The Yeast Fre1p/Fre2p Cupric Reductases Facilitate Copper Uptake and Are Regulated by the Copper-modulated Mac1p Activator*

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Fre1p and Fre2p are ferric reductases which account for the total plasma membrane associated activity, a prerequisite for iron uptake, in Saccharomyces cerevisiae. The two genes are transcriptionally induced by iron depletion. In this communication, we provide evidence that Fre2p has also cupric reductase activity, as has been previously shown for Fre1p (Hassett, R., and Kosman, D.J. (1995) J. Biol. Chem. 270, 128–134). Both Fre1p and Fre2p enzymes are functionally significant for copper uptake, as monitored by the accumulation of the copper-regulated CUP1 and CTR1 mRNAs in fre1Δ, fre2Δ, and fre1afre2Δ mutant strains. However, only Fre1p activity is induced by copper depletion, even in the presence of iron. This differential copper-dependent regulation of Fre1p and Fre2p is exerted at the transcriptional level of the two genes. We have shown that Mac1p, known to affect the basal levels of FRE1 gene expression (Jungmann, J., Reins, H.-A., Lee, J., Romeo, A., Hassett, R., Kosman, D., and Jentsch, S. (1993) EMBO J. 12, 5051–5056), accounts for both the copper-dependent induction of FRE1 and down-regulation of FRE2 gene. Finally, Mac1p transcriptional activation function is itself modulated by the availability of copper.

Iron and copper are elements that have many similar chemical properties. Both are transition metals with several oxidation states, have close atomic and ionic radii numbers, and have very similar electronegativity. They are relatively homogeneously distributed on the planet and have thus been integrated in many biochemical reactions during evolution. Both are essential for life in almost every species (bacteria, fungi, plants, mammals) (1, 2). Excess quantities of iron and copper exert similar amount-dependent cytotoxicity, both favoring the formation of hydroxyl radicals which are disastrous to the cell (3). Iron presents a particular problem for its uptake, since in formation of hydroxyl radicals which are disastrous to the cell exert similar amount-dependent cytotoxicity, both favoring the coupled function of the cell surface Fet3p multicopper oxidase, which catalyzes the conversion of Fe(II) to Fe(III) extracellularly (6), and the recently reported Ftr1p permease (7) are also required for high affinity iron uptake. Thus, a link between iron and copper metabolism was first noted by the isolation of the the FET3 gene in a scheme aiming to clone the ferrous transporter (8). Fet3p requires copper to function, and therefore high affinity iron uptake requires copper (8, 9). Mutations either in the high affinity copper transporter gene CTR1 or in the CCA2 gene, encoding a member of the family of P-type ATPases proposed to transport cytosolic copper into the lumen of a secretory organelle, results in iron deficiency in the cell (9, 10). A similar mechanism has been postulated in mammals for the release of newly absorbed iron from intestine to blood involving the plasma glycoprotein ceruloplasmin, a copper-binding protein with ferrous oxidase activity (11, 12). Finally, the iron regulated transcription of the yeast genes FRE1, FRE2, FET3, CCA2, FTR1, and FTH1 (FTR1 homologue of unknown function) is affected by the Aft1p transcriptional activator which recognizes a specific consensus sequence on their promoters (13, 14). Moreover, expression of FRE1 and CTR1 mRNAs depends on the nuclear protein Mac1p, which is involved in iron and copper utilization (15).

We have previously shown that although Fre1p and Fre2p have seemingly redundant functions in S. cerevisiae, the two genes are up-regulated by the absence of extracellular iron in a kinetically different way, implying that they are subject to distinct regulation (5). Since there is evidence that copper reduction might be a component of copper uptake (16) and that Fre1p is also a copper-repressible cupric reductase (15–17), in this study we have investigated the participation of FRE1 and FRE2 genes products in copper metabolism. Our data clearly point to a role of both activities in copper uptake, although the two genes are differentially affected by the function of Mac1p under conditions of copper (and iron) depletion. Mac1p transcriptional function is itself modulated by the availability of copper, being higher in its absence.

