The yeast vacuole is the storage depot for cellular iron. In this report we quantify the import-export balance in the vacuole because of the import of iron by Ccc1p and to export by the combined activity of Smf3p and the ferroxidase, permease pair of proteins, Fet5p and Fth1p. Our data indicate that the two efflux pathways are equally efficient in trafficking iron out of the vacuole. A major focus of this work was to identify the ferrireductase(s) that supplies the FeII for efflux whether by Smf3p or vacuole. A major focus of this work was to identify the ferrireductase(s) that supplies the FeII for efflux whether by Smf3p or the Fet5p-Fth1p complex. Using a combination of flameless atomic absorption spectrophotometry to quantify vacuolar and whole cell iron content and a reporter assay for cytoplasmic iron we demonstrate that Fre6p supplies FeII to both efflux systems, while Fre7p plays no role in Fe-efflux from the vacuole. Enzymatic assay shows the two fusions to have similar reductase activity, however. Confocal fluorescence microscopy demonstrates that Fre6p:GFP localizes to the vacuolar membrane; in contrast, Fre7p:GFP fusions exhibit a variable and diffuse cellular distribution. Demonstrating a role for a vacuolar ferrireductase in Fe-efflux supports the model that iron is stored in the vacuole in the ferric state.

Across all superkingdoms, transition metal ion homeostasis is strongly dependent on the regulation of nutrient accumulation rather than on excess nutrient excretion (1–5). Metal ion storage appears to be a complement of this regulatory pattern, storage that is illustrated by ferritin, the metallothioneins, and compartmentalization into cell organelles such as the mitochondria and vacuole. Although in some cases, this storage provides a detoxification mechanism (e.g. of heavy metals by the thioneins), it is as likely to play a dynamic role in cell and/or organismal metal ion homeostasis. This is certainly the case with ferritin in regards to vertebrate cell iron handling (2), and it is very likely to be the case with the vacuoles found in the cells of many diverse eukaryotic genera; the vacuole in fungi and plants is a dynamic organelle that plays a significant role in the overall nutritional status of the organism (3–5). Vacuoles provide a storage depot for newly arrived nutrients as well as being the site of macromolecular degradation and nutrient recycling (6, 7). In regards to metal metabolism the vacuoles in plants and fungi have been associated with the handling of copper, iron, manganese, and zinc, in addition to magnesium and calcium; the yeast vacuole is essential for the detoxification of several first row transition metals (8, 9).

Iron metabolism in the yeast Saccharomyces cerevisiae has served as a paradigm for iron handling in many other fungi, plants and even humans (1, 4, 5, 10). Under normal nutrient conditions ([Fe]environmental = 1 μM) iron uptake is mediated by the high affinity iron uptake complex consisting of the multicopper ferroxidase Fet3p and iron permease Ftr1p. Homologous iron uptake complexes are likely to be found in most fungi and plants (10–12). Uptake by yeast of environmental FeIII is preceded by ferric iron reduction to FeII by plasma membrane reductase activity supplied principally by Fre1p; reduction of environmental FeIII (and CuII) is paradigmatic of the uptake of these metal ions by all aerobic organisms (3, 11, 13, 14). Given the reduction potential of the cytoplasm, iron is undoubtedly present there as FeII; based on the GSH/GSSG ratio quantified in a wild-type yeast cell, this potential can be as low as −300 mV (15–17). This is likely to be the case in the cytoplasm where a major fraction of this GSH/GSSG is localized; in contrast, because of the overall non-equilibrium distribution of GSH in the cell, the reduction potential for the endoplasmic reticulum has been estimated at around −200 mV (17). Also, the GSH/GSSG reduction potential is strongly pH-dependent: ΔE/ΔpH is +60 mV for a decrease in pH = 1.0 (17). Thus, relatively acidic compartments like endosomes and the vacuole will be oxidizing in comparison to the cytoplasm; the presence of iron in the yeast vacuole as a ferric (poly)phosphate is consistent with this electrochemical condition (18).

