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Copper(II) protects yeast against the toxicity of cisplatin independently of the induction of metallothionein and the inhibition of platinum uptake

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Abstract

We have made the unexpected discovery that copper sulfate protects *Saccharomyces cerevisiae* from the toxic effects of cisplatin. Addition of copper to the culture medium of yeast cells at concentrations above 0.1 μM significantly reduced the toxicity of cisplatin. Since a high-affinity copper transporter, Ctr1, has been reported to play a major role in the uptake of cisplatin, we examined the effects of copper on the cellular uptake of cisplatin. We found that the cellular concentration of platinum was not significantly affected by treatment of cells with 1 μM copper. It is known that mammalian metallothionein is induced by copper and is involved in acquired resistance to cisplatin. Copper significantly increased the level of mRNA for yeast metallothionein at a concentration that has effectively reduced the toxicity of cisplatin. However, the toxicity of cisplatin in cells with a disrupted gene for ACE1, a factor that regulates transcription of the yeast gene for metallothionein, was also significantly reduced by treatment with copper. These results suggest that copper protects yeast cells from cisplatin toxicity independently of induction of the synthesis of metallothionein and of the inhibition of platinum uptake. Since copper is one of the trace elements that are essential for cell function and since a relatively low concentration of copper (0.1 μM) significantly reduced cisplatin toxicity, it is possible that copper might play an important role in the expression of cisplatin toxicity.

Keywords: Cisplatin; Resistance; Copper; Metallothionein; Yeast

Cisplatin [*cis*-diamminedichloroplatinum(II)] is an effective antitumor agent for several types of cancer [1,2], but the effectiveness of chemotherapy with cisplatin is often limited by the development of resistance to this drug [3]. Various mechanisms of cisplatin resistance have been described and they include decreased accumulation of cisplatin [4–7], enhanced intracellular detoxification by a system that involves glutathione [8–12] or metallothionein [13–16], altered patterns of DNA platination [17], and increased repair of damaged DNA [18–22]. However, the details of the mechanism of cisplatin resistance remain to be clarified.

We have been searching for cellular factors that confer resistance to cisplatin in yeast because yeast has been established as a model organism in which powerful genetic techniques can be used to elucidate fundamental but complex eukaryotic processes. Previously, we identified *CIN5* and *YDR259c* as genes that confer resistance to cisplatin in yeast [23]. We described here the unexpected finding that copper has a powerful protective effect against cisplatin toxicity. Copper is one of the trace elements that is essential for cell function and a relatively low concentration of copper (0.1 μM) significantly reduced cisplatin toxicity. Thus, copper might play an important role in the expression of cisplatin toxicity.

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Materials and methods

Yeast and media. *Saccharomyces cerevisiae* W303B (*MATα his3 can1-100 ade2 leu2 trp1 ura3*) was grown in yeast extract–peptone–adenine–dextrose (YPAD) medium (1% yeast extract, 2% peptone, 0.004% adenine, and 2% glucose) or in synthetic dextrose (SD) medium supplemented with amino acids [24].

Quantitation of the toxicity of cisplatin in yeast. Yeast cells were cultured (1×10^4 cells/200 μl) in SD medium that contained cisplatin (Nippon Kayaku, Tokyo, Japan) at various concentrations. After a 48-h incubation, we measured the absorbance of the culture at 620 nm to quantify cell growth. We treated yeast cells with metal compounds by incubating them with copper sulfate, silver nitrate, cadmium chloride or zinc chloride for 12 h. After the 12-h incubation, we washed the yeast cells once with SD medium and then resuspended them in fresh SD medium that contained cisplatin, as indicated.

Disruption of the ACE1 gene in yeast. The *ACE1* gene of *S. cerevisiae* W303B was disrupted as described previously [25]. For construction of the *ace1::HIS3* vector, the *HIS3* gene was amplified by PCR with the following oligonucleotides as primers: 5′-ATG GTCGTAATTAACGGGGTCAAATATGCCTGGAAACGTGTA TC-3′and 5′-TTATTGTGAATGTGAGTTATGCGAAGATACTTG TTTGTATAGCTC-3′. The product of PCR was introduced into W303B cells for construction of the *ACE1* disruptant (*ace1Δ*), and disruption of the gene was verified by PCR and subsequent analysis of the products.