EXPERIMENTAL PROCEDURES

Yeast Strains, Media, and Growth Conditions—The yeast strains used in this study are all derivatives of the S288C strain. The wild type and fre1Δ, fre2Δ, and fre1afre2Δ strains have been previously described (5). For the MAC1 gene disruption in the yeast genome, a 480-base pair Sty1 fragment (codons 41–201) was replaced by the 1.1-kilobase HindIII fragment of the URA3 gene on a MAC1 (–100 to 963) pBluescript recombinant phagemid. The resulted insertion fragment was excised and used to transform the desired ura3–52 strains (18). Uracil prototrophy was used for the selection of transformants. Transformants able to grow on glycerol only in the presence of 50 μM copper (15) were

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Further confirmed for the MAC1 gene deletion by DNA blot hybridization.

The yeast strain used for transcriptional induction and repression assays of the LexA-Mac1p fusion proteins to lacZ reporters was L9F5T5, a derivative of FY105 (MATα, his3Δ1, leu2::LexAopHIS3 leu2::PET56 ura3-52 trp1-63). The growth media were SD (2% glucose, 0.67% yeast nitrogen base; Difco) or SD supplemented either with 100 μM bathophenanthroline disulfonic acid-Na₂ salt (BPS) (Fe(II) and Cu(II) chelator) or with 100 μM bathocuproine disulfonic acid-Na₂ salt (BCS) (Cu(I) chelator) (16, 19, 20). For all experiments described, cells were grown to saturation in SD medium, subsequently resuspended in the same medium, and grown for 8–10 generations (exponential phase). They were then shifted to the desired medium (SD, SDB18, or SDBP5) at a concentration of 4–5 × 10⁶ cells/ml. Aliquots were removed from each culture every 2 h and assayed for copper and iron reducing activities simultaneously.

**Genes and Plasmids** — The FRE2/lacZ and FRE1/lacZ fusion plasmids were constructed starting from the low copy number terminal codons of b Escherichia coli lacZ containing YCP50 vector carrying a 6.3-kilobase 9,5 mids were constructed starting from the low copy number terminal codons of b Escherichia coli lacZ containing YCP50 vector carrying a 6.3-kilobase 9,5 mids were constructed starting from the low copy number terminal codons of b Escherichia coli lacZ containing YCP50 vector carrying a 6.3-kilobase.

For the construction of the LexA-Mac1p fusion proteins, the MAC1 gene sequence from +4 to +1249 nucleotides was synthesized by polymerase chain reaction and subcloned into the Smal-Kpn1 site of plasmid YCp91, a derivative of pRS314 (TRP1) containing the ADH1 promoter and 5′ untranslated region followed by a T7 RNA polymerase and sequences encoding the SV40 nuclear localization signal, the HA1 epitope from influenza virus, and the LexA binding domain (21). The oligonucleotide sequences used for the polymerase chain reaction are 5′-TCCCCGG-GATAATATTTAATGGGAACA-3′, and 5′-GAGGATCCATTGAGTAGCTG-GCACGGCGA-3′. The reporter plasmids used for the transcriptional assay were correspondingly pK103, in which LexA operators upstream of the GAL1 TATA element control the lacZ gene transcription (21, 22), and pK1621, in which four LexA operators, two upstream activation sequences, and TATA element of the CYC1 gene control the lacZ gene transcription (23, 24).

The sequences of the antisense CTR1, CUP1, and EFL1 synthetic oligonucleotides used as probes in the RNA blot hybridization analyses are 5′-CCTGCAACCTTTGGGCAATCTCAGAGAAGTC-3′, 5′-GTTACGCGGAGCCATTTGGCTGAT-3′, and 5′-CCAGAATGGATGACGATCCATAACAGC-3′, respectively. End-labeling of these oligonucleotides with γ[32P]ATP was performed by DNA kinase (22). The described oligonucleotides were synthesized by the Institute of Molecular Biology and Biotechnology MIcrochemistry group (Heraklion, Crete, Greece). The RNA and DNA blot hybridization analyses — Samples of total RNA and yeast DNA were prepared and blot-hybridized according to standard procedures (22).