One hallmark of iron metabolism in yeast is, therefore, redox cycling between FeIII (exocytoplasmic, vesicular) and FeII (substrate for uptake, cytoplasmic) (1, 6, 11). As noted, Fre1p supports this cycling at the plasma membrane where exocytoplasmic ferric iron needs to be reduced prior to ferroxidation and uptake by the Fet3p, Ftr1p complex. The FeII/FeIII ratio within the cell is likely determined by the overall reduction potential which, as noted, is strongly dependent on the [glutathione]total and pH. In addition, the GSH/GSSG ratio will be sensitive to the level of dioxygen; indeed, this ratio is a measure of cellular oxidative stress (17, 19).

The differential redox speciation of iron in the cytoplasm and vacuole provides the context for the mechanism of the cycling of iron between these two compartments. Some of the proteins involved in this cycling in S. cerevisiae have been identified. Ccc1p is localized to the vacuolar membrane and appears to function as a divalent metal ion transporter; Ccc1p has been...
associated with calcium, manganese, and iron homeostasis in yeast (20–22). Smf3p is a member of the Nramp divalent metal ion transporter family; Smf3p also exhibits relatively little selectivity among first row transition ions (23, 24). Smf3p localizes to the vacuolar membrane (24). Both transporters are thought to support vectorial iron flux with Cc1p supporting import (21) and Smf3p supporting export of iron from the vacuolar lumen (24) although neither activity has been directly demonstrated. A complex of Fet5p and Fth1p is likely to support export of vacuolar iron also; this complex is localized to the vacuolar membrane with an orientation that is identical to that of its homologues, Fet3p and Ftr1p (25). In other words, both complexes transport iron into the cytoplasmic compartment: Fet3p, Ftr1p from the exocytoplasmic space, and Fet5p, Fth1p from the vacuolar one.

As is the case for Fet3p, Ftr1p permeation, the substrate for Fet5p, Fth1p transport is most reasonably FeII. However, as noted above, the valence state of vacuolar iron is likely FeIII. In parallel with plasma membrane iron import that starts with ferrireduction, so, too, does Fet5p, Fth1p export begin with the same reduction step. Of the seven yeast genes that encode reductase proteins, FRE6 is one of those up-regulated via the iron-responsive transcription factor, Aft1p (26) although neither activity has been directly demonstrated. Smf3p localizes to the vacuolar membrane (24). Both transporters are thought to support vectorial iron flux with Cc1p supporting import (21) and Smf3p supporting export of iron from the vacuolar lumen (24) although neither activity has been directly demonstrated. A complex of Fet5p and Fth1p is likely to support export of vacuolar iron also; this complex is localized to the vacuolar membrane with an orientation that is identical to that of its homologues, Fet3p and Ftr1p (25). In other words, both complexes transport iron into the cytoplasmic compartment: Fet3p, Ftr1p from the exocytoplasmic space, and Fet5p, Fth1p from the vacuolar one.

As is the case for Fet3p, Ftr1p permeation, the substrate for Fet5p, Fth1p transport is most reasonably FeII. However, as noted above, the valence state of vacuolar iron is likely FeIII. In parallel with plasma membrane iron import that starts with ferrireduction, so, too, does Fet5p, Fth1p export begin with the same reduction step. Of the seven yeast genes that encode reductase proteins, FRE6 is one of those up-regulated via the iron-responsive transcription factor, Aft1p (26) although neither activity has been directly demonstrated. Smf3p localizes to the vacuolar membrane (24). Both transporters are thought to support vectorial iron flux with Cc1p supporting import (21) and Smf3p supporting export of iron from the vacuolar lumen (24) although neither activity has been directly demonstrated. A complex of Fet5p and Fth1p is likely to support export of vacuolar iron also; this complex is localized to the vacuolar membrane with an orientation that is identical to that of its homologues, Fet3p and Ftr1p (25). In other words, both complexes transport iron into the cytoplasmic compartment: Fet3p, Ftr1p from the exocytoplasmic space, and Fet5p, Fth1p from the vacuolar one.

