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Enhanced Expression of High-affinity Iron Transporters via H-ferritin Production in Yeast

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Our heterologous expression system of the human ferritin H-chain gene (*hfH*) allowed us to characterize the cellular effects of ferritin in yeasts. The recombinant *Saccharomyces cerevisiae* (YGH2) evidenced impaired growth as compared to the control, which was correlated with ferritin expression and with the formation of core minerals. Growth was recovered via the administration of iron supplements. The modification of cellular iron metabolism, which involved the increased expression of high-affinity iron transport genes (*FET3* and *FTR1*), was detected via Northern blot analysis. The findings may provide some evidence of cytosolic iron deficiency, as the genes were expressed transcriptionally under iron-deficient conditions. According to our results examining reactive oxygen species (ROS) generation via the fluorescence method, the ROS levels in YGH2 were decreased compared to the control. It suggests that the expression of active H-ferritins reduced the content of free iron in yeast. Therefore, present results may provide new insights into the regulatory network and pathways inherent to iron depletion conditions.

Keywords: Expression, High-affinity iron transporter, H-ferritin, Yeast

Introduction

Ferritin is the principal intracellular iron storage protein encountered in most living organisms, from bacteria to mammals. All ferritins are composed of 24 subunits being arranged in a hollow protein shell (apoferritin) with the capacity storing up to 4,500 Fe^{3+} atoms (Harrison and Arosio, 1996; Chasteen and Harrison, 1999). In the majority of vertebrates, ferritins in various tissues consist of two main subunits, the heavy (known as heart type, or H) and light (known as liver type, or L) subunits. The results of *in vitro* studies have indicated that the H subunit facilitates iron oxidation at a dinuclear iron ferroxidase site, whereas the L subunit expedites protein stabilization and core formation (Santambrogio *et al*., 1993; Levi *et al*., 1994). Although tissue isoferritins exhibit functional differences that may be associated with variations in subunit composition, the functional differences of the two subunits *in vivo* have yet to be clearly defined. The ability of this protein to sequester iron confers upon ferritin the dual functions of iron reserve and iron supply. Another cellular function of the protein is the protection of the cells against intracellular free iron-induced toxicity (Aust, 1995; Schafer *et al*., 1996). Excess iron can potentially prove harmful, as it can catalyze the formation of reactive oxygen species (ROS) via the Fenton reaction (Cadenas, 1989). Therefore, iron metabolism involving iron uptake, transport, and mobilization must be tightly controlled.

The yeast, *Saccharomyces cerevisiae,* is an ideal organism for the elucidation of the biological functions of ferritin subunits and intracellular iron mobilization in eukaryotic cells. In general, iron uptake is facilitated via two different transport systems: a high-affinity system expressed in the iron-limited cells and a low-affinity system being active in the iron-replete cells (Lesuisse *et al*., 1987; Eide *et al*., 1992). No iron-rich protein, such as ferritin, has been detected within the cytoplasm of *S. cerevisiae*. The vacuole, rather than ferritin, is the major compartment for iron storage in *S. cerevisiae* (Lesuisse *et al*., 1987; Anderson *et al*., 1994; Eide, 1998).

In recent years, several studies have been carried out regarding the *in vivo* functions of ferritin. The expressions of recombinant H-ferritins (H-chain ferritins) in stable transfectants of the mouse erythroleukemic cell line (Picard *et al*., 1996; 1998; Epsztejn *et al*., 1999) and HeLa cells (Cozzi *et al*., 2000; Orino *et al*., 2001) were found to induce iron-deficient

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phenotypes and reduced the oxidative damage associated with free iron. It was, therefore, postulated that the ferritin H subunit plays a pivotal role in the regulation of iron availability, via the activity of its ferroxidase catalytic site. Nevertheless, the mammalian cell lines themselves harbor their own ferritin genes, and the possible exaggerations ensuing from that fact must be carefully avoided.