Northern blotting analysis. Cells (5×10^6 cells/ml) were cultured in 30 ml of SD medium that contained a metal compound for 12 h. Total RNA was prepared as described elsewhere [26]. The probe for the CUP1 gene was obtained by PCR with the yeast genome as template- and gene-specific oligonucleotides. Northern blotting was performed using the digoxigenin system from Roche Applied Science (Indianapolis, IN) in accordance with the manufacturer's instructions.

Quantitation of the cellular accumulation of platinum. W303B cells (1×10^6 cells/ml) were cultured in SD medium (30 ml) that contained 150 μM cisplatin for 4 h at 30 °C. The cellular platinum content was determined by ICP-MS (HP4500; Yokokawa Analytical Systems, Tokyo, Japan).

Results and discussion

As shown in Fig. 1A, copper significantly protected yeast cells against cisplatin toxicity at concentrations of copper above 0.1 μM and the effect of copper was dose-dependent. The effective concentration of copper that reduced cisplatin toxicity was considerably lower than the concentration at which copper is cytotoxic (more than 1 mM; Fig. 1B).

The involvement of copper in cisplatin toxicity has been explained in two ways. One way involves the regulation of the high-affinity copper transporter Ctr1. Ctr1 plays a major role in the uptake of cisplatin by yeast and mammalian cells [27,28]. The level of Ctr1 falls in the presence of copper [29,30], and thus, copper has an inhibitory effect on the uptake of cisplatin [27,28]. We monitored the cellular uptake of cisplatin after treatment of yeast cells with copper at a concentration (1 μM) that effectively prevented the cytotoxic effects of cisplatin under our conditions. However, the cellular concentration of platinum was not significantly affected by treatment of cells with 1 μM copper as shown in Fig. 1C. Ishida et al. [28] reported that 100 μM copper (II) significantly decreased the accumulation of platinum in yeast cells treated with cisplatin, but copper at 10 μM had only a minimal effect. In the present study, 1 μM copper significantly reduced the toxicity of cisplatin without affecting the uptake of cisplatin (Figs. 1A and C). These results suggest that a relatively low

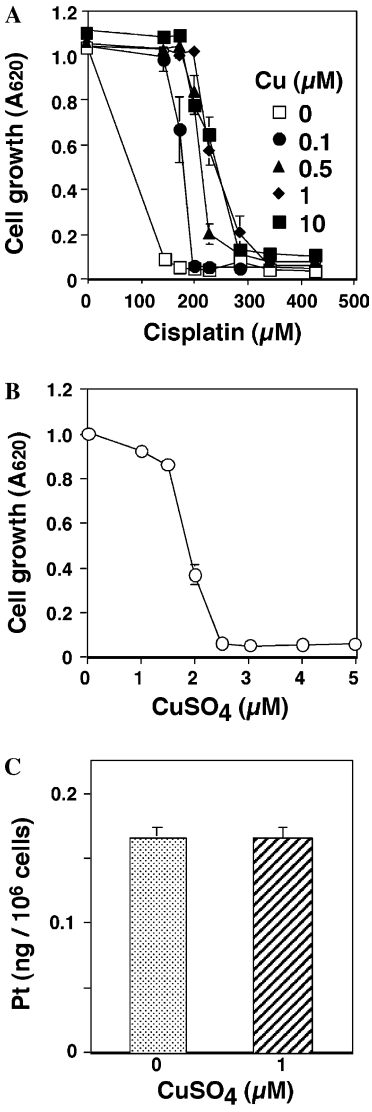

Fig. 1. (A) Effects of copper on the sensitivity of yeast cells to cisplatin. Yeast cells (W303B; 1×10^4 cells/200 μl/well) were grown in SD medium that contained cisplatin and copper sulfate as indicated. After a 48-h incubation, absorbance at 620 nm was measured spectrophotometrically. (B) Effects of copper sulfate on the growth of yeast cells. Yeast cells (W303B; 1×10^4 cells/200 μl/well) were grown in SD medium that contained copper sulfate as indicated. After a 48-h incubation, absorbance at 620 nm was measured spectrophotometrically. (C) Effects of copper sulfate on the uptake of platinum. Yeast cells (W303B; 1×10^6 cells/ml) were cultured in SD medium (30 ml) that contained 150 μM cisplatin for 4 h and then cellular platinum was quantitated. Each result is shown as the mean value with SD of results from three cultures. The absence of a bar indicates that the SD falls within the symbol.

