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Genetic Evidence that Ferric Reductase Is Required for Iron Uptake in *Saccharomyces cerevisiae*†

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The requirement for a reduction step in cellular iron uptake has been postulated, and the existence of plasma membrane ferric reductase activity has been described in both procaryotic and eucaryotic cells. In the yeast *Saccharomyces cerevisiae*, there is an externally directed reductase activity that is regulated by the concentration of iron in the growth medium; maximal activity is induced by iron starvation. We report here the isolation of a mutant of *S. cerevisiae* lacking the reductase activity. This mutant is deficient in the uptake of ferric iron and is extremely sensitive to iron deprivation. Genetic analysis of the mutant demonstrates that the reductase and ferric uptake deficiencies are due to a single mutation that we designate *fre1-1*. Both phenotypes cosegregate in meiosis, corevert with a frequency of 10^{-7}, and are complemented by a 3.5-kilobase fragment of genomic DNA from wild-type *S. cerevisiae*. This fragment contains *FRE1*, the wild-type allele of the mutant gene. The level of the gene transcript is regulated by iron in the same was as the reductase activity. The ferrous ion product of the reductase must traverse the plasma membrane. A high-affinity (K_m = 5 μM) ferrous uptake system is present in both wild-type and mutant cells. Thus, iron uptake in *S. cerevisiae* is mediated by two plasma membrane components, a reductase and a ferrous transport system.

Iron is required by virtually all organisms for essential biological processes. For example, cellular respiration requires cytochromes and DNA synthesis requires ribonucleotide reductase. These proteins contain iron as the ferrous (Fe^{2+}) ion. Although iron is abundant in the crust of the earth, its predominant form is the ferric (Fe^{3+}) salt. This ion is very insoluble; the maximum concentration of Fe^{3+} in water at pH 7 is 10^{-17} M, so organisms are faced with "famine in a land of plenty." Ferrous iron is more soluble (up to 10^{-1} M) but is very unstable, oxidizing readily under atmospheric conditions. Thus, the problems that organisms must solve in the metabolism of iron are its solubilization, transport across the cell membrane into the cytosol, and at least one reduction step from the ferric to the ferrous form.

Two distinct solutions to the problem of capture and reduction of iron have evolved. In many microorganisms, fungi, and plants, ferric iron is solubilized by being bound to high-affinity chelators called siderophores (20, 21). Ferrisiderophore complexes are recognized by specific membrane receptors which mediate the transmembrane movement of the metal chelate. Iron reduction then takes place intracellularly. An alternative mechanism has been proposed in which environmental ferric iron is initially solubilized by reduction, and the ferrous iron is then transported across the plasma membrane (9, 25). A plasma membrane reductase activity able to reduce ferric iron has been observed in some bacterial strains, fungi, and plant and animal cells (3, 5, 16, 19, 22, 29, 30); however, it has not been possible to link the observed reductase activities with iron uptake. The latter is important because ferric reductase can be utilized in the plasma membrane for functions other than iron uptake. For instance, in *Alteromonas putrefaciens* and other bacteria, the ferric reductase is involved in respiration. This enzyme couples the oxidation of organic substrates to the reduction of iron (16, 19). Another problem with the postulated role of reductase in iron uptake has been the lack of a molecular characterization of its components.

To address these problems, we studied iron uptake in the yeast *Saccharomyces cerevisiae* as a model system that can be easily manipulated both metabolically and genetically. These cells have an externally directed ferric reductase activity in the plasma membrane that is regulated by the concentration of iron in the growth medium (5, 15). We isolated a mutant lacking this plasma membrane reductase activity. The mutant was also deficient in the uptake of ferric iron. Genetic analysis demonstrated that both phenotypes are due to the mutation of a single gene. We cloned the wild-type allele of this gene, *FRE1*. Finally, we observed a saturable ferrous uptake system present in both the reductase-deficient mutant and the wild-type cells. These results allow us to propose a model for the mechanism of iron uptake in *S. cerevisiae* that can probably be extended to other eucaryotic cells.

MATERIALS AND METHODS

Strains and media. The strains of *S. cerevisiae* used had the following genotypes: F113 (*MAT***a** *ura3-52 ino1-1*), H746 (*MAT*α *leu2-3 leu2-112*). Yeast cells were grown in yeast extract-peptone-glucose (YPD) medium, synthetic glucose (SD) medium (28), or minimum defined (MD) medium. The latter is a modification of that described by van Steveninck and Booij (32). A solution was made containing 0.1 M Tris hydrochloride (pH 7.3), 0.1 M glucose, 8 mM KH_2PO_4, 75 mM citric acid, 3.7 mM potassium citrate, 22 mM ammonium sulfate, and 50 ml of basal medium Eagle vitamin solution (GIBCO Laboratories, Grand Island, N.Y.) per liter. This solution was treated twice with 50 g of Chelex 100 resin (Bio-Rad Laboratories, Richmond, Calif.) per liter for 24 h with agitation to remove contaminating iron. The liquid was decanted from the resin, salts were added back to a final concentration of 2.4 mM $MgSO_4$, 13 mM KCl, 2.0 mM $CaCl_2$, 6.0 mM $MnCl_2$, 0.86 mM NaCl, and 0.36 mM $ZnCl_2$, and the solution was sterilized by microfiltration. For growth

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† This paper is dedicated to the memory of Mari Angeles.

on solid medium, 2% agar was added to YPD, SD, or MD medium. In all cases, the nutrients required for growth of auxotrophic strains were added at the final concentrations indicated by Sherman et al. (28).