We previously characterized the heterologous expression of the H-chain of human ferritin in yeast. Recombinant H-ferritin was active in the formation of the iron core (Seo *et al*., 2003). This study, then, was conducted in an effort to characterize further the cellular effects of the expression of the H subunit. We reported for the first time in this study that the expression of the human ferritin H-chain modifies cellular iron metabolism in yeast, which results in reduced cell growth, increased high-affinity iron transporter gene expression, and reduced ROS production upon oxidative stress induced by H_2O_2.

Materials and Methods

Yeast strains and growth conditions. *S. cerevisiae* 2805 (*MATα pep4::HIS3 prb1-δ can1 GAL2 his3 ura3-52*) was employed as a host strain for the heterologous expression of the human ferritin H-chain gene (*hfH*). *S. cerevisiae* cells expressing *hfH* were referred to as strain YGH2. The YGT strain was used as a control, the cells of which harbor the YEp352 plasmid, which contains the promoter and terminator regions (Seo *et al*., 2003). For the expression of *hfH*, recombinant yeasts were grown aerobically at 30°C in uracil-deficient selective medium (ura$^-$ selective; 0.67% yeast nitrogen base without amino acids, 0.5% casamino acids, 0.03 g l^{-1} adenine and tryptophan) supplemented with 2% galactose as an inducer. The recombinant yeasts were grown in ura$^-$ selective media for 3 days at 30°C in various ferric citrate concentrations.

Gel electrophoresis. In order to determine the expression of the human ferritin H-chain in yeast, YGH2 cells were harvested via 10 min of centrifugation at 5,000 × *g* (4°C) and washed twice in distilled water and once in 20 mM Tris/HCl buffer (pH 7.4). The cells were then disrupted using a bead beater (Biospec Products Inc.) for 3 min, and the lysates were centrifuged for 10 min at 10,000 × *g* (4°C). The supernatant was examined via 12% (w/v) SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE samples were heated for 10 min at 100°C in SDS gel-loading buffer. The total amount of ferritin generated in the cells was analyzed via densitometry (Molecular Dynamics PD-120). The subunit assembly and core formation were characterized via 7.5% PAGE in non-denaturing gels. Apart from the iron contained in the culture medium, no extra iron was added to the cell lysates. Partially purified samples were prepared via 10 min of heat denaturation at 75°C. The gels were stained for proteins using 0.2% Coomassie brilliant blue, and stained for iron using $K_4Fe(CN)_6$ (2%) and HCl (2%), mixed (1 : 1, v/v) immediately prior to use. The protein concentrations were then determined via the Lowry method, with some modifications (Hess *et al*., 1978).

Total RNA isolation and Northern blot analysis. Cells were grown in ura$^-$ selective medium (2% galactose) and harvested at the indicated times. Total RNA was isolated using conventional glass bead/phenol-chloroform extract. RNA was quantified via spectrophotometry. Approximately 20 μg of total RNA was electrophoresed on 1.2% formaldehyde agarose gel, and transferred to nylon membranes (Amersham Pharmacia Biotech). Northern blot analyses were conducted, as described by Sambrook *et al.* (1989). The DNA probes for *hfH*, *FET3*, *FTR1,* and *ACT1* were radiolabeled with [α-^{32}P]dCTP, using a random primer-labeling kit (Promega). The probe DNA was prepared via the PCR amplification of the cDNA. The following primer pairs were employed for PCR: *FET3* (5'-ATGAC TAACGCTTTGCTCT CT-3' and 5'-TTAGAAGAACCGTTTGGC TTTA-3'), *FTR1* (5'-ATGCCTAACAAAGTGTTTAAC-3' and 5'-TCAAAGAGAGTCGGCTTTAAC-3') and *ACT1* (5'-ATGGATTC TGAGGTTGCT-3' and 5'-TTAGAAACACTTGTGGTGAA-3'). *ACT1* was used as an internal standard. The cells were also grown for 24 h and treated for 1 h with 2,2'-bipyridyl (BIP) or ferrous ammonium sulfate (FAS). Cell samples were collected and analyzed via Northern blotting.