**TABLE 1**

Strains constructed in this work

| Strain | Description |
|--------|-------------|
| 1457Δfet5 | MATa ade6 can1 his3 leu2 trp1 ura3 6-furfuryl-thio-TRP1 |
| 1457Δccc1 | MATa ade6 can1 his3 leu2 trp1 ura3 ccc1::LEI12 |
| 1457Δmot1::LEU2 (L) | MATa ade6 can1 his3 leu2 trp1 ura3 smf3::LEI12 |
| 1457Δmot1::HIS3 (H) | MATa ade6 can1 his3 leu2 trp1 ura3 smf3::HIS3 |
| 1457Δfre6 | MATa ade6 can1 his3 leu2 trp1 ura3 trp1::FET5-His3 |
| 1457Δfre7 | MATa ade6 can1 his3 leu2 trp1 ura3 fre6::KanMX4 gre7::TRP1 |
| 1457Δfre6Δfre7 | MATa ade6 can1 his3 leu2 trp1 ura3 fre6::KanMX4 gre7::TRP1 |
| 1457Δfre5::pFET3::lacZ | MATa ade6 can1 his3 leu2 trp1 ura3 tet5::TRP1 pFET3::lacZ (URA3) |
| 1457Δccc1::FET5::lacZ | MATa ade6 can1 his3 leu2 trp1 ura3 ccc1::LEI12 pFET3::lacZ (URA3) |
| 1457Δmot1::pFET3::lacZ | MATa ade6 can1 his3 leu2 trp1 ura3 smf3::LEI12 pFET3::lacZ (URA3) |
| 1457Δfre6::pFET3::lacZ | MATa ade6 can1 his3 leu2 trp1 ura3 fre6::KanMX4 pFET3::lacZ (URA3) |
| 1457Δfre7::pFET3::lacZ | MATa ade6 can1 his3 leu2 trp1 ura3 fre6::KanMX4 gre7::TRP1 pFET3::lacZ (URA3) |

---

**MATERIALS AND METHODS**

**Strain Construction**—The yeast strains employed in this study were derived from DEY1457 (MATa ade6 can1 his3 leu2 trp1 ura3) and are listed in Table 1. The fet5Δ strain was a kind gift from D. Eide. The smf3Δ strain was generated by using the HindIII-linearized smf3Δ::LEU2 plasmid pJS409, a kind gift from V. Culotta. Transformation of yeast with this linear plasmid resulted in deletion of the chromosomal SMF3 sequence from +116 to +1313, which was verified by PCR. The ccc1Δ strain was generated by using the SacI-digested ccc1Δ::HIS3 plasmid (also a kind gift from V. Culotta) to transform DEY1457. The fre6Δ strain was constructed by amplifying the KANMX4 sequence from the plasmid pFAKanMX4 (29); primers were used that contained sequences from the 5′- and 3′-flanking regions from the FRE6 locus. The resulting PCR fragment was transformed into DEY1457. Genomic DNA was analyzed by PCR for the gene disruptions using a primer flanking the FRE6 ORF and a primer in the integrated KAN gene. The strains for quantification of FET3 promoter activity (for Aft1p activation) were generated by transforming DEY1457 and various deletion strains derived from it, with the Apal-digested FET3::lacZ plasmid (kindly provided by A. Dancis), which was integrated at the URA3 locus.

**Growth Media**—Stocks of yeast were maintained on standard yeast extract-peptone-dextrose media. The cultures for experimental analysis were grown in a synthetic minimal media (6.67 g/liter yeast nitrogen base without amino acids, 2% glucose, and appropriate mix of amino acids). The experiments involving the MET3 promoter were performed using the synthetic complete (SC)2 media supplemented with methionine (3.75 mM) and cysteine (5 mM) for repression and a Met- and Cys-free medium for promoter activation.

**Plasmid Construction**—The methionine promoter plasmids were constructed by amplifying the 450-bp MET3 promoter inserting Apal/Sall sites and cloning this fragment into pRS423, pRS424, pRS425, and pRS426 (30). The open reading frames of SMF3, CCC1, FTH1, and FRE6 including 400 base pairs of 3′-UTR were amplified from yeast chromosomal DNA while the FET5 ORF and 3′-UTR was amplified from the Ylp-FET5HA plasmid, a kind gift from D. Eide. These amplified genes were cloned into SalI/NotI sites in the MET3 promoter pRS series plasmids. The FRE6-GFP plasmid was constructed by amplifying FRE6 from chromosomal DNA and cloning it into the pMET3-424 plasmid at the SalI/NotI sites. The GFP ORF was inserted following amino acid residue 236 in the pMET3-FRE6 plasmid modified to include XbaI/AattI sites for this purpose. This placed the GFP in a loop predicted topologically to be in the cytoplasm. In the case of pMET3-FET7, the GFP ORF was inserted at four locations: in three predicted loop

---

2 The abbreviations used are: SC, synthetic complete (media); UTR, untranslated region; ORF, open reading frame; GFP, green fluorescent protein; PIPES, piperazine-1,4-bis-(2-ethanesulfonic acid); FAA5, flameless atomic absorption spectrophotometry; DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; ONPG, o-nitrophenylgalactopyranoside; PM, plasma membrane; ER, endoplasmic reticulum; PBS, phosphate-buffered saline.
regions after residues 101, 150, and 227, respectively, and just prior to the FET7 termination codon to generate a C-terminal GFP fusion.