The *Escherichia coli* strain used was DH5α (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Bacteria were grown in Luria-Bertani (LB) medium (17). When required, ampicillin was added at 10 μg/ml final concentration.

Microbiological procedures for yeasts and bacteria. Cells of strain F113 were mutagenized by exposure to 3% ethyl methanesulfonate for 2 h, yielding a survival of 0.02%. Transformation by the lithium acetate protocol, crosses of haploid strains, sporulation, tetrad dissection, and all other microbiological procedures with yeasts were performed by the methods described by Sherman et al. (28) unless otherwise indicated. Methods for *E. coli* were as described by Maniatis et al. (17).

Plasmids. YCp50 is a shuttle vector derived from pBR322 with origins of replication for *E. coli* and *S. cerevisiae*. It contains the yeast *ARS1* sequence and *CEN4* centromere sequence that maintains the copy number at one to two per cell. This vector also contains the *URA3* gene for selecting transformants of yeast *ura3* auxotrophs and ampicillin and tetracycline resistance genes for selection of transformants in *E. coli* (26). A library made by *Sau*3A partial digestion of wild-type yeast genomic DNA, size selection, and cloning into the unique *Bam*HI site located in the tetracycline resistance gene of YCp50 (26) was used to clone the *FRE1* gene by complementation of the *fre1-1* mutation.

Subclones of the complementing plasmid pWDC9 were constructed by utilizing convenient restriction enzyme cleavage sites in the insert C17 and parent vector YCp50. pWDC10 was constructed by digestion of pWDC9 with *Sac*I, which cleaves near the center of the insert (see Fig. 6), and *Sal*I, which cleaves in the vector to the right of the insert. pWDC11 was constructed from pWDC9 by digestion with *Bst*EII and *Sal*I. pWDC13 was constructed from pWDC10 by *Sac*II and *Hin*dIII digestions. In each of the above constructs, the free ends of the plasmid were rendered blunt with T4 DNA polymerase and then religated. pWDC12 was constructed by digesting pWDC9 with *Eco*RI, gel isolating the 5.5-kilobase (kb) fragment, and inserting it into the *Eco*RI cloning site of the vector.

YIp5 is a shuttle vector which lacks the *CEN4* and *ARS1* sequences and so can be maintained in yeasts only by integration into the genome. The 5.5-kb *Eco*RI fragment of C17 was inserted into this vector in the *Eco*RI cloning site. The resulting plasmid, pWDC14, was linearized at the unique *Sac*I site in the yeast DNA insert to direct integration to the homologous site in the yeast genome and used to transform wild-type strain F113 to uracil prototrophy. One of the transformants was crossed with strain W104 (*MATα fre1-1 ura3-52*). The frequency of recombination between *FRE1* and the *URA3* gene integrated on pWDC14 was analyzed by sporulation of the diploid, tetrad dissection, and assay of the clones arising from the spores for reductase and uracil auxotrophy. Inositol auxotrophy and mating type were also analyzed as controls for 2:2 segregation.

Reductase assay. Reductase activity was measured by a modification of the procedure of Avron and Shavit (1). We followed two protocols, a quantitative assay of cells in suspension and a qualitative assay of cells grown on solid medium. For the fluid-phase assay, cells were collected by centrifugation at 1,000 × *g*, washed twice with distilled water, and incubated at 30°C in assay buffer consisting of 0.05 M sodium citrate (pH 6.5) with 5% glucose. After a 15-min incubation, bathophenanthrolene disulfonate (BPS) and $FeCl_3$ were added to a final concentration of 1 mM and the incubation was continued for an additional 15 to 30 min. At this time, the cells were removed by centrifugation and the optical density at 520 nm of the supernatant was measured. The amount of Fe^{2+} produced was estimated with a calibration curve constructed from solutions of known ferrous ion concentrations. Background values were calculated in parallel incubations performed without cells. When the cells had grown on solid medium, nylon filters (1.2-μm pore size; Biotrans, ICN, Irvine, Calif.) were used to lift replicas from the plates. The inverted replicas were incubated on Whatman 3MM filter paper soaked in assay buffer (50 mM sodium citrate, 5% glucose, pH 6.5). At the end of a 5-min incubation, the filters were transferred for 5 min to a second paper soaked in assay buffer plus 1 mM BPS and 1 mM $FeCl_3$. During this incubation, the colonies with reductase activity stained the filter in their vicinity, producing a red spot. The replica was then dried and compared with the original plate to identify colonies with or without reductase activity. Ferricyanide was also used as a substrate in a colorimetric assay of plasma membrane ferric reductase as described by Crane et al. (5), although this reagent is not suitable for use in a solid-phase enzyme assay.