Measurement of intracellular oxidation levels. The oxidant-sensitive probe, dihydrorhodamine 123 (Sigma D1054), was utilized in the measurement of intracellular oxidation levels in yeast, in accordance with the method described by Madeo *et al.* (1999). The recombinant cells were cultured for 24 h in ura$^-$ selective medium supplemented with 2% galactose. The cells were washed and resuspended in phosphate-buffered saline (10 mM phosphate buffer, 0.15 M NaCl, pH 7.0). The cells were then incubated for 1 h with dihydrorhodamine 123 (2.5 μg/ml). Hydrogen peroxide was added as indicated, and the cells were incubated for an additional 20 min. The cells were washed and resuspended in phosphate-buffered saline. They were then analyzed using a FACS Caliber system (Becton Dickinson). Dihydrorhodamine 123 fluorescence was excited at a wavelength range of 450-490 nm, and emission was monitored at 515-565 nm (filter FL1). The experiments were repeated three times, with approximately 10,000 cells per assay.

Results

Ferritin expression and cellular growth. The episomal expression vector Yep352, which harbors the GAL1 promoter, has previously been used to express *hfH* (Seo *et al*., 2003). Recombinant human H-ferritin was found to be active in iron storage in *S. cerevisiae*. When the growth of the recombinant strain was evaluated, no significant differences were observed between YGT and YGH2 grown in YEP (1% yeast extract and 2% peptone) medium supplemented with 2% galactose (Seo, 2002). In this study, the effects of *hfH* expression were examined further with regard to its cellular metabolism. Cell growth, *hfH* expression and subunit assembly were detected in ura$^-$ selective medium supplemented with 2% galactose at the culture times (12, 24, 36, 48, 60 and 72 h) as shown in Fig. 1. The growth of YGH2 after 12 h culture deviated from the

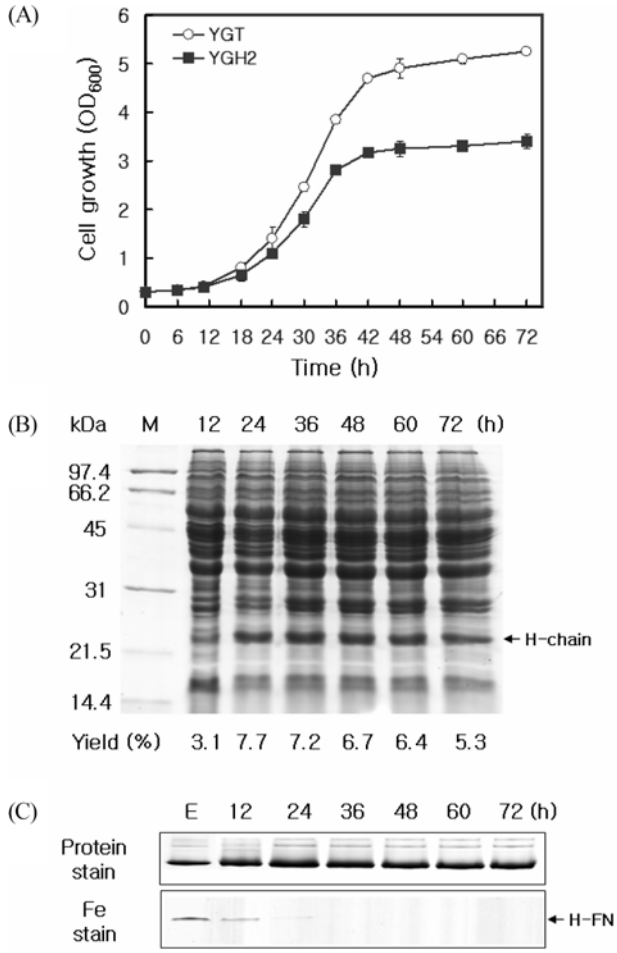

Fig. 1. Cell growth and time course expression of recombinant human ferritin in YGH2. The YGT strain was used as a control. (A) The yeast cells were cultured in uracil-deficient (ura^-) selective medium containing 2% galactose. (B) 12% SDS-PAGE analysis of human ferritin H-chain expressed in YGH2. The cell extracts obtained at each culture time were separated via SDS-PAGE, and stained with Coomassie blue R-250. Expression yields and relative H subunit contents were estimated via densitometric analysis. (C) 7.5% non-denaturing gel of H-ferritin expressed in YGH2. Heat-labile proteins were removed via 10 min of heat treatment at 75°C. The protein samples were loaded with 10 μg per lane of the gel, stained for protein with Coomassie blue, and stained for iron with Prussian blue. The lanes are: M, size marker; 12-72, each number indicates culture time in hours; E, purified *E. coli*-derived recombinant H-ferritin.