**Vacuole Isolation**—Vacuoles were prepared from 600 ml of cells (OD$_{660} \approx 1–1.5$) grown in SC media. The cells were collected by centrifugation at 1800 × g for 5 min, resuspended in 30 ml of 0.1 M Tris-SO$_4$ (pH 9.3) and 10 mM dithiothreitol and incubated for 10 min at 30 °C. The cells were washed once with spheroplasting buffer (1.2 M sorbitol, 20 mM potassium phosphate, pH 7.4) and incubated with 20 µg/ml lyticase for 45 min at 30 °C. Spheroplasts were collected by centrifugation at 3500 × g for 5 min and resuspended in 3.5 ml of 15% Ficoll buffer (15% Ficoll in 0.2 M sorbitol, 10 mM PIPES-KOH, pH 6.8). DEAE-Dextran (50 µg/ml) was added to the spheroplasts, and the sample incubated for 3 min on ice and for 5 min at 30 °C. The lysate (3.5 ml) was transferred to two SW41 tubes (Beckman Instruments) and overlaid sequentially with 3 ml of 8% Ficoll, 4 ml of 4% Ficoll, and 1 ml of buffer (0.2 M sorbitol, 10 mM PIPES-KOH, pH 6.8). The tubes were centrifuged at 110,000 × g for 90 min. The vacuolar fraction was collected from the 0–4% interface. Microscopic examination (see below) showed this fraction to contain >90% intact, sealed vacuoles with little contamination with other vesicular bodies or membrane fragments. The iron content of acid digested vacuoles was quantified by flameless atomic absorption spectrophotometry (faAAS) on a Perkin Elmer graphite furnace instrument. All analyses were performed in triplicate on samples from two independent experiments. The means were performed in triplicate on samples from two independent cultures derived from separate transformants for each plasmid. Each condition was measured in triplicate using three separate log phase cells (1 ml) were resuspended in 650 µl of Z buffer (0.1 M sodium phosphate buffer, pH 7.0, 10 mM KCl, 1 mM MgSO$_4$) containing 100 µl of Y-PER reagent (Pierce) and 2 µl of β-mercaptoethanol. The mixture was vortexed for 5 min at room temperature and 150 µl of ONPG (4 mg/ml) was added. The reaction was stopped by addition of 400 µl of 1 M Na$_2$CO$_3$. The reaction mixture was centrifuged for 10 min at high speed at 4 °C. The absorbance of the supernatants was read at 420 nm in a Spectronic 20. The enzyme activity was calculated in Miller units (31). The second vacuolar Fe-efflux pathway. The role of Fre6p in this stepwise relationship between import, ferrireduction and export was examined in more detail by use of the regulated overproduction of Ccc1p and Smf3p in wild type and fre6Δ cells. Using pMET3 as the promoter in the presence (repressing) and absence of methionine (activating), we examined the role of Fre6p in the balance between import and export of iron in the vacuole. Overproduction of Ccc1p in wild-type cells resulted in a 4-fold increase in steady-state vacuolar iron accumulation whereas overproduction of Smf3p alone reduced this iron accumulation by a factor of two (Fig. 2, open bars, compare with Fig. 1). Overproduction of Smf3p along with Ccc1p reduced by 50% the excess iron accumulation because of Ccc1p alone. In all cases, vacuolar Fe-accumulation in the fre6Δ strain was increased (Fig. 2, shaded bars); the most dramatic increases (>2-fold) were in cells overproducing Smf3p, which in Fre6p wild type had the effect of counteracting the accumulation because of Ccc1p. The data indicate Fre6p is upstream of Smf3p in the Fe-export pathway from the vacuole.

