Journal of Microbiology and Biotechnology
Vol. 4, No. 1, 30-35, 1994

Characterization of a Cadmium-resistant Yeast Strain in Response to Cadmium or Heat Shock Stress

HUH, NAM-EUNG*, NACK-SHICK CHOI, YOUNG-KYO SEO, TAE-SHICK YU[2] AND HYUN-SOOK LEE[1]

**Department of Biology, [1]Department of Microbiology, Kyeongsang National University, Chinju 660-701, Korea*
[2]Department of Microbiology, Keimyung University, Taegu 750-701, Korea

A varient strain of budding yeast, *Hansenula anomala* B-7 which had been identified to be highly resistant to cadmium ions, were observed by transmission electron microscopy. It was shown that the cells accumulated excess amounts of cadmium ions throughout inside the cell rather than on the cell surface. The cell growth in response to cadmium or heat shock stress has also been investigated. It was observed that the cells precultured in the presence of 500 μg/ml of Cd ions grew slower than those precultured at 1,000 μg/ml of the metal ions, when they were cultivated in the media containing 1,000 μg/ml of the metal ions. Heat shock, however, stimulated the cell growth transiently, when the cells were allowed to grow in the presence of 1,000 μg/ml of the metal ions. But the cells given heat shock for more than 100 min received permanent damage to growth. Effects of both stresses on budding rate was also examined. It revealed that the stresses did not change the budding ratio much, which was contradictory to that observed from the same budding yeast, *Saccharomyces cerevisiae*. Furthermore, the cells treated with 1,000 μg/ml of the metal ions not only induced, but also switched off the expression of several new proteins. Some of the cadmium stress-inducible proteins were estimated to be also induced by heat shock stress.

From yeast to human, the synthesis of a metal binding protein, metallothionein (MT), as well as heat shock proteins (hsp) in response to heavy metals and elevated temperature, respectively, has been observed in most every species examined so far. The synthesis of such stress-responsible proteins are induced very rapidly to protect the cells against toxicity caused by the stresses, thereby the organism can survive in harmful environmental conditions (2, 6, 10, 13). It has been demonstrated that in *Saccharomyces cerevisiae*, the expression of the metallothionein, which is a cysteine-rich, metal-binding protein, is induced transcriptionally by Cu and Ag, but not by Cd. The protein has been speculated to play a role in detoxification and storage of these metals (6). It has been reported, however, that high concentrations of those metals can not be accumulated in the baker's yeast (4). It has been also known that the yeast cells synthesize various hsp in response to heat shock treatment (15-16). Furthermore, there has been a crucial report that transcription of the MT gene in *Saccharomyces cerevisiae* can be under control of heat shock transcription factor (HSF) (14, 17). The report also presents the data to support that the MT biosynthesis is important in response to heat shock stress. Normally, however, HSF mediates the transcriptional induction of hsp in eukaryotes and the transcription of MT is induced by metal ions.

Hansenula anomala, the same budding yeast as *Saccharomyces cerevisiae* but a cadmium-resistant variant, can accumulate cadmium as much as 5.8 mg/gr cell and has been shown to synthesize a cysteins-rich MT-like protein (18-19). In this report, we present data obtained from transmission electron microscopy showing that cadmium ions were accumulated throughout inside of the cells, but neither on the cell surface nor in the membrane. However, we observed that the precultured cells in the 500 ppm of cadmium ions were slower in growth than those precultured in 1,000 ppm of the metal ions, when they were inoculated into the media containing 1,000 ppm of cadmium ions. Heat shock, however, stimulated the cell growth transiently, although the cells given the heat shock for more than 100 min were not able to recover normal growth pattern perma-

*Corresponding author
Key words: Cadmium-tolerant yeast, heat shock stress, cadmium shock, stress-inducible

nently.