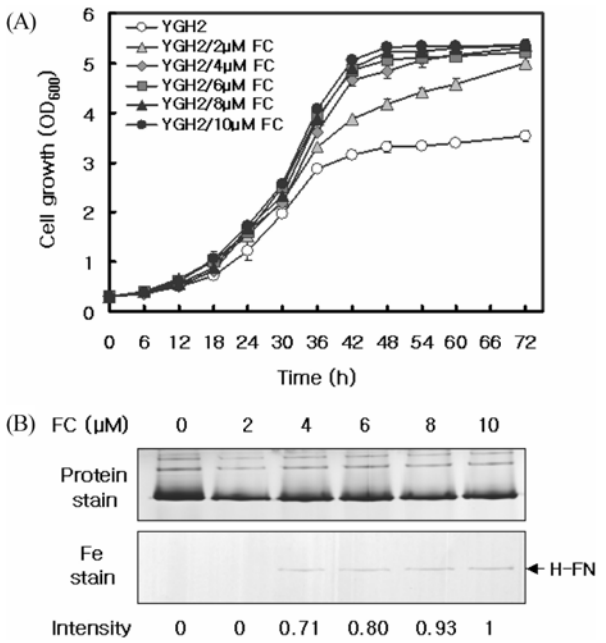

Fig. 2. Recovery of cell growth in the presence of ferric citrate. (A) The growth of the YGH2 strain was examined in ura^- selective medium containing 2% galactose, and various concentrations of ferric citrate (FC). (B) 7.5% non-denaturing gel of H-ferritin in YGH2 cultured for 72 h in ura^- selective medium (2% galactose). Heat-labile proteins were removed via 10 min of heat treatment at 75°C. The protein samples were loaded at 10 μg per lane. Each number indicates the ferric citrate concentration. The band intensity of the iron stain gel was estimated via densitometric analysis.

control strain (YGT). After 72 h culture, the final cell densities achieved an OD_{600} of just over 3 as compared to the 5 OD_{600} observed with YGT (Fig. 1A). In order to assess *hfH* expression, crude cell extracts of YGH2 were analyzed via 12% SDS-PAGE. The extracts obtained at the culture times (12, 24, 36, 48, 60 and 72 h) evidenced the band for the H subunit (21 kDa), which represented about 3.1, 7.7, 7.2, 6.7, 6.4, and 5.3% of the total soluble proteins, respectively, as analyzed via densitometry (Fig. 1B). The expression of the recombinant ferritin H-chain showed a maximum at approximately 24 h. The band of the expressed proteins was confirmed as the ferritin H subunit via Western blotting (Seo *et al*., 2003). The expressed ferritin subunits were assembled spontaneously into holoproteins, which were observed on non-denaturing gels (Fig. 1C). For the experiment, heat-denatured, partially purified samples were employed in order to eliminate unnecessary protein bands. The recombinant H-ferritins migrated in a fashion similar to that of the purified *E. coli*-derived recombinant H-ferritins. Iron incorporation was verified at 12 h of culture. Iron bands also appeared at 24 h of culture, but no further (Fig. 1C). Apart from the iron contained in the ura^- selective medium, no extra iron was added to the cell lysates, implying that the recombinant H-ferritin had spontaneously formed a stable iron core *in vivo* as the protein was generated.

