibition by Cu^{2+}, however, was recovered as shown in (Figures 4 and 5). Therefore, the effect of Mn^{2+} on copper accumulation was examined. Figure 6 shows the copper content of cells grown in 1.0 mM Cu-containing medium with or without Mn^{2+}. The copper content was 23.8 nmol/mg dry weight of cells grown for 24 h in 1.0 mM Cu-containing medium. When Mn^{2+} was added at 0.01 mM to the 1.0 mM Cu-containing medium, the copper content was at or below 20% of that of the Mn^{2+}-free control. With prolonged culture from 24 h to 48 h, the cell yield and copper content increased from 0.67 mg to 0.95 mg dry weight per ml of culture and 23.8 nmol to 24.8 nmol/mg dry weight of cells, respectively. Furthermore, the culture in 1.0 mM Cu-containing medium with Mn^{2+} tended to show a growth yield similar to that of the control and also showed a slight increase in copper content (data not shown).

Discussion

The data presented in this study demonstrate that Cu-induced expression of the *CUP1*p/*lacZ* fusion gene was inhibited by adding low concentrations of Cd^{2+} or Mn^{2+}, accompanied by lower accumulation of MT. Coexistence of Cd^{2+} in the Cu-medium also led to a toxic effect on the growth, but not with Mn^{2+}.

The increased metal sensitivity due to the combination of Cu^{2+} and Cd^{2+} appears to be caused not by increased incorporation (or uptake) of Cu^{2+} or Cd^{2+} but from interaction at the transcriptional level of Cu-induced MT synthesis, although it was reported that ACE1-mediated expression of *CUP1*p/*lacZ* was specific for Cu^{2+} and that the addition of 20 μM Cd^{2+} did not inhibit the observed induction with 5 μM Cu^{2+} (Thorvaldsen *et al.* 1993). It was well-known that Ace1p, which acts as a transcription factor in the *CUP1* gene, also has high affinity for Cd^{2+} *in vitro*, but the Ace1p-Cd complex fails to bind at the UAS existing upstream sequence of the *CUP1* gene (Dameron *et al.* 1993). Thus, Cd^{2+} salts are unable to induce expression of the MT genes. Furthermore, rapid metal exchange occurs between Zn- or Cd-Ace1p and Cu^{+} donors *in vitro* (Dameron *et al.* 1993). Therefore, even *in vivo*, inhibition of Cu-induced MT synthesis by Cd^{2+} appears to occur via interaction with Ace1p.

Moreover, it has been reported that the uptake of Cu^{2+} by yeast *S. cerevisiae* competed with that of Cd^{2+} (Yu *et al.* 1996) and that Cu-MT is not directly involved in Cu^{2+} uptake in yeast (Lin & Kosman 1990). Further studies on the transcriptional level for Cu-induced MT synthesis are needed in the presence of Cd^{2+} *in vivo*.

As another possibility, growth inhibition by the increased accumulation of Cd^{2+} uptake by Cu^{2+} was expected. However, because effective concentration of Cd^{2+} that inhibited Cu-induced MT synthesis was only 1.0 μM in 1.0 mM Cu-medium, it is unlikely that the cells accumulated a large amount of Cd^{2+}; besides, the yeast *S. cerevisiae* XTY-14 used in this experiment was a Cd-resistant phenotype carrying a functionally unknown *CAD2* gene and is able to proliferate without any growth inhibition in the presence of 100 μM $CdSO_4$ (Tohoyama *et al.* 1990).

Within a few years, much information has been reported for Cu^{2+} homeostasis in yeast *S. cerevisiae* where the concentration of intracellular free copper was strictly limited by a copper chaperone or trafficking in the nutritional level of Cu^{2+} (Pufahl *et al.* 1997; Valentine & Gralla 1997; Yuan *et al.* 1997; Casareno *et al.* 1998; Srinivasan *et al.* 1998; Rae *et al.* 1999) or copper-scavenging and/or sequestering systems involved in metallothionein (Welch *et al.* 1983; Ecker *et al.* 1986), vacuoles (Ramsay & Gadd 1997; Szcypka *et al.* 1997) or copper sulfide mineralization (Yu *et al.* 1996) at an excess level of Cu^{2+}. Especially, metallothionein is thought to play a central role in the protection against Cu^{2+} toxicity (Butt *et al.* 1984; Ecker *et al.* 1986; Yu *et al.* 1996). Cu-MT is also stable in the yeast cells (Weser *et al.* 1986; Felix *et al.* 1989; Pena *et al.* 1998). Therefore, this suggests that a profound growth inhibition by the combination of large amounts of Cu^{2+} and Cd^{2+} appears to be responsible for the reduced MT synthesis as a result of decreased *CUP1* gene expression by Cd^{2+}, allowing increased free copper ion in the cytosol to inhibit intracellular metabolisms.

In contrast, although the presence of Mn^{2+} in yeast cultures led to a decrease in Cu-induced expression of the *CUP1*p/*lacZ* fusion gene and intracellular MT accumulation, Cu^{2+} toxicity is reversed rather than increased. Because the accumulation of Cu^{2+} was markedly decreased by adding Mn^{2+} to the medium (Figure 6), a possible mechanism for this recovery from the growth inhibition appears to be competition of both the metal ions. In yeast-like cells of *Aureobacidium pullulans*, the intracellular Cu^{2+} influx was reduced about 80% by Mn^{2+} under conditions containing the same metal concentration at 40 μM, but surface binding was not reduced (Gadd and Mowll 1985). In *S. cerevisiae*, however, the copper uptake system through a high affinity copper transporter (Ctr1p) was specific; uptake was not competitive with that of Mn^{2+}, Co^{2+}, Ni^{2+} or Zn^{2+} (Dancis *et al.* 1994b). Moreover, expression of the *CUP1*p/*lacZ* fusion gene was not induced in the *CTR1* gene deletion strain, even at high concentrations of copper in the medium (Dancis *et al.* 1994a). These facts were inconsistent with the reduced accumulation of Cu^{2+} by Mn^{2+} also observed in this experiment. As shown in Figure 5, however, the extent of decreased copper content of the cells grown in Cu-medium with Mn^{2+} also seems too sufficient to relieve growth inhibition by Cu^{2+}. This evidence suggests the decreasing penetration of Cu^{2+} into the cells.

Inhibition of Cu-induced expression of the *CUP1*p/*lacZ* fusion gene by cations tested other than Cd^{2+} is unlikely to be a direct interaction with the processes of MT-synthesis, because relatively high concentrations of the cation are required. Probably, the reduced Cu-induced expression of the *CUP1*p/*lacZ* fusion gene by Co^{2+}, Ni^{2+} or Zn^{2+} appears to be due to insufficient concentration of Cu^{2+} entering the yeast cells as well as is the case with Mn^{2+}.

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