**Antisense RNA Probe Preparation** — For the FRE1 gene-specific probe, a PouII-SacI genomic DNA fragment containing the coding region of the FRE1 gene (~680 to +2280) was subcloned into the EcoRV site of the pbU5cset I gene (21, 22). The recombinant plasmid was linearized by restriction at the SpeI site on the FRE1 gene (+1893) and a 444-base (including polylinker sequences) single-stranded homogeneously radiolabeled antisense RNA probe was synthesized by the T3 RNA polymerase in the presence of α−[32P]UTP (21). From the 444-base FRE1 probe, a 298-base fragment was protected, which defined the 3′ end of the RNA 135 bases downstream of the translation termination. A protected doublet was observed with this probe due to a region of seven consecutive thymidines preceding the A/III site.

For the HIS3 gene-specific probe, a HindIII genomic DNA fragment covering the coding region of the HIS3 gene from +305 to +591 was subcloned into the HindIII site of the phagemid described for the FRE1-specific probe. The recombinant plasmid was linearized by restriction at the AhaII site on the FRE2 gene (+1682) and a 712-base probe was produced by the same procedure as the FRE1-specific probe. From the 712-base FRE2 probe, 654 bases were protected, i.e. the size of the whole probe omitting the polylinker sequences (the FRE2 mRNA 3′ is downstream the A/III site used for subcloning). A protected doublet was observed with this probe due to a region of seven consecutive thymidines preceding the A/III site.

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**RESULTS**

**Fre1p and Fre2p Both Are Cupric Reductases but Only Fre1p Is Induced by Copper Deprivation** — It has been shown previously that Fre1p ferric reductase is also cupric reductase induced by copper depletion (16). In this report, we have examined Fre2p ferric reductase for cupric reducing activity asayed in a fre2Δ strain (5). We have first used iron depletion culture conditions known to induce Fre2p (5) to obtain measurable amounts of the enzyme. For comparison, we have assayed three additional strains, wild type, fre2Δ (Fre1p activity), and fre1Δfre2Δ, in parallel according to the conditions described under “Experimental Procedures.” The results presented in Table I show that Fre2p as well as Fre1p have cupric reductase activity.

Since we had previously observed that the two enzymes follow kinetically distinct induction by iron deprivation (5), we have assayed the same strains under conditions of copper depletion during 12 h of exponential growth. Our results (Table I) show that Fre1p was induced under copper depletion at similar levels as by iron depletion. In contrast, Fre2p was not detectably modulated by copper depletion. Fre2p activity was not induced even in the presence of the reducing agent sodium ascorbate (up to 500 μM), excluding the possibility of inefficient retention of the Cu(I)II ion in the minimal medium by the Cu(I)-specific chelator BCS (data not shown). We have obtained the same results (activity observed only in the fre2Δ strain) by using medium reconstituted from its ingredients with the omission of copper (data not shown).

**Data in Table I show residual copper-reducing activity in the fre1Δfre2Δ strain which was not affected by metal depletion and accounted for about 80% of the activity in noninduction conditions. We have not detected similar residual ferric reducing activity in this fre1Δfre2Δ strain (5) (Table I).**

Although Fre2p did not show induction within 12 h in copper-depleted fre1Δ cultures, this was achieved later between 12 and 15 h in repeatedly diluted copper-depleting medium and remained relatively constant during the exponential growth of the cells (Fig. 1). We suggest that, according to the proposed model of iron-copper connection (8, 9), prolonged depletion of copper intracellularly created also iron depletion, which in turn induced Fre2p activity.