In YGH2, cell growth was evaluated via the addition of various concentrations of iron (2-10 μM ferric citrate) to ura^- selective medium supplemented with 2% galactose. Cell growth was gradually restored in conjunction with the increase in iron concentration, and closely approached the level of the control above 4 μM ferric citrate (Fig. 2A). Ferritin expression and iron incorporation were determined at 72 h of culture. The H-ferritin bands stained positively for iron *in vivo* above 4 μM

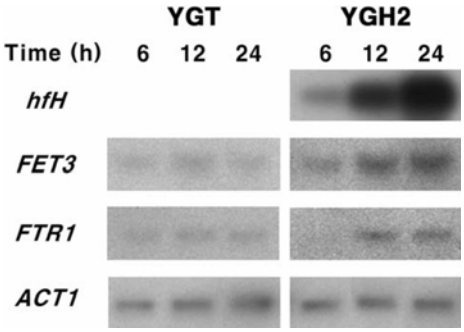

Fig. 3. Time course of the expression of iron transport genes via Northern blot analysis. Cells were grown in ura$^-$ selective medium (2% galactose). Total RNA was prepared from all cell samples, size-fractionated on agarose, and probed with random-primed DNA fragments taken from the respective genes. *ACT1* was employed for the control of the constitutively-expressed genes. The lanes are: 6-24, each number indicates culture time in hours.

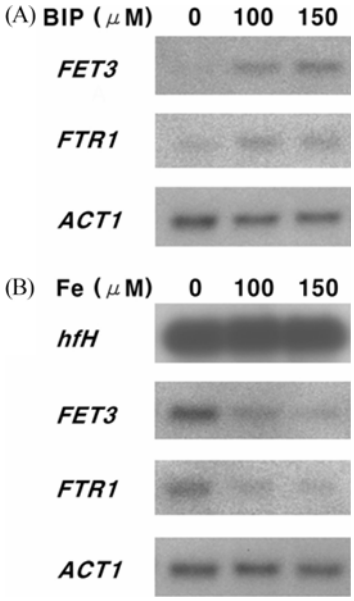

Fig. 4. Effects on the expression of iron transport genes via the administration of iron chelator (A) or supplement (B). Cells were grown in ura$^-$ selective medium (2% galactose) for 24 h and then treated with 2,2'-bipyridyl (BIP; 100 and 150 μM) or ferrous ammonium sulfate (FAS; 100 and 150 μM) for 1 h. Cells were collected and analyzed via Northern blotting.

ferric citrate, and the intensity of the stain increased gradually from a level of 0.71 up to control levels, as the iron concentration increased from 4 μM to 10 μM (Fig. 2B). However, no iron-stained bands were detected at 2 μM ferric citrate, where cell growth was not recovered to control levels.

Ferritin expression and cellular iron metabolism. In order to determine the effects of H-ferritin expression on changes in cellular iron metabolism involving iron uptake systems, we evaluated the transcription of the high-affinity iron transport genes (*FET3* and *FTR1*) via Northern blot analysis (Fig. 3). We determined the amounts of *FET3* and *FTR1* mRNAs in the recombinant yeasts grown in ura$^-$ selective medium (2% galactose). In YGT, limited but not pronounced amounts of *FET3* and *FTR1* transcripts were exhibited over 6-24 h culture. For YGH2, however, the quantity of *hfH* mRNA increased between 6 to 24 h culture. In the case of *FET3* and *FTR1* mRNAs, a different expression pattern was noted, as compared to that observed in YGT. The *FET3* and *FTR1* transcripts of YGH2 increased directly with increases in *hfH* mRNAs. The results were further verified via the treatment of the cells with iron chelators or supplements (Fig. 4). In order to induce iron depletion in YGT, the cells were grown for 24 h and treated for 1 h with BIP. The membrane-permeating iron chelator BIP has the potential to pass into cells and alter intracellular iron distribution. The BIP concentrations of 100 and 150 μM induced the expression of both the *FET3* and *FTR1* genes compared to the untreated cells (Fig. 4A). In the case of YGH2, the cells were grown for 24 h and then treated for 1 h with FAS. The Fe^{2+} salt FAS provides soluble elemental iron to the yeast. As shown in Fig. 4B, iron additions of 100 or 150 μM FAS induced reductions in the expression of both the *FET3* and *FTR1* genes. The administration of iron supplement did not alter the amount of *hfH* transcripts, as *hfH* expression is independent from iron-mediated cellular regulation. Our results show that the generation of active recombinant H-ferritin induced changes in the expression of the genes (*FET3* and *FTR1*) associated with cellular iron uptake.