In case of *Saccharomyces cerevisiae*, heat shock causes proliferating populations of the yeast cells to accumulate transiently as unbudded cells. These cells then spontaneosly recover even under heat shock conditions (12). In the course of our study, however, it was noticed that neither cadmium treatment nor heat shock stress changed budding ratio significantly. Interestingly, both high concentration of cadmium ion and heat shock treatment induced a protein with the same molecular weight (42 kd). These studies provide evidence that the regulatory mechanism in *H. anomala*, which is involved in responding to harmful environmental conditions is different from that in the same budding yeast, *Saccharomyces cerevisiae*. The studies also suggest the possibility that the same set of proteins is induced by different stresses.

MATERIALS AND MEHTODS

Cells, Growth Conditions and Heat Shock Protocol

A strain of highly cadmium tolerant yeast variant, *Hansenula anomala* B-7, used in this work was obtained from the department of Microbiology, Keimyung University. The cells were grown in the medium as described by Yu *et al.* (18) at 28°C unless otherwise specified. The cultivated cells were harvested by centrifugation at 3,000×g for 10 min and washed three times with ice-cold deionized water as described (19). The cell growth was determined with optical density measured at 600 nm. To give the cells heat shock stress, the yeast cells were grown at 23°C with constant agitation (150 rpm) and were transferred to a glass flask prewarmed to 37°C in a shaking water bath and further incubated for the times indicated in the text.

Assessment of Cell Number and Budding

The cells grown to early log phase (5×10^6 cells per ml) cultured in the standard media containing either 0 ppm or Cd ions of the various concentrations indicated in the figure legends were used for assessment of cell morphology. Budding was assessed visually by direct microscopic inspection (12). The cells were fixed in Formalin and sonicated briefly to disrupt any clumps, and at least 200 cells were scored for each determination (7).

Transmission Electron Microscopy

Cells cultured to a density of mid-log phase in the standard media containing either 0 ppm or 1,000 ppm of Cd ions were used for the experiment. Preparation of the cells and transmission electron microscopy were done as described (13).

Protein Gels

Extraction of the yeast proteins was carried out as described previously with the following modifications (19). The cell pellets washed with ice-cold deionized water were resuspended in three volumes of cell disruption buffer (200 mM Tris HCl, pH 8.0, 10% glycerol, 10 mM $MgClA_2$, 10 mM β-mercaptoethanol, 1 mM PMSF) (5). Then, 4 volumes of chilled acid-washed glass beads were added. The cells were disrupted by vortexing with the maximum speed at 4°C until 30% of the cells were broken. The cell debris were removed by centrifugation at 12,000×g for 60 min. Protein concentrations were determined by the Bradford assay (Bio-Rad). SDS polyacrylamide gel electrophoresis was performed by a modification of the procedure of Laemmli (9).

RESULTS AND DISCUSSION

Hansenula anomala B-7, a variant of budding yeast, has been identified to be tolerent even upto 2,700 ppm of the cadmium ions and can grow relatively well in the liquid medium containing 1,000 ppm of the metal ions (18). Yu et al. have also purified two polypeptides which can bind Cd and have rationally high content of cysteine as well as a paucity of aromatic amino acid residues and low molecular weights (19). These characteristics are hallmarks of the metallothionein (MT) expressed in the same budding yeast, *S. cerevisiae*, or other eukaryotes examined so far (6). However, the localization of the Cd ions internalized in the *H. anomala* has not been revealed yet. Therefore, we sought to determine the distribution of cadmium in the cells by transmission electron microscopy. Fig. 1 shows that there is no trace of the metals in the cells cultured in the liquid media free of Cd (Fig. 1 A). In contrast, the cells grown in the presence of 1,000 ppm of Cd ions accumulate excessive amounts of the metal ions throughout the cells (Fig. 1B). However, it is hardly believed that the amount of MT normally synthesized in the cell is enough to sequester all of such excessively internalized Cd ions. Therefore, we can not rule out the possibility of induction of other molecule(s) by Cd ions which take(s) part in sequestering the excess amount of the Cd ions transported into the cells. Actually, *Candida glabrata* expresses both MT and γ-glutamyl peptides and their biosynthesis is regulated by copper and cadmium, respectively, in metal-specific manner. Furthermore, both are capable of binding a variety of heavy metal ions (11).