**Copper Reduction by Fre1p and Fre2p Cupric Reductases Facilitates Copper Uptake** — While our results showed that both Fre1p and Fre2p are cupric reductases, they do not an-
Assays of reductase activities were performed in aliquots of the indicated strain cultures at different time points of growth in minimal (SD), copper-depleted (SDBCS), and iron-depleted (SDBPS) media. The average unit values (multiplied by a factor of 10) from five identical experiments obtained at 4 and 10 h of growth (when peak activities of the two reductases were detected) are shown. Units are defined as Cu(I) or Fe(II) nmol/h/10^6 cells.

| Medium | Strain        | Cupric reductase units | Ferric reductase units |
|--------|---------------|------------------------|------------------------|
|        |               | 4 h                     | 10 h                   | 4 h                      | 10 h                     |
| SD     | WT            | 5.3 ± 0.8               | 6.3 ± 2.0              | 4.5 ± 1.3                | 5.5 ± 2.7                |
|        | fre1Δ         | 5.0 ± 0.8               | 4.8 ± 0.4              | 1.2 ± 1.0                | 1.4 ± 0.9                |
|        | fre2Δ         | 4.2 ± 1.0               | 4.6 ± 0.5              | 6.1 ± 3.5                | 4.2 ± 1.9                |
|        | fre1Δfre2Δ    | 4.7 ± 2.0               | 3.6 ± 0.3              | 0.8 ± 1.4                | 1.4 ± 1.1                |
| SDBCS  | WT            | 38.5 ± 4.5              | 18.8 ± 1.8             | 102.5 ± 26.1             | 48.8 ± 7.1               |
|        | fre1Δ         | 4.2 ± 0.0               | 5.3 ± 1.3              | 1.9 ± 1.8                | 3.4 ± 1.8                |
|        | fre2Δ         | 35.5 ± 5.1              | 15.8 ± 1.8             | 104.6 ± 15.9             | 45.8 ± 4.8               |
|        | fre1Δfre2Δ    | 4.2 ± 0.4               | 4.7 ± 0.6              | 2.0 ± 1.7                | 3.2 ± 1.0                |
| SDBPS  | WT            | 40.2 ± 3.8              | 47.0 ± 2.0             | 120.7 ± 35.0             | 91.2 ± 31.8              |
|        | fre1Δ         | 4.4 ± 2.7               | 32.0 ± 1.1             | 1.4 ± 1.4                | 53.6 ± 17.0              |
|        | fre2Δ         | 31.8 ± 7.9              | 12.0 ± 4.1             | 103.7 ± 20.9             | 33.6 ± 18.2              |
|        | fre1Δfre2Δ    | 5.2 ± 0.7               | 3.8 ± 0.1              | 1.8 ± 1.6                | 1.8 ± 1.3                |

**Fig. 1.** Induction of Fre1p/Fre2p reductases during prolonged growth in copper- and iron-depleted media. Ferric reductase assays were performed in fre1Δ and fre2Δ strains during growth in SDBCS and SDBPS media. Ferric reductase activity was assayed in preference to cupric reductase activity to avoid the interference of the residual noninduced activity and to obtain higher values. The assay and growth conditions were as described under "Experimental Procedures," except that the cultures were diluted in fresh medium to a density of 4.5 × 10^6 cells/ml at 6-h intervals (arrows) to avoid saturation. The reductase assays for these particular time points were performed immediately prior to dilution. The results of one of two identical experiments are shown.

The Availability of Copper Affects the Transcription of FRE1 and FRE2 Genes—The differential induction profiles of the two reductases in copper depleted cells led us to investigate the levels of FRE1 and FRE2 mRNAs under these conditions. Using an RNase A protection assay we were able to simultaneously detect and quantitatively compare FRE1, FRE2, and HHS3 (as an internal control) mRNAs in total RNA of a wild type strain. Fig. 3A (left panels) shows that FRE1 mRNA accumulated under copper depletion and FRE2 mRNA was barely detectable (only at very long film exposures) at all time points, as described for the induction of the corresponding gene.
enzymatic activities. In parallel experiments using iron-depleted cultures, FRE1 and FRE2 mRNAs accumulated both following the time-dependent induction observed for the corresponding reductase activities (5) (Table I).