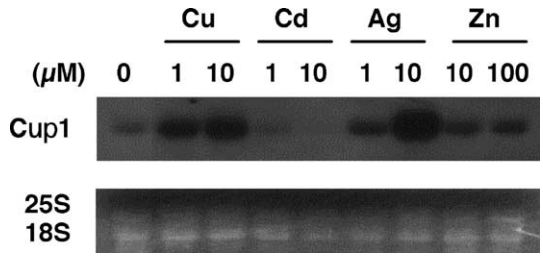

Fig. 2. Effects of metal compounds on the expression of Cup1 mRNA in yeast cells. Each lane was loaded with 25 μg of total RNA that had been extracted from cells after treatment with copper sulfate (Cu), cadmium chloride (Cd), silver nitrate (Ag), or zinc chloride (Zn), as indicated. The bands of 25S and 18S rRNA (lower panel) provide an indication of the amount of total RNA loaded in each lane.

concentration of copper can prevent cisplatin toxicity independently of suppression of the expression of Ctr1.

Synthesis of mammalian metallothionein, a protein that protect cells against the toxicity of certain drugs, metals, and oxidative stresses, is induced by heavy metals, such as copper, zinc, cadmium, and silver [31]. Copper protects mice against the lethal toxicity of cisplatin as effectively as other metallothionein-inducing metals [15]. Metallothionein is one of the major factors involved in the resistance to cisplatin in mammalian cells [13,32–34]. Transcription of the yeast gene for metallothionein, *CUP1*, is also induced by copper and silver, but not by zinc and cadmium [35–37]. The protective effect of yeast metallothionein (Cup1) against cisplatin toxicity has not been examined but the possibility exists that copper might reduce the toxicity of cisplatin via induction of the synthesis of Cup1 in yeast. To examine this possibility, we investigated the relationship between the effects of copper on the level of Cup1 mRNA and on cisplatin toxicity. As shown in Fig. 2, the level of Cup1 mRNA was significantly elevated by treatment of cells with copper sulfate at concentrations at which this salt efficiently reduced the toxicity of cisplatin (see Fig. 1A). We also examined the effects of other metals, namely, silver, cadmium, and zinc, on the toxicity of cisplatin and the level of Cup1 mRNA. Cadmium and zinc slightly reduced the toxicity of cisplatin (Figs. 3B and C). However, the effective concentrations of these metals for depression of the toxicity of cisplatin (1 μM for cadmium and 10 μM for zinc) did not significantly affect the level of Cup1 mRNA (Fig. 2). Silver induced the synthesis of Cup1 mRNA at concentrations above 1 μM (Fig. 2), but treatment with silver at concentrations of 0.1 and 1 μM significantly enhanced the toxicity of cisplatin (Fig. 3A). This result suggests that not all metal compounds protect the yeast cells from the toxicity of cisplatin and that the induction of Cup1 mRNA does not always reduce the toxicity of cisplatin.

Transcription of the *CUP1* gene is regulated by ACE1, a copper-dependent transcription factor [38]. To investigate the role of Cup1 in the effects of copper on

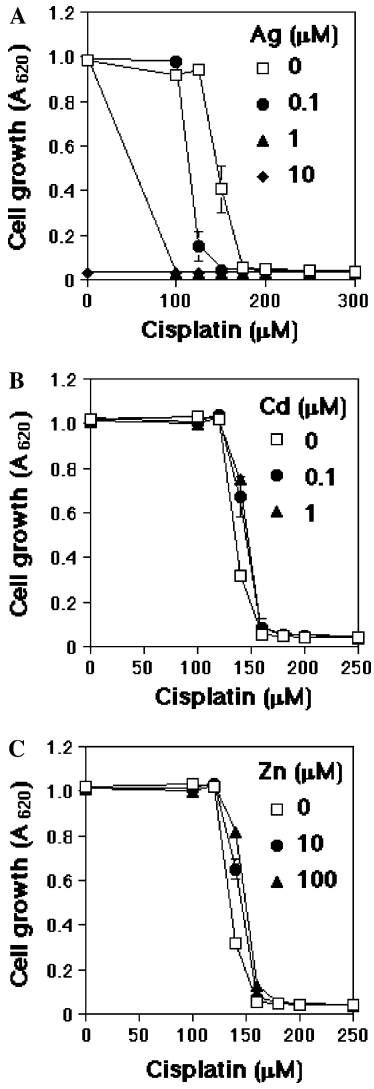

Fig. 3. The effects of metal compounds on the sensitivity of yeast cells to cisplatin. Cells (W303B; 1×10^4 cells /200 μl/well) were grown in SD medium that contained cisplatin plus silver nitrate (A), cadmium chloride (B), and zinc chloride (C) at indicated concentrations. After a 48-h incubation, absorbance at 620 nm was measured spectrophotometrically. Each point and bar represent the mean value and SD of results from three cultures. The absence of a bar indicates that the SD falls within the symbol.