Ferritin expression and oxidative stress. In order to investigate the role of recombinant H-ferritin under oxidative stress, we determined ROS levels via the incubation of cells with dihydrorhodamine 123. Free iron has the capacity to participate in the formation of ROS under aerobic conditions (Cadenas, 1989). The probe, dihydrorhodamine 123, accumulates in the cells, in which it is oxidized by ROS to the fluorescent chromophore, rhodamine. The level of ROS thus reflects that of cellular free iron (Yuan *et al.*, 2004). ROS generation in the recombinant yeast cells was assessed via flow cytometry (Fig. 5). The YGT cells accumulated a comparatively large number of oxygen radicals in a reaction with 5-15 mM H_2O_2, as compared to the untreated cells. In the case of YGH2 cells, the inhibition of ROS production was found to be significant in a reaction with 5-15 mM H_2O_2. Our results suggested that the YGH2 cells expressing the H-ferritins possessed reduced amounts of free iron in the cells due to an accumulation of iron within a core.

Discussion

This study attempted to determine the cellular effects of *hfH* expression in *S. cerevisiae*, in which neither ferritin nor bacterioferritin is generated within the cytosol. Recombinant human H-ferritins have previously been successfully produced,

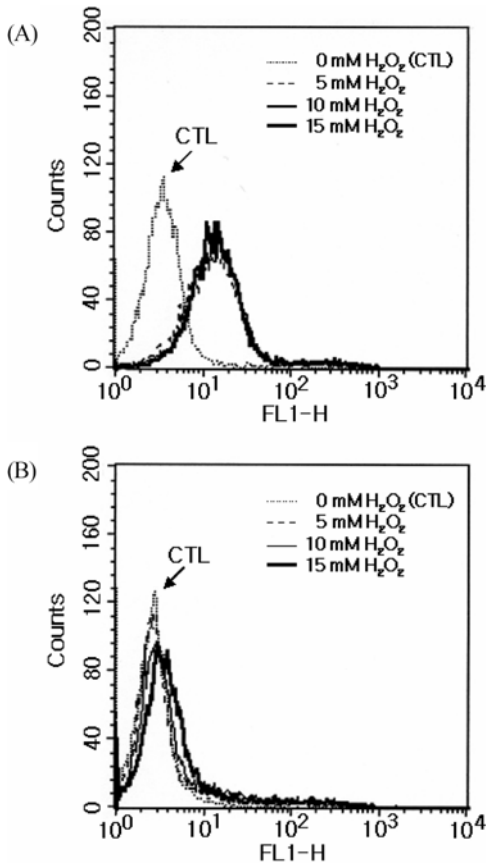

Fig. 5. Comparison of ROS generation under oxidative stress conditions with H_2O_2 in YGT (A) and YGH2 (B). Yeasts were grown in ura$^-$ selective medium (2% galactose) for 24 h. The cells were incubated with 2.5 μM dihydrorhodamine 123 for 1 h and treated with various concentrations of H_2O_2 for 20 min.