As noted, the Fet5p, Fth1p ferroxidase-permease pair offers a second vacuolar Fe-efflux pathway. The role of Fre6p in this
pathway was assessed in a similar fashion (Fig. 3). Although overproduction of Fet5p and Fth1p had little effect on iron accumulation in parental, wild-type cells (Fig. 1), it reduced by 50% the excess iron accumulation because of Ccc1p overproduction (Fig. 3, open bars). This reduction was absent in the fre6Δ strain indicating that Fre6p was upstream of Fet5p, Fth1p-mediated export as well (Fig. 3, shaded bars).

Cytosolic Iron as a Monitor of the Vacuolar Iron Import-Export Cycle—The balance between cytosolic and compartmentalized iron in yeast has commonly been monitored by use of a reporter plasmid in which the lacZ gene is placed under control of the Fe-responsive Aft1p transcription factor (21, 24, 25). When cytosolic iron is (relatively) low, Aft1p traffics to the nucleus where it activates transcription of genes in the Aft1p regulon; in (relatively) high cytosolic Fe, Aft1p returns to the cytoplasm (33). Using this reporter, the dependence of vacuolar iron accumulation on the overproduction of Ccc1p and Smf3p in wild type and fre6Δ could be quantified. These data are shown in Fig. 4. In 4-h post-induction, wild-type cells overproducing Ccc1p exhibit an 8-fold increase in Aft1p activity; this increase is unaffected by the absence of Fre6p (Fig. 4 compare first two sets of open and shaded bars). This parallels the vacuolar iron data shown in Fig. 3. Co-production of Smf3p reduces by 40% the increase in Aft1p transcriptional activity because of vacuolar Fe-ac-
Vacuolar Iron Cycling in Yeast

Cooperation of Plasma and Vacuolar Membrane Iron Trafficking—We recognized that of the two measures of vacuolar membrane iron trafficking we used, vacuolar iron content, and the trans-activation of genes regulated by cytosolic iron, the latter assay was compromised by the extent to which plasma membrane iron trafficking contributed to the steady-state level of cell iron. For example, an increased vacuolar Fe-uptake because of overproduction of Ccc1p would decrease cytoplasmic iron and thereby stimulate Aft1p-dependent transcription that, in the end, would result in an increased Fe-uptake into the cytoplasm thus suppressing the signal obtained from our reporter assay. In a complementary fashion, the signal from a Smf3p overproducing culture would over-report on the increase in vacuolar iron efflux (seen as a decrease in reporter plasmid read-out) since the increase in cytoplasmic iron would decrease uptake of iron into the cell as well. We tested these predictions by measuring whole cell iron levels in the various cultures used in these studies. The data did, in fact, illustrate this balancing of plasma membrane Fe-uptake and cycling of iron out of and into the vacuole (Fig. 6).

Thus, in comparison to parental wild-type cells (indicated by the arrow) total cell iron was increased in a fre6Δ but not in a fre7Δ strain (first two open and shaded bars, respectively); total cell iron in the fre7Δ strain and wild type was the same. This result is consistent with a model in which the reduced Fe-efflux into the cytoplasm in the fre6Δ background in comparison to the fre7Δ one resulted in a stronger activation of Fe-uptake at the PM. This relative difference was seen with overexpression of either vacuolar Fe-uptake (Ccc1p) or efflux (Smf3p or Fet5, Fth1p) activities (last three sets of bars), data that support the conclusion that Fre6p but not Fre7p is upstream from both pathways for vacuolar Fe-export.
Fre6p and Fre7p Exhibit Reductase Activity Comparable to Fre1p—To demonstrate if either Fre6p or Fre7p had a reductase activity, we prepared membrane extracts from a host fre1/H9004 yeast strain producing GFP fusions of Fre1, Fre6, or Fre7 from a high copy vector under control of the MET3 promoter. The cells were grown in the presence of 10 μM copper and 100 μM iron to repress expression of endogenous reductase genes. In these cells under these growth conditions we expected that the major fraction of the reductase activity measured would result from episomal expression of a specific reductase gene. With such extracts it was not possible to perform metalloreductase assays with FeII or CuI as substrate (because of nonspecific metalloreduction) but we were able to detect reductase activity using as substrate a standard one-electron acceptor for Fre proteins, INT (34). The results of this experiment are presented in Fig. 7, panel A and demonstrate that in this protocol an equivalent amount of INT reductase activity is produced irrespective of the reductase gene fusion being expressed. Although not quantitative, the Western blot shown in Fig. 7, panel B indicates that the GFP fusions were produced at comparable levels in the three cell samples. On this basis, Fre7p is indistinguishable as an INT reductase (at the least) in comparison to either Fre1p or Fre6p.