Fig. 1 also shows that the cell wall integrity of *H. anomala* cultured in the presence of 1,000 ppm of Cd ions is severely damaged in comparison with that grown in the Cd-free media. However, it remains to be identified whether the damage of the cell wall can

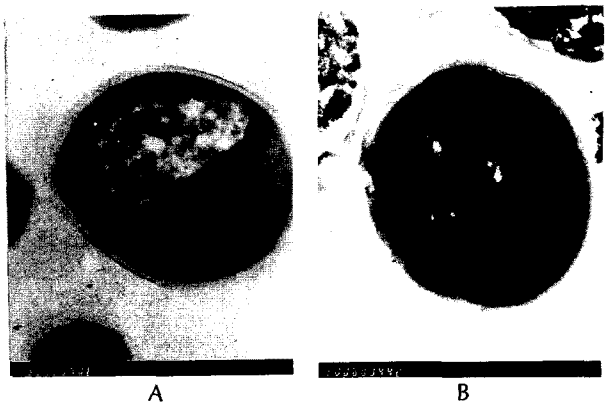

Fig. 1. Transmission electron microscopy (TEM) of the yeast cells, *Hansenula anomala* B-7, cultured in the absence or presence of high concentration of cadmium ions.
A culture of mid log cells (5×10^6/ml, or $OD_{600}=0.4-0.8$) grown in the standard media containing 0 ppm (pannel A), or 1,000 ppm (pannel B) of cadmium ions was harvested to fix with glutaraldehyde and cacodylate as described (14). The procedure for taking the fictures was followed as recommended by the manufacturer. The transmission electron microscope used in the experiment was a model of Hitachi 600.

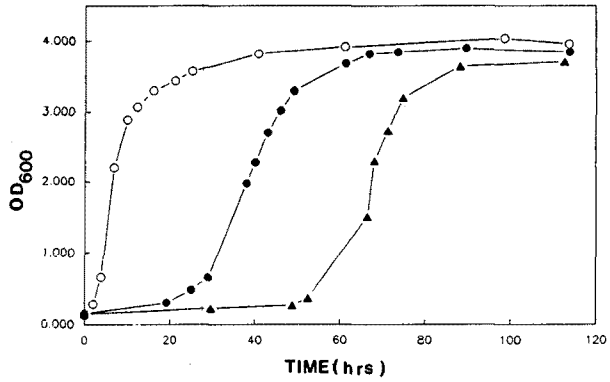

Fig. 2. Effect of pre-culture at various concentrations of Cd ions on the adaptation of the *H. anomala* cells to 1,000 ppm of the metal ions.
The cells pre-cultured to mid-log phase at 0 ppm (○), 500 ppm (▲) and 1,000 ppm (●), respectively, were transferred to the Cd-free media (○), or to the ones containing 1,000 ppm of the Cd ions (▲, ●) for further culture. The cell growth was measured by optical density at 600 nm with an aliquot harvested at various time intervals.

be recovered if the cells were transferred to the Cd-free media. If the damage is not recovered, it may suggest that high concentration of Cd ions causes permanent damage to the cell wall resulting in loss of ability of the cells to transport the metal ions selectively. This loss may allow Cd ions to influx into the cells. Therefore, it may be of intereat to examine whether other metal ions usually not accumulated in the cells can also be influxed into the cells along the Cd ions, if they were added together with 1,000 ppm of cadmium.

We examined the effect of pre-culturing in the presence of 500 ppm of Cd ions on adaptation of the cells to 1,000 ppm. For this examination, an aliquot of the cells previously grown in 500 ppm of Cd ions was transferred to the standard media containing 1,000 ppm of the metal ions and the cell growths were detected as described in Methods. As a control, the cells pre-cultured with 1,000 ppm of Cd ions were transferred as inoculum to the media containing 1,000 ppm of the metal ions. The data in Fig. 2 shows that the cells pre-shocked with 500 ppm reached mid-log phase 30 hours later than the control cells, which were pre-shocked with 1,000 ppm. However, the cells under being Cd-shocked in the presence of 1,000 ppm of Cd ions required at least 2 weeks to reach the mid-log phase, whereas the cells under being pre-shocked in the presence of 500 ppm needed only 4 to 5 days to grow to the same density. Therefore, the pre-shocking with 500 ppm could reduce half of the growth time that the control cells took to reach the mid-log phase. These studies provide the possibility that pre-shocking will be helpful to increase economic value of the cells, when they are used to remove Cd ions from industrial pollutants.