Measurements of β-galactosidase activities driven by the FRE1 or FRE2 promoters in wild type cells grown under copper and iron depletion indicated clearly that the FRE1 promoter was able to confer both copper- and iron-regulated expression of β-galactosidase, whereas the FRE2 promoter conferred only iron-regulated expression (Fig. 4).

The Function of Mac1p Accounts for the Differential Copper-dependent Regulation of FRE1 and FRE2 Gene Expression—The only known nuclear protein implicated in both copper and iron metabolism, affecting basal expression of the FRE1 gene is Mac1p (15). mac1 mutant cells suffer from copper and, possibly, iron deficiency, since addition of copper or iron (partly) can complement their phenotypes (15). Mac1p contains a region homologous to the copper-dependent transcription factor Ace1p (15). We have followed the response of FRE1 and FRE2 genes to copper depletion in a MAC1 gene-deleted strain during 12 h
promoter and more when driven by the FRE1 promoter. The results obtained from cultures of wild type (open bars) or mac1Δ (hatched bars) strains transformed with the chimeric plasmids FRE1/lacZ and FRE2/lacZ and grown for 7 h in minimal (SD), copper-depleted (SDBCS), and iron-depleted (SDBPS) media.

of growth. The results shown in Fig. 3A (right panels) revealed that FRE2 mRNA was detectable in mac1Δ cells grown in minimal medium (SD) and substantially induced following copper depletion. In contrast, FRE1 mRNA basal levels were significantly lower in the mac1Δ uninduced cells (SD medium) compared with the wild type levels and accumulated at very reduced levels in the copper-depleted cells. Following iron depletion of the mac1Δ strain, both FRE1 and FRE2 mRNAs were induced. However, by comparison to the induction patterns obtained in wild type cells, we found a temporal shift of the FRE1 mRNA induction to later time points in the exponentially growing mac1Δ culture, in contrast to a temporal shift of the FRE2 mRNA accumulation to earlier time points (Fig. 3, A and B).

β-Galactosidase activity levels produced by the FRE1/lacZ and FRE2/lacZ plasmids in a mac1Δ strain grown under copper or iron depletion conditions showed that the differential accumulation of FRE1 and FRE2 mRNAs resulted from the transcriptional response of the two genes (Fig. 4). Comparing these results to the RNA accumulation profiles, we should note that at 7 h of growth (when the assays were performed) the accumulation of β-galactosidase was less in the mac1Δ strain compared with the wild type strain when driven by the FRE1 promoter and more when driven by the FRE2 promoter. The results of reductase assays on copper- and iron-depleted fre1 mac1Δ and fre2 mac1Δ cultures were in agreement with the induction patterns of their transcripts (data not shown).

Therefore, under copper limitation, Mac1p function has a negative role on FRE2 gene regulation, while it affects positively FRE1 gene. In the absence of Mac1p (in mac1Δ strain) under copper limitation, additional inducing factors might exist for both genes, since FRE2 and FRE1 mRNA levels are higher than those detected in the noninducing culture (minimal medium). Furthermore, as presented in the diagrams shown in Fig. 3B, Mac1p seems to be involved in the temporally differential expression of FRE1/FRE2 genes under iron limitation. These observations are elaborated further under “Discussion.”

Mac1p Transactivating Activity Is Modulated by Copper—The observed copper-dependent regulation of FRE1/FRE2 genes affected by Mac1p function led us to investigate the copper dependence of Mac1p protein function. First we have tested the entire Mac1p coding sequence fused to the LexA binding domain for its ability to activate transcription of a β-galactosidase reporter following binding to the LexA operator (21, 22). As shown in Fig. 5, Mac1p protein was capable of transactivating the β-galactosidase reporter in cells grown in minimal medium. This activity was significantly increased when cells were depleted from copper, which suggests that the Mac1p protein was activated at these conditions. Mac1p mRNA levels were unaffected under copper-depleting conditions (data not shown). These results implicate a direct effect of copper on Mac1p modulating its transcriptional activation function. Testing of the LexA-Mac1p fusion for repression function on a CYC1 promoter-driven lacZ transcription (23, 24) showed inducing rather than repressing effect on the constitutive expression of this reporter (Fig. 5), indicating that Mac1p probably does not have repressing activity. This result is relevant for the explanation given for its different role on the expression of the FRE1/2 genes (see under “Discussion”).