Fre6p but Not Fre7p Localizes to the Vacuolar Membrane—Neither Fre6p nor Fre7p has been localized in the yeast cell. We examined the locale of the GFP fusions noted above by confocal fluorescence microscopy. By this criterion, Fre6:GFP localized exclusively to the vacuolar membrane (Fig. 8). This was confirmed by co-staining with a dye specific for the vacuolar membrane, FM4–64. The lack of any fluorescence from the vacular lumen indicated that little if any of the fusion protein was being internalized and degraded within the vacuole indicating that the localization observed was not a result of the overproduction; pMET3 was chosen expressly to avoid this type of false positive result (35).

In contrast to the distinct and reproducible localization of the Fet6:GFP fusion to the vacuolar membrane, none of the four
Fre7:GFP fusions constructed (see “Materials and Methods”) exhibited a specific localization; fluorescence from these protein species was diffuse and primarily cytoplasmic with little if any accumulation in any cellular membrane, e.g. the vacuolar or plasma membranes (supplemental Fig. S1). Because all four Fre7p fusions, including the GFP insertion at residue 227 (homologous to the Fre6:GFP fusion), gave this result we infer that it was not due to the insertion of the GFP domain; all of these fusions were detectable in the cell by Western blot analysis (Fig. 7, panel B). When our Fre6p and Fre7p fusions were produced under control of their own promoters (under inducing low [Fe] and [Cu] conditions) they were undetectable by Western blot using anti-GFP antibody (data not shown).

**DISCUSSION**

Vacuolar iron in yeast is metabolically active; 70% of iron stores are mobilized from vacuoles in yeast switched from glucose to ethanol as carbon source, i.e. from fermentation to respiration (18). Yeast strains lacking a putative vacuolar iron import or export complex lag behind wild-type in this growth adaptation to a non-fermentable carbon source (25). There are two activities in the yeast vacuolar membrane shown to be involved in vacuolar iron mobilization. These include the Fet5p-Fth1p complex (25) and Smf3p, one of the three yeast homologs of the Nramp mammalian divalent metal transporters (24). Because the redox state of iron present in the cell is strongly dependent upon the GSH/GSSG ratio, which in turn is strongly pH-dependent (17), the relatively acidic vacuole is likely to be oxidizing in comparison to the cytoplasm; thus vacuolar iron is likely stored as a ferric (hydr)oxide and/or phosphate (18). In as much as FeIII is the substrate for the Fet5p-Fth1p complex and for Smf3p, ferrireduction is required for the mobilization of iron from the vacuole. Of the seven genes that encode reductase proteins in yeast, Fre6E is up-regulated by iron deprivation along with *FET5*, *FTH1*, and *SMF3* (28). Our data indicate that Fre6p supplies (some of) the luminal reductase activity required to support the mobilization of FeII from the vacuolar ferric iron stores and the export of iron from the vacuole via Smf3p and the Fet5p-Fth1p complex.

We have localized a GFP fusion of Fre6p specifically to the vacuolar membrane. In the background we used for our experiments Fre7:GFP does not co-localize to the vacuole; at the level of expression of the fusion because of p*MET3* no specific cellular localization could be discerned for this reductase. On the other hand, at this level of expression Fre6p and Fre7p exhibited similar reductase activity, one that was equivalent to that measured for the plasma membrane metalloreductase, Fre1p.

In a recent, complementary report, Rees and Thiele (36) demonstrated a plasma membrane localization for a Fre7:GFP fusion protein, but only in an *rps5-I* background. *RPS5* encodes a ubiquitin ligase that is involved in marking endosomal membrane proteins for degradation; *rps5-I* is a temperature sensitive allele that is inactivated at 37 °C (37). In this background, plasma membrane localization of Fre7:GFP was observed clearly but only at the non-permissive temperature. This protein supported a PM metalloreductase activity under those conditions like that exhibited by Fre1p. Whether this PM localization resulted from suppression of ER quality control in the *rps5-I* background and the default trafficking of the fusion to the PM, or to a stabilization of the protein in the PM remains an open question.