The stress caused by exposure of proliferating cells of the budding yeast *S. cerevisiae* to temperatures well above normal growth temperatures results in rapid loss of viability. However, the yeast cells can adapt to and survive this usually lethal treatment, if they are first subjected to a heat shock, a procedure in which cells are incubated at an elevated but otherwise nonlethal growth temperature (1). It has also been revealed that stress causes proliferating *S. cerevisiae* to accumulate transiently as unbudded cells. These heat-shocked cells then spontaneously recover, even under heat shock conditions and the cells resume proliferation (8, 12). Therefore, it will be of interest to investigate whether heat shock treatment instead of lethal temperature treatment of *H. anomala*, which is the same budding yeast as *S. cerevisiae*, can increase the adaptability of the cells to high concentration of Cd ions, or whether both stresses can cause the cells to accumulate as unbudded cells as does in the baker's yeast.

To examine the effect of heat shock on the growth of *H. anomala* in the presence of high concentration of Cd ions, the cells previously grown in the Cd-free media were subjected to heat shock for 45 min immediately after being inoculated into the media containing 1,000 ppm of Cd ions (Fig. 3). The data in Fig. 3 demonstrate that heat shock causes stimulation of the cell growth transiently in the presence of extremely high concentration of Cd salts (1,000 ppm). But further incubation results in suppression of cell proliferation

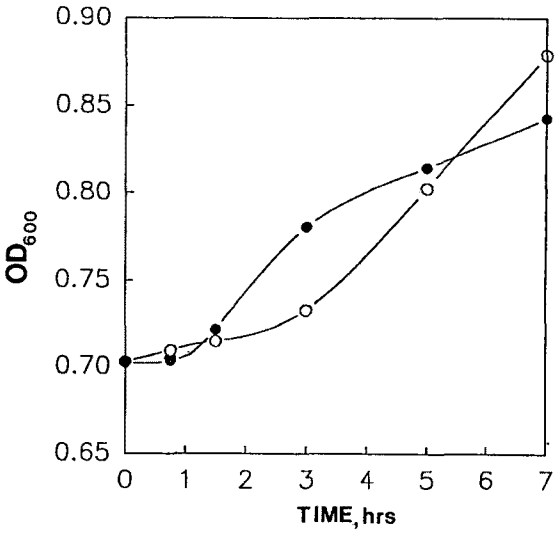

Fig. 3. Effect of heat shock on the cell growth at high concentration of Cd salts.
The *H. anomala* cells grown to mid-log phase at Cd-free media were harvested by centrifugation. The cell pellets were resuspended in the fresh medium containing 1,000 ppm of Cd salts to make the cell density to OD_{600}=0.7. An aliquot the cell suspensions was either subjected to heat shock at 43°C for 45 min as described in the text (●), or kept at the normal growth temperature as a control (○). Then the cells were transferred to 28°C for further growing. The cell growth was determined as described in the methods.

in comparison with those not received thermal stress.

We also examined the kinetics of thermal effect on the inhibition of the cell proliferation. The cells given heat shock at 43°C for various times were shifted to 28°C for cultivating additionally for 500 min. The results are shown in Fig. 4. The curve is biphasic, and consist of an initial rapid decline followed by arrest of the cell proliferation. Furthermore, the cells which received heat shock for more than 90 min could not recover the damage in the growth, even though they were allowed to grow at normal temperature for 500 min in the Cd-free media (Fig. 4).