cisplatin toxicity, we generated a yeast strain with a disrupted *ACE1* gene (*ace1Δ*). As shown in Fig. 4, the extent of the copper-dependent induction of *CUP1* mRNA in *ace1Δ* yeast cells was very low compared with that in the wild-type yeast W303B cells. The mutant yeast cells exhibited slight resistance to cisplatin, but treatment with copper significantly reduced the toxicity of cisplatin even in these *ace1Δ* cells (Fig. 5). Our results suggest that induction of the synthesis of Cup1 does not play a major role in the protection by copper against cisplatin toxicity. Although mammalian metallothionein protects cells against cisplatin toxicity [13,32,34], the

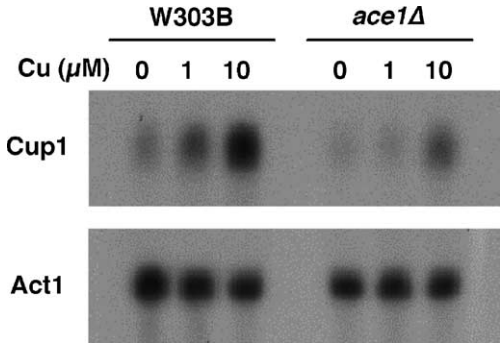

Fig. 4. Effects of copper on the expression of Cup1 mRNA in wild-type yeast (W303B) and yeast with disrupted *ACE1* gene (*ace*1*Δ*). Each lane was loaded with 25 μg of total RNA that had been extracted from cells after treatment with copper sulfate for 12 h. The bands of actin (Act1) RNA (lower panel) provide an indication of the amount of total RNA loaded in each lane.

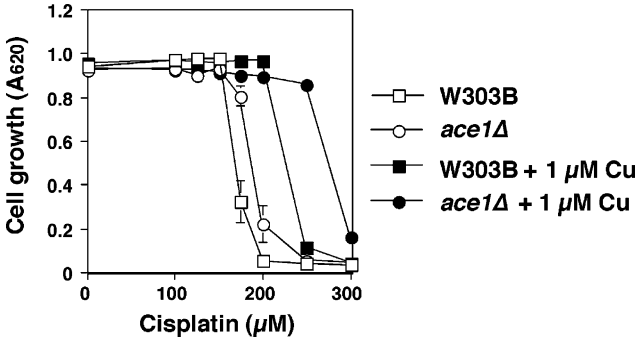

Fig. 5. Effects of copper on the sensitivity of wild-type (W303B) and *ace*1Δ yeast cells to cisplatin. Yeast cells (1×10^4 cells/ 200 μl/well) were grown in SD medium that contained cisplatin with or without 1 μM copper sulfate. After a 48-h incubation, absorbance at 620 nm was measured spectrophotometrically. Each point and bar represent the mean value and SD of results from three cultures. The absence of a bar indicates that the SD falls within the symbol.

yeast metallothionein Cup1 might have only limited ability to depress the toxicity of cisplatin.

In the present study, we found that a relatively low concentration of copper provided protection against cisplatin toxicity independently of the induction of the synthesis of metallothionein and of the inhibition of platinum uptake. Although the mechanism of such protection is unknown, this study provides new insight into the biological function of copper, an essential trace element, in the protection of eukaryotic cells against an anticancer drug, cisplatin.