Endogenous Fre6p and Fre7p differ in their regulation with the former produced under control of the Fe-sensitive transcriptional system of Aft1p/Aft2p and the latter under control of the Cu-sensitive, Mac1 protein. With their common regulation, the reductase/permease group of proteins that includes Fre6p, Smf3p, Fet5p, and Fth1p might be expected to represent a redundant metabolic pathway directed toward the mobilization of ferric iron from the vacuole. In this simple model, one could suggest that being Cu-regulated, Fre7p might play a role in the redox management of vacuolar copper, presumably reducing CuII to CuI as the plasma membrane reductases, Fre1p and Fre2p, do in support of cell Cu-uptake via the Cu-transporter, Ctr1p (38). Ctr2p is the vacuolar counterpart to Ctr1p, transporting copper out of the vacuole (39, 40).

The complementary work by Rees and Thiele (36), however, indicates that Ctr2p-dependent Cu-efflux from the vacuole is, like Fe-efflux, dependent on Fre6p. Thus, Fre6p is similar to Fre1p in that both proteins function upstream from the iron and copper transport components in the vacuolar and plasma membranes of yeast, respectively. The two metalloreductases do, however, differ in their regulation since *FRE1* and not *FRE6* is under control of both the Fe-dependent Aft1p and Cu-dependent Mac1p transcription factors (28). Thus, whereas Fre1p activity is increased when either iron or copper is limiting Fre6p activity is increased only when cytoplasmic iron is low. This regulatory pattern suggests that Cu-efflux from the vacuole is stimulated only when an increase in Fe-uptake is signaled. This pattern can be rationalized in that the copper recycled from the vacuole can be used to activate the Cu-dependent Fet3p ferroxidase needed in support of Fe-uptake at the plasma membrane (11).

Last, we suggest our observations indicate the role of Fre7p as a metalloreductase will be found in some pathway other than membrane transport. One possibility is that as the endosomal, Steap3 reductase in mammals is required for efficient release of iron from diferric transferrin (41, 42), Fre7p could be involved in the release of iron, as FeIII, from an intracellular ferric iron chelate. In as much as *S. cerevisiae* can accumulate environmental iron from siderophores (43), it is a reasonable hypothesis that these chelates could be the substrates for this reductase that as of now remains without a function.

**Acknowledgments**—We thank Dr. Wade Sigurdson for continuing assistance in the use of confocal fluorescence microscopy in the examination of protein localization in yeast and to Drs. Erin Rees and Dennis Thiele for discussions about our shared work on metal ion efflux from the vacuole.

**REFERENCES**

1. Finney, L. A., and O’Halloran, T. V. (2003) Science 300, 931–936
2. Aisen, P., Enns, C., and Wessling-Resnick, M. (2001) Int. J. Biochem. Cell Biol. 33, 940–959
3. Curie, C., and Briat, J. F. (2003) Annu. Rev. Plant Biol. 54, 183–206
4. De Freitas, J., Wintz, H., Kim, J. H., Poynton, H., Fox, T., and Vulpe, C. (2003) Biometals 16, 185–197
Vacuolar Iron Cycling in Yeast