**DISCUSSION**

Iron and copper are metals whose biological importance and common properties have been emphasized frequently. The cellular mechanisms by which living organisms exploit them to fulfill their needs and simultaneously protect themselves are now being unravelled (12). The externally directed enzymes Fre1p and Fre2p are situated at the “beginning” of the pathway that links extracellular to intracellular iron communication, leading to nuclear events that alter gene expression, as well as at the “end” of such a pathway, being themselves regulated by iron to control its influx into the cell. Although a common iron-related transactivator, Aft1p, has been identified (13, 14),
the two reductase genes are differentially induced (Ref. 5 and this paper).

In this study we have examined the participation of the ferric reductases Fre1p and Fre2p in copper metabolism, and we have revealed elements of their metal-regulated expression. Previous reports have presented the ferrireductase plasma membrane activity (17) and Fre1p (16) as being repressed by copper. We have demonstrated that Fre2p is a copper reductase, as is Fre1p, but only Fre1p is induced in copper starvation, accounting for 80–90% of the plasma membrane activity. The residual copper-specific reducing activity, which is not modulated by copper, probably corresponds to the Cu(II)-specific reductase, not repressible by iron, described by Hassett and Kosman (16). This reductase is possibly encoded by another FRE-homologous gene.2

We have further shown that the distinct response of the two cupric reductases to copper depletion is reflected from the differential transcriptional regulation of the two genes. While FRE1 gene was transcriptionally up-regulated, FRE2 gene did not seem to respond to copper depletion. Furthermore, we have shown that Mac1p nuclear protein contributed directly or indirectly to this differential response of the two reductase genes. FRE1 gene basal and induced expression were highly dependent on its presence, in agreement with the notion that Mac1p is involved in the communication of the copper starvation signal to the FRE1 gene. In contrast, FRE2 gene basal and copper-induced expression were only observed in the absence of Mac1p. An explanation for this phenomenon, which needs to be proven, may lie in the following observations. FRE1 promoter contains a pyrimidine-rich directly repeated sequence, previously shown to mediate iron-regulated transcription of a reporter gene (4), distinct from the Aft1p binding consensus, which could be responsible for the Mac1p effects, as discussed by Yamaguchi-Iwai et al. (14). Similar pyrimidine-rich repeated sequences are also found on the FRE2 (5) and CTR1 (9) promoters in different orientations. CTR1 gene expression is also dependent on the presence of Mac1p (15).1 Only on the FRE2 promoter are these sequences situated next to the TATA box between positions −54 and −80. (The transcription start site of FRE2 gene is at position −5 from the first coding AUG and the potential TATA box is at position −89.) If these sequences were indeed affected by Mac1p, they might play different roles on the different promoters. While affecting positively the expression of FRE1 and CTR1 genes, Mac1p may result in steric hindrance of the formation of the transcriptional complex on the FRE2 promoter (28). This hypothesis is also in agreement with our results indicating that Mac1p plays a major role in the temporally different induction of the two reductase genes by the use of the Fe/Cu chelator (BPS medium). We can conceive that upon metal deprivation Mac1p facilitates FRE1 expression and inhibits FRE2 expression. Accumulation of Aft1p and/or appearance of another epistatic FRE2-specific inducer results in the FRE2 late induction.