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References

[1] G. Chu, Cellular responses to cisplatin. The roles of DNA-binding proteins and DNA repair, J. Biol. Chem. 269 (1994) 787–790.
[2] M. Yoshida, A.R. Khokhar, Z.H. Siddik, Biochemical pharmacology of homologous alicyclic mixed amine platinum(II) complexes in sensitive and resistant tumor cell lines, Cancer Res. 54 (1994) 3468–3473.
[3] L.A. Zwelling, K.W. Kohn, Mechanism of action of *cis*-dichlorodiammineplatinum (II), Cancer Treat. Rep. 63 (1979) 1439–1444.
[4] R. Fujii, M. Mutoh, K. Niwa, K. Yamada, T. Aikou, M. Nakagawa, M. Kuwano, S. Akiyama, Active efflux system for cisplatin in cisplatin-resistant human KB cells, Jpn. J. Cancer Res. 85 (1994) 426–433.
[5] D. Shen, I. Pastan, M.M. Gottesman, Cross-resistance to methotrexate and metals in human cisplatin-resistant cell lines results from a pleiotropic defect in accumulation of these compounds associated with reduced plasma membrane binding proteins, Cancer Res. 58 (1998) 268–275.
[6] R.A. Hromas, J.A. North, C.P. Burns, Decreased cisplatin uptake by resistant L1210 leukemia cells, Cancer Lett. 36 (1987) 197–201.
[7] W.R. Waud, Differential uptake of *cis*-diamminedichloroplatinum(II) by sensitive and resistant murine L1210 leukemia cells, Cancer Res. 47 (1987) 6549–6555.
[8] M.E. Anderson, A. Naganuma, A. Meister, Protection against cisplatin toxicity by administration of glutathione ester, FASEB J. 4 (1990) 3251–3255.
[9] B. Arrick, C.F. Nathan, Glutathione metabolism as a determinant of therapeutic efficacy: a review, Cancer Res. 44 (1984) 4424–4432.
[10] K. Ikeda, K. Miura, S. Himeno, N. Imura, A. Naganuma, Glutathione content is correlated with the sensitivity of lines of PC12 cells to cisplatin without a corresponding change in the accumulation of platinum, Mol. Cell. Biochem. 219 (2001) 51–56.
[11] T. Ishikawa, C.D. Wright, H. Ishizuka, GS-X pump is functionally overexpressed in *cis*-diamminedichloroplatinum (II)-resistant human leukemia HL-60 cells and down-regulated by cell differentiation, J. Biol. Chem. 269 (1994) 29085–29093.
[12] A.K. Godwin, A. Meister, P.J. O'Dwyer, C.S. Huang, T.C. Hamilton, M.E. Anderson, High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis, Proc. Natl. Acad. Sci. USA 89 (1992) 3070–3074.
[13] S.L. Kelley, A. Basu, B.A. Teicher, M.P. Hacker, D.H. Hamer, J.S. Lazo, Overexpression of metallothionein confers resistance to anticancer drugs, Science 241 (1988) 1813–1815.
[14] A. Naganuma, M. Satoh, N. Imura, Effect of copper pretreatment on toxicity and antitumor activity of *cis*-diamminedichloroplatinum in mice, Res. Commun. Chem. Pathol. Pharmacol. 46 (1984) 265–270.
[15] A. Naganuma, M. Satoh, N. Imura, Protective effect of metallothionein inducing metals on lethal toxicity of *cis*-dimminedichloroplatinum in mice, Toxicol. Lett. 24 (1985) 203–207.
[16] A. Naganuma, M. Satoh, N. Imura, Prevention of lethal and renal toxicity of *cis*-diamminedichloroplatinum(II) by induction of metallothionein synthesis without compromising its antitumor activity in mice, Cancer Res. 47 (1987) 983–987.
[17] S.W. Johnson, P.A. Swiggard, L.M. Handel, J.M. Brennan, A.K. Godwin, R.F. Ozols, T.C. Hamilton, Relationship between platinum–DNA adduct formation and removal and cisplatin cytotoxicity in cisplatin-sensitive and -resistant human ovarian cancer cells, Cancer Res. 54 (1994) 5911–5916.
[18] M. Dabholkar, E. Reed, Cisplatin, Cancer Chemother. Biol. Response Modif. 16 (1996) 88–110.
[19] M. Gosland, B. Lum, J. Schimmelpfennig, J. Baker, M. Doukas, Insights into mechanisms of cisplatin resistance and potential for its clinical reversal, Pharmacotherapy 16 (1996) 16–39.