and their activity regarding the formation of stable iron cores has been verified (Seo *et al*., 2003). It is observed for the first time that the cell growth of the yeast grown in ura$^-$ selective medium was reduced as the result of heterologous *hfH* expression with ca. 8% of the total soluble proteins. However, the recombinant yeasts that express the human ferritin L-chain gene up to 21% of the total soluble proteins were not shown to modify cell growth (data not shown). This suggests that the observed suppression of cell growth may be associated with the function of H-ferritin itself. The *hfH* expression began early and its level reached 3.1% of total soluble proteins at 12 h culture. At this stage, the H-ferritin holoprotein spontaneously assembled and accumulated iron within a core. Simultaneously, the rate of cell growth began to decline (Fig. 1). While *hfH* expression increased rapidly the intensity of the iron band decreased gradually and was not detected after 36 h culture. It may have reached the detection limit of the iron staining. Otherwise, this phenomenon may be attributable to the fact that the iron contained in the culture medium was limited, and that the intracellular free iron was also limited to sequestration into the protein. This was further examined by adding iron and the recovery of growth was also confirmed, as shown in Fig. 2. Ferritins accumulated more irons as iron content increased from 4 μM to 10 μM. The occurrence of the iron band indicated that iron was available to the cells above 4 μM ferric citrate, and the iron was accumulated into the recombinant H-ferritins. Cell growth was almost completely recovered to control levels at 4 μM ferric citrate. An increase in the iron-band intensity with the iron concentrations implies that the accumulation of iron in the ferritins occurred continuously, as the H-ferritin expression level remained constant. The implication is that the phenomenon of iron release from the ferritins remains unclear at this point, but may be negligible.

The yeast, *S. cerevisiae,* exploits several iron transport pathways, which involve a low affinity transporter encoded for by *FET4* (Dix *et al*., 1994) and high affinity transporters. The two genes, *FET3* and *FTR1*, are direct requirements for high-affinity iron transport (Askwith and Kaplan, 1997; Stearman *et al*., 1996). These genes are not expressed normally in iron-rich media, but its expression is transcriptionally upregulated in iron-limited cells (Askwith and Kaplan, 1997; Eide *et al*., 1992; Stearman *et al*., 1996; Yamaguchi-Iwai *et al*., 1995). An increase in *FET3* and *FTR1* transcript levels was observed in YGH2, whereas only a low level of transcripts was detected in YGT, suggesting that the transcript level may be reflective of the iron conditions within the cells. Increased gene expression was observed after the YGT cells were treated with the iron chelator BIP, indicating that the chelator reduced the bioavailable iron pool via the binding of intracellular free iron. It is, therefore, suggested that the recombinant H-ferritins may behave similarly to cellular iron chelators in yeast. The expression of the two genes was reduced in YGH2 cells treated with iron supplements, which also, incidentally, confirms the iron-depleted conditions of the cells induced by the expression of H-ferritins.

Compared to YGT, reduced ROS formation was observed in YGH2. Because the level of ROS is reflective of the free iron level in the cells, this result implied that the YGH2 cells that expressed H-ferritins possessed far less free iron in their cells than the controls. Previously, comparable results were reported by Orino *et al*. (2001) and others (Cozzi *et al*., 2000), where the overexpression of H-ferritins in HeLa cells was observed to effect a reduction in cell growth and ROS accumulation in response to H_2O_2 challenge. It has also been reported that the overexpression of ferritin H-chains in a mouse erythroleukemic cell line demonstrated the capacity of H-ferritin to sequester cellular iron and to regulate the levels of intracellular free iron (Picard *et al*., 1998).

In summary, H-ferritins are generated as a result of the heterologous expression system. Protein expression and iron accumulation occur independently of iron-mediated cellular regulation. As such, the recombinant H-ferritins bind irons within the core, notwithstanding the depletion of free iron in the cell. This resulted in a reduction of cell growth. The modification of cellular iron metabolism involving the increased expression of high-affinity iron transport genes (*FET3* and *FTR1*) was detected via Northern blot analysis. The findings

may provide evidence of cytosolic iron deficiency, as the genes are transcriptionally expressed under iron-deficient conditions. The ROS production was assessed via the fluorescence method, and ROS levels were far lower in YGH2 than the control, indicating that the expression of active H-ferritins reduced free iron concentrations in yeast.

Iron depletion normally induces iron-deficient anemia, which constitutes a significant worldwide health problem (Umbreit, 2005). Nevertheless, the mechanism by which iron deficiency directly affects the cells remains largely unknown. Cellular iron deficiency may result in the arrest of cell growth, and may eventually culminate in cell death. In this study, heterologous *hfH* expression induced cytosolic iron depletion in yeast, and the result may provide us with new insight into the regulatory network and pathways inherent to iron deficiency.

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