For *S. cerevisiae*, the presence of a bud reflects the cell cycle position: cells in the G_1 interval of the cell cycle are unbudded. The yeast cells respond to heat shock by transiently accumulating in the unbudded (G_1) interval of the cell cycle (1, 12). However, the data described in Fig. 5 demonstrate that under the heat shock conditions, the *H. anomala* cells did not showed transient accumulation of the proliferating budded cells as unbudded cells. They also did not show variation on the ratio of budded to unbudded cells, even though they were allowed to grow further at the normal growth temperature for 300 min. On the other hand, the Cd-shock derived from 1,000 ppm of the metal ions caused the cells to accumulate transiently as budded cells, although the accumulation was not

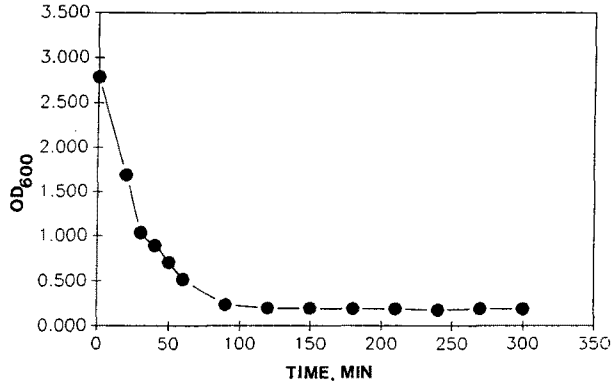

Fig. 4. Level of growth recovery in response to heat shocked time.
The cells grown to mid-log phase at 28°C were heat shocked at 43°C for various times from 0 to 300 min (●). The heat shocked cells were shifted to 28°C for further cultivation for 500 min and then the cell density was measured by OD_{600}.

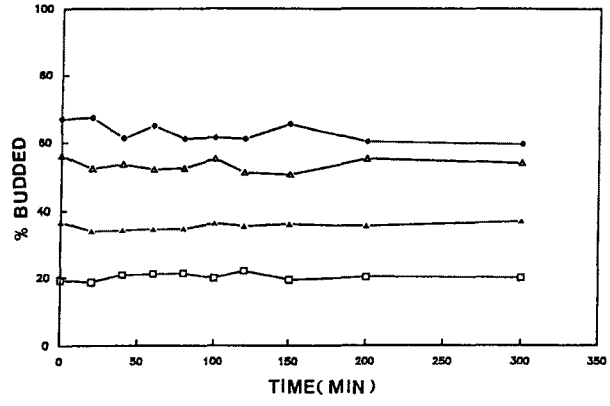

Fig. 5. Effect of heat shock on accumulation of budded cells.
The cadmium-resistant budding yeast, *H. anomala*, grown to the various densities was shifted to 37°C from 23°C for heat shock. An aliquot of the cells was removed at the intervals for assessment of bud morphology. The densities of the cells starting given heat shock were 0.75 (●), 1.09 (△), 2.81 (▲) and 3.62 (□), respectively. Budding was assessed visually by microscopic inspection as described in the methods.

so prominent as those observed in *S. cerevisiae* (Fig. 6). However, the *H. anomala* cells showed no variation on the cell proliferation (or the ratio of budded to unbudded cells), when they were allowed to grow further at 1,000 ppm. Therefore, these observations make us suggest that the stresses caused by heat shock as well as Cd treatment can make the cells to arrest in the G_1 interval of the cell cycle.

It has been demonstrated that the MT gene in *Saccharomyces cerevisiae* is transcribed in response to heat shock, but the induction of MT from the gene by the heat shock stress can be indirect (14). Yang et al. also provided evidence for multiple pathways of MT gene transcriptional regulation in *S. cerevisiae*