[20] G.M. Lai, R.F. Ozols, J.F. Smyth, R.C. Young, T.C. Hamilton, Enhanced DNA repair and resistance to cisplatin in human ovarian cancer, Biochem. Pharmacol. 37 (1988) 4597–4600.
[21] H. Masuda, R.F. Ozols, G.M. Lai, A. Fojo, M. Rothenberg, T.C. Hamilton, Increased DNA repair as a mechanism of acquired resistance to *cis*-diamminedichloroplatinum (II) in human ovarian cancer cell lines, Cancer Res. 48 (1988) 5713–5716.
[22] N. Masumoto, S. Nakano, H. Fujishima, K. Kohno, Y. Niho, v-src induces cisplatin resistance by increasing the repair of cisplatin-DNA interstrand cross-links in human gallbladder adenocarcinoma cells, Int. J. Cancer 80 (1999) 731–737.
[23] T. Furuchi, H. Ishikawa, N. Miura, M. Ishizuka, K. Kajiya, S. Kuge, A. Naganuma, Two nuclear proteins, Cin5 and Ydr259c, that confer resistance to cisplatin in *Saccharomyces cerevisiae*, Mol. Pharmacol. 59 (2001) 470–474.
[24] A. Naganuma, N. Miura, S. Kaneko, T. Mishina, S. Hosoya, S. Miyairi, T. Furuchi, S. Kuge, GFAT as a target molecule of methylmercury toxicity in *Saccharomyces cerevisiae*, FASEB J. 14 (2000) 968–972.
[25] G.W. Hwang, T. Furuchi, A. Naganuma, A ubiquitin–proteasome system is responsible for the protection of yeast and human cells against methylmercury, FASEB J. 16 (2002) 709–711.
[26] T. Furuchi, G.W. Hwang, A. Naganuma, Overexpression of the ubiquitin-conjugating enzyme Cdc34 confers resistance to methylmercury in *Saccharomyces cerevisiae*, Mol. Pharmacol. 61 (2002) 738–741.
[27] X. Lin, T. Okuda, A. Holzer, S.B. Howell, The copper transporter CTR1 regulates cisplatin uptake in *Saccharomyces cerevisiae*, Mol. Pharmacol. 62 (2002) 1154–1159.
[28] S. Ishida, J. Lee, D.J. Thiele, I. Herskowitz, Uptake of the anticancer drug cisplatin mediated by the copper transporter Ctr1 in yeast and mammals, Proc. Natl. Acad. Sci. USA 99 (2002) 14298–14302.
[29] A. Dancis, D. Haile, D.S. Yuan, R.D. Klausner, The *Saccharomyces cerevisiae* copper transport protein (Ctr1p). Biochemical characterization, regulation by copper, and physiologic role in copper uptake, J. Biol. Chem. 269 (1994) 25660–25667.
[30] C.E. Ooi, E. Rabinovich, A. Dancis, J.S. Bonifacino, R.D. Klausner, Copper-dependent degradation of the *Saccharomyces cerevisiae* plasma membrane copper transporter Ctr1p in the apparent absence of endocytosis, EMBO J. 15 (1996) 3515–3523.
[31] M. Webb, The Chemistry, Biochem. Biol. Cadmium (1979) 195–266.
[32] A. Bakka, L. Endresen, A.B. Johnsen, P.D. Edminson, H.E. Rugstad, Resistance against *cis*-dichlorodiammineplatinum in cultured cells with a high content of metallothionein, Toxicol. Appl. Pharmacol. 61 (1981) 215–226.
[33] L. Endresen, A. Bakka, H.E. Rugstad, Increased resistance to chlorambucil in cultured cells with a high concentration of cytoplasmic metallothionein, Cancer Res. 43 (1983) 2918–2926.
[34] Y. Okazaki, N. Miura, M. Satoh, N. Imura, A. Naganuma, Metallothionein-mediated resistance to multiple drugs can be induced by several anticancer drugs in mice, Biochem. Biophys. Res. Commun. 245 (1998) 815–818.
[35] T.R. Butt, E.J. Sternberg, J.A. Gorman, P. Clark, D. Hamer, M. Rosenberg, S.T. Crooke, Copper metallothionein of yeast, structure of the gene, and regulation of expression, Proc. Natl. Acad. Sci. USA 81 (1984) 3332–3336.
[36] M.T. Carri, F. Galiazzo, M.R. Ciriolo, G. Rotilio, Evidence for co-regulation of Cu, Zn superoxide dismutase and metallothionein gene expression in yeast through transcriptional control by copper via the ACE 1 factor, FEBS Lett. 278 (1991) 263–266.
[37] M. Karin, R. Najarian, A. Haslinger, P. Valenzuela, J. Welch, S. Fogel, Primary structure and transcription of an amplified genetic locus: the CUP1 locus of yeast, Proc. Natl. Acad. Sci. USA 81 (1984) 337–341.
[38] D.J. Thiele, ACE1 regulates expression of the *Saccharomyces cerevisiae* metallothionein gene, Mol. Cell. Biol. 8 (1988) 2745–2752.