5. Luk, E., Jensen, L. T., and Culotta, V. C. (2003) *J. Biol. Inorg. Chem.* 8, 803–809
6. Kucharczyk, R., and Rytka, J. (2001) *Acta Biochim. Pol.* 48, 1025–1042
7. Herman, E. M., and Larkins, B. A. (1999) *Plant Cell* 11, 601–614
8. Ramsay, L. M., and Gadd, G. M. (1997) *FEMS Microbiol. Lett.* 152, 293–298
9. Szczypka, M. S., Zhu, Z., Silar, P., and Thiele, D. J. (1997) *Yeast* 13, 1423–1435
10. Kaplan, J. (2002) *Cell* 111, 603–606
11. Kosman, D. J. (2003) *Mol. Microbiol.* 47, 1185–1197
12. Hell, R., and Stephan, U. W. (2003) *Planta* 216, 541–551
13. Andrews, N. C., Fleming, M. D., and Gunshin, H. (1999) *Nutr. Rev.* 57, 114–123
14. Schroder, I., Johnson, E., and de Vries, S. (2003) *FEMS Microbiol. Rev.* 27, 427–447
15. Muller, E. G. (1996) *Mol. Biol. Cell* 7, 1805–1813
16. Grant, C. M. (2001) *Mol. Microbiol.* 39, 533–541
17. Schafer, F. Q., and Buettner, G. R. (2001) *Free Rad. Biol. Med.* 30, 1191–1212
18. Raguzzi, F., Lesuisse, E., and Crichton, R. R. (1988) *FEBS Lett.* 231, 253–258
19. Wheeler, G. L., and Grant, C. M. (2004) *Physiol. Plant.* 120, 12–20
20. Lapinskas, P. J., Lin, S. J., and Culotta, V. C. (1996) *Mol. Microbiol.* 21, 519–528
21. Li, L., Chen, O. S., McVey Ward, D., and Kaplan, J. (2001) *J. Biol. Chem.* 276, 29515–29519
22. Fu, D., Beeler, T., and Dunn, T. (1994) *Yeast* 10, 515–521
23. Nelson, N. (1999) *EMBO J.* 18, 4361–4371
24. Portnoy, M. E., Liu, X. F., and Culotta, V. C. (2000) *Mol. Cell. Biol.* 20, 7893–7902
25. Urbanowski, J. L., and Piper, R. C. (1999) *J. Biol. Chem.* 274, 38061–38070
26. Martins, L. J., Jensen, L. T., Simon, J. R., Keller, G. L., and Winge, D. R. (1998) *J. Biol. Chem.* 273, 23716–23721
27. De Freitas, J. M., Kim, J. H., Poynton, H., Su, T., Wintz, H., Fox, T., Holman, P., Loguinov, A., Keles, S., van der Laan, M., and Vulpé, C. (2004) *J. Biol. Chem.* 279, 4450–4458
28. Rutherford, J. C., Jaron, S., and Winge, D. R. (2003) *J. Biol. Chem.* 278, 27636–27643
29. Wach, A., Brachat, A., Pohlmann, R., and Philippson, P. (1994) *Yeast* 10, 1793–1808
30. Sikorski, R. S., and Heiter, P. (1989) *Genetics* 122, 19–27
31. Miller, J. H. (1972) *Experiments in Molecular Genetics*, pp. 352–355, Cold Spring Harbor, NY
32. Shatwell, K. P., Dancis, A., Cross, A. R., Klausner, R. D., and Segal, A. W. (1996) *J. Biol. Chem.* 271, 14240–14244
33. Yamaguchi-Iwai, Y., Ueta, R., Fukunaka, A., and Sasaki, R. (2002) *J. Biol. Chem.* 277, 18914–18918
34. Lesuisse, E., Casteras-Simon, M., and Labbe, P. (1996) *J. Biol. Chem.* 271, 13578–13583
35. Mao, X., Hu, Y., Liang, C., and Lu, C. (2002) *Curr. Microbiol.* 45, 37–40
36. Rees, E. M., and Thiele, D. J. (2007) *J. Biol. Chem.* 282, 21629–21638
37. Wang, G., Yang, J., and Hubrechts, D. J. (2007) *Mol. Cell. Biol.* 29, 342–352
38. Puig, S., and Thiele, D. J. (2002) *Curr. Opin. Chem. Biol.* 6, 171–180
39. Portnoy, M. E., Schmidt, P. J., Rogers, R. S., and Culotta, V. C. (2001) *Mol. Genet. Genomics* 265, 873–882
40. Rees, E. M., Lee, J., and Thiele, D. J. (2004) *J. Biol. Chem.* 279, 54221–54229
41. Ohgami, R. S., Campagna, D. R., Greer, E. L., Antiochos, B., McDonald, A., Chen, J., Sharp, J. I., Fujiwara, Y., Barker, J. E., and Fleming, M. D. (2005) *Nat. Genet.* 37, 1264–1269
42. Ohgami, R. S., Campagna, D. R., McDonald, A., and Fleming, M. D. (2006) *Blood* 108, 1388–1394
43. Yun, C.-W., Bauler, M., Moore, R. E., Kebba, P. E., and Philpott, C. C. (2001) *J. Biol. Chem.* 276, 10218–10223