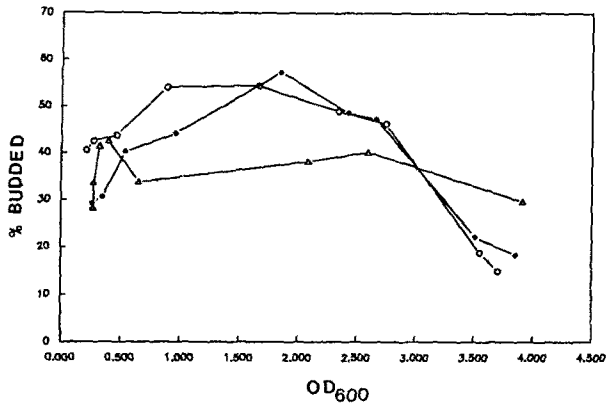

Fig. 6. Effect of cadmium shock on the accumulation of budded cells.
The *H. anomala* cells grown in the media containing 0 ppm (○ and ●), or 1,000 ppm (△) of cadmium ions were removed at the various time intervals for assessment of bud morphology. The inoculum used in the cadmium-free media was derived directly from a slant culture containing 100 ppm of the metal ion (●), or from the ones allowed to proliferate in the Cd-free media for three generations (○).

Fig. 7. SDS-PAGE of the soluble proteins prepared from the cadmium-shocked *Hansenula anomala* cells.
The yeast cells were grown in the media containing 0, 1, 10, 100, 500 and 1,000 ppm (lanes 1~6), respectively, and a culture of mid-log cells (at the density of 1.0 as OD_{600}) was harvested to extract proteins as described in the methods. The proteins were resolved in 15% SDS-PAGE by the standard method. Size markers included phosphorylase B (97.4 kd), bovine serum albumin (66.2 kd), egg white ovalbumin (45 kd), bovine carbonic anhydrase (31.0 kd), soybean trypsin inhibitor (21.5 kd) and egg white lysozyme (14.4 kd), respectively. Indicated is the signal expected to be induced by the high concentration of cadmium in the *H. anomala* cells.

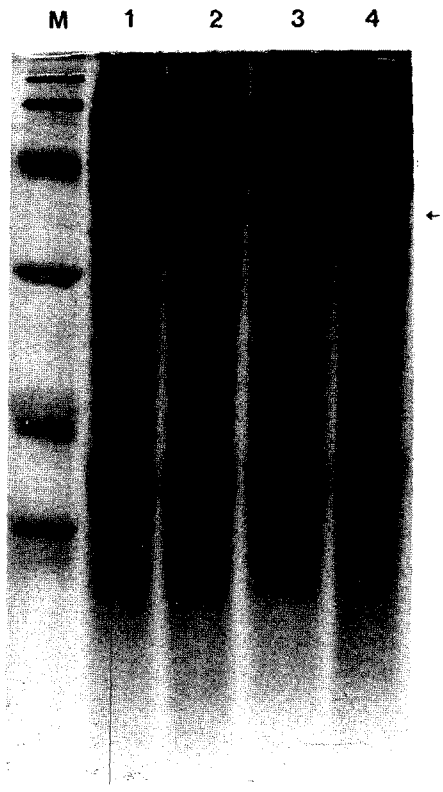

Fig. 8. SDS-PAGE for the comparison of proteins extracted from Cd-shocked cells with the ones obtained from heat shocked cells.
The cells were grown in the absence (lanes 1~3), or presence (lane 4) of 1,000 ppm of cadmium salts (lane 4), respectively. The cells cultured in absence of the metal ion were shifted from 23°C to 37°C and further incubated for 0, 15 and 45 min, respectively, for heat shock treatment (lanes 1~3). The soluble proteins were prepared from the cells as described above, and were resolved in 13% SDS-PAGE. Size markers were the same ones used in Fig. 7. The location of 42 kd is indicated by the arrow.

(17). These observations together with the data described above make us to predict that both stresses can activate expression of other polypeptide(s) contributing to cellular resistance to stress toxicity. In order to investigate the possibitity, the *H. anomala* cells were subjected to cadmium stress in the presence of various concentrations of Cd salts and the harvested cells were disrupted to extract cellular proteins. The proteins were resolved on a SDS polyacrylamide gel with size markers (Fig. 7). More than 1,000 ppm of Cd salts induced the synthesis of a polypeptide, molecular weight of which was estimated to be 42 kd. The synthesis of the polypeptide was very prominent. We also investigated whether heat shock stress could also induce the synthesis of a polypeptide with the same molecular weight. Fig. 8 demonstrated that the thermal stress given for more than 15 min also induced the expres-

sion of the polypeptide with the same MW (Fig. 8, lanes 2~3). Furthermore, the level of the expression induced by each stress was consistent with each other. These observations suggest that two different stresses may induce a common polypeptide(s). However, further investigation by more accurate approaches will be necessary to verify the suggestion. It also remains to be elucidated whether these two proteins are transcribed from the same gene or not.