This scheme is in agreement with our additional findings on the role of Mac1p. First, Mac1p was able to transactivate a reporter gene (lacZ) when artificially brought to its promoter (by the LexA binding domain). Second, Mac1p did not show any repression function on a constitutively expressed reporter gene (CYC1(UAS)-lacZ). Third, an important finding that directly related Mac1p function to the cupric reductases was the fact that its transactivation function was modulated by the availability of copper, being increased in its absence. The precise role of Mac1p will be revealed by the detailed study of its functional domains as well as examination of the interacting elements of the transcriptional complexes in which it participates.

Our last finding concerns the role of the Fre1p/Fre2p cupric reductases on the uptake of copper. Asking the question whether copper reduction is necessary for its cellular uptake, the profit from such a reaction is not immediately obvious since Cu(II) ions, unlike Fe(III), are found in a soluble form in water-dominated environments. Evidence as to whether copper enters the cell as Cu(II) or Cu(I) was indirect. Several studies in plants have shown a concomitant increase in Fe(III) and Cu(II) reducing activities in response to depletion of metals from the soil (29, 30). These observations could be explained either by introducing the notion of a general role of reductases in regulating the redox state of the plasma membrane which would affect channel gating and ion influx (17, 30) or a more specific role in copper uptake which would take into account environmental factors, e.g. the availability of Cu(II) ions which form very stable complexes with various environmental ligands. Hassett and Kosman (16) presented evidence for copper reductive assimilation in S. cerevisiae, since Pt inhibition (Fre1p inhibitor) of copper uptake was relieved when the reducing agent ascorbate was included in the uptake assay. Very recently, Knight et al. showed that FRE1 gene is necessary for the Ctr1p and Ctr3p function in 64Cu uptake (31).

We have obtained evidence for the importance of reduction for copper uptake by using the CTR1 and CUP1 mRNAs as reporters to monitor entrance and utilization of copper in the cell. We have demonstrated that Fre1p/Fre2p activities assure higher intracellular copper levels, as shown by the increased CTR1 mRNA quantities in the fre1Δfre2Δ strain compared with wild type cells. This activity was not an absolute prerequisite for copper uptake, possibly due to the entrance of Cu(II) ions or to Cu(I) produced by the residual plasma membrane copper-reducing activity mentioned above. Our results provide evidence that mostly Fre1p but also Fre2p have a major contribution to the uptake of copper when we used a chelator capturing its oxidized form (BPS). This contribution might be very useful for the organism, since there are probably many chelators of Cu(II) in the ecosystem of S. cerevisiae (e.g. citrate in fruits). The involvement of the two ferric reductases in copper and iron metabolism appears distinct and specific, particularly since Fre1p and Fre2p do not respond to molybdenum, zinc, or manganese deprivation.1 In reference to the iron-copper connection already discussed, it seems inevitable that ferric reductases have evolved to participate also in copper metabolism. Since iron cannot enter the cells in conditions of copper starvation, induction of the ferric reductases by iron depletion would not be of any use if copper entrance was not facilitated simultaneously. On the other hand, when copper is depleted, Fre1p is first induced, able to assume most part of copper import into the cells, and if, for some reason, copper continues to be rare, Fre2p comes to its aid, being induced much later indirectly because of iron deprivation. A clear contribution of Fre2p was seen both under prolonged copper starvation and in the mac1Δ strain. Our results show clearly that reduction of copper facilitates its entrance into the cells. We propose that Fre1p and Fre2p, although not indispensable for copper uptake, are actively and catalytically participating in this process, especially under natural conditions.

In conclusion, the Fre1p/Fre2p system, involving two genes distally related in primary structure (5) functioning in close collaboration for iron and copper handling but with clearly differentiated controlling mediators, is a very promising experimental model in revealing “fine tuning” mechanisms that have evolved in living organisms to assure profitable metal homeo-

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2 Internet data bases (http://speedy.mips.biochem.mpg.de/mips/yeast and http://genome-www.stanford.edu/Saccharomyces).
stasis. It is interesting to note that, as revealed by the completed sequence of the *S. cerevisiae* genome, six additional open reading frames share similarities with the Fre1p and Fre2p sequences and possibly are involved in other specific membrane reductase activity functions.

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