Acknowledgement

This work was supported by the research grant from the Korea Sanhack Foundation.

REFERENCE

1. Barnes, C.A., G.C. Johnston and R.A. Singer. 1990. Thermotolerance is independent of induction of the full spectrum of heat shock proteins and of cell cycle blockage in the yeast *Saccharomyces cerevisiae*. *J. Bacteriol.* **172**: 4352-4358.
2. Blom, A., W. Harder and A. Martin. 1992. Unique and overlapping pollutant stress proteins of *Escherichia coli*. *Appl. Envi. Microb.* **58**: 331-334.
3. Byers, B. and L. Goetsch. 1991. Prepation of yeast cells for thin-section electron microscopy. *Methods Enzymol.* **194**:.602-608.
4. Church, J.F., G. Scholz and K. Kwok. 1989. Activation of pyridoxal kinase by metallothionein. *Biochim. Biophys. Acta.* **996**: 181-186.
5. Gallo, G., Schuetz, T. and R. Kingston. 1991. Regulation of heat shock factor in *Schizosaccharomyces pombe* more closely resembles regulation in mammals than in *Saccharomyces cerevisiae* *Mol. Cell. Biol.* **11**: 281-288.
6. Hamer, D. 1986. Metallothionein. *Ann. Rev. Biochem.* **55**: 913-951.
7. Hartwell, L.H. 1970. Periodic density fluctuation during the yeast cell cycle and the selection of synchronous cultures. *J. Bacteriol.* **104**: 1280-1285.
8. Johnston, G.C. and R.A. Singer. 1980. Ribosomal precursor RNA metabolism and cell division in the yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **178**: 357-360.
9. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227**: 680-685.
10. Lindquist, S. and E.A. Craig. 1988. The heat-shock proteins. *Ann. Rev. Genet.* **22**: 631-77.
11. Mehra, R.K., E.B. Tarbet, W.R. Gray and D.R. Winge. 1988. Metal-specific synthesis of two metallothioneins and γ-glutamyl peptides in *Candida glabrata*. *Proc. Natl. Acad. Sci.* **85**: 8815-8819.
12. Rowley, A., G.C. Johnston, B. Butler, M. Werner-Washburne, R.A. Singer. 1993. Heat shock-mediated cell cycle blockage and G1 cyclin expression in the yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **13**: 1034-1041.
13. Schlesinger, M.J. 1990. Heat shock proteins. *J. Biol. Chem.* **265**: 12111-12114.
14. Silar, P., G. Butler and D.J. Thiele. 1991. Heat shock transcription factor activates transcription of the yeast metallothionein gene. *Mol. Cell. Biol.* **11**: 1232-1238.
15. Sorger, P.K. and H.R.B. Pelham. 1987. Purication and characterization of a heat shock element binding protein from yeast. *EMBO J.* **6**: 3035-3041.
16. Stone, D.E. and E.A. Craig. 1990. Self-regulation of 70-kilodolton heat shock proteins in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **10**: 1622-1632.
17. Yang, W., W. Gahl and D. Hamer. 1991. Role of heat shock transcription factor in yeast metallothionein gene expression *Mol. Cell. Biol.* **11**: 3676-3681.
18. Yu, T.S., H.I. Song and K.T. Chung. 1989. Intracellular accumulation of cadmium by intact cadmuim tolerant yeast cells. *Kor. J. Appl. Microbial. Biotech.* **18**: 233-238.
19. Yu, T.S. and B. Koo. 1990. Purification and characteristics of cadmium-binding protein from *Hansenula anomala*. *Kor. J. Microbiol.* **28**: 258-263.

(Received December 28, 1993)