Iron uptake. To measure ferric iron uptake, cells were collected by centrifugation at 1,000 × *g*, washed twice in MD medium, and suspended in 10 mM sodium citrate (pH 6.5)–5% glucose containing 20 μM $^{55}FeCl_3$ (95 Ci/mol; Amersham Corp., Arlington Heights, Ill.). The suspension was incubated at 30°C, and at different times the cells were collected on glass filters and washed first with 10 ml of 0.25 M EDTA (pH 6.5) and then with 10 ml of distilled water, after which the cell-associated ^{55}Fe was determined by counting β emissions in a scintillation counter. The ferrous uptake was determined in the same way, but the citrate buffer was replaced by 0.1 M sodium ascorbate (pH 4)–1 mM dithiothreitol–5% glucose for the labeling. $^{55}FeSO_4$ (220 Ci/mol; Dupont, NEN Research Products, Boston, Mass.) was present in the incubation media at different final concentrations.

DNA isolation and Southern blot analysis. Yeast genomic DNA was extracted from spheroplasts (33) and digested with the restriction enzyme *Eco*RI, *Bam*HI, or *Hin*dIII (New England BioLabs, Inc., Beverly, Mass.). Digested DNA (0.5 μg) was separated by electrophoresis on a 0.8% agarose gel and transferred to a nylon membrane. The membrane was hybridized with a DNA fragment labeled with [α-^{32}P]dCTP by the random priming procedure (10) and then washed at high stringency (65°C, 0.2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) (17). The hybridizing species were identified by autoradiography with Kodak XAR 5 film.

RNA isolation and Northern (RNA) blot analysis. RNA was isolated from yeast cells (28) grown in high- or low-iron media. A 15-μg portion of total RNA or 5 μg of oligo(dT)-selected poly(A)$^+$ RNA (28) was separated by electrophoresis on a 1% agarose gel and electrophoretically transferred to a nylon membrane (Biotrans). The membrane was processed as described above for the Southern blot analysis. When hybridization with a second probe was required, the nylon membrane was washed at 75°C in 1× SSC–50% formamide (17) to remove the first probe and then hybridization, washing, and autoradiography were performed as before.

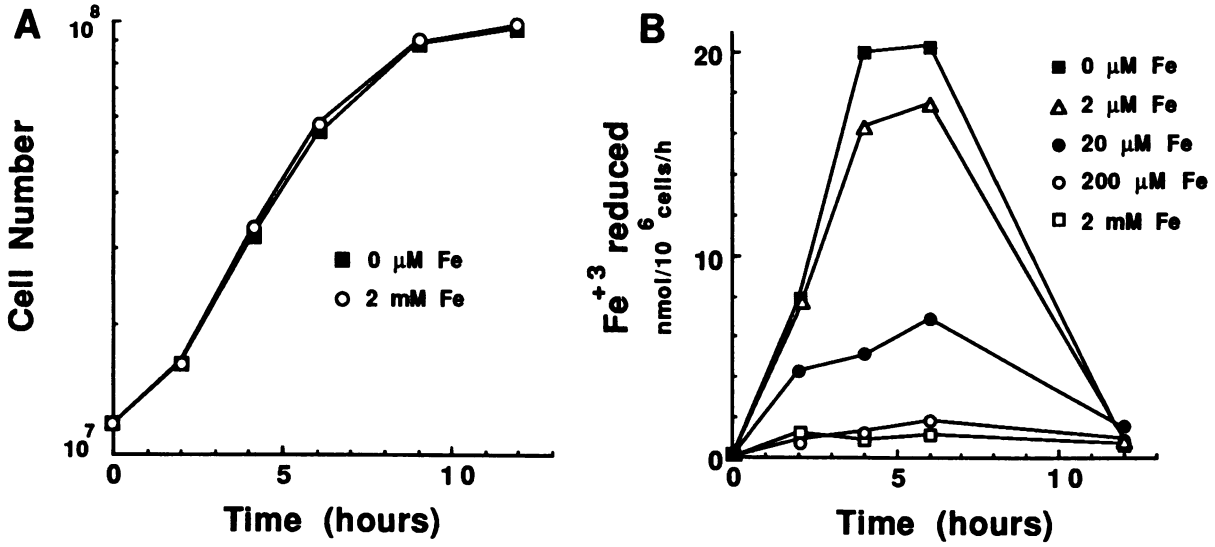

FIG. 1. Ferric reductase activity in *S. cerevisiae* grown in media with different iron concentrations. Cells of strain F113 were grown to the stationary phase in complete (YPD) medium at 30°C with agitation and aeration. Cells were then diluted 50-fold and grown for 4 h in MD medium with 2 mM $FeCl_3$ added. At time zero, the cells were collected by centrifugation, washed twice with MD medium, and resuspended at a cell density of 10^7/ml in MD medium with the indicated concentration of $FeCl_3$. At different times, samples were removed from the cultures for determination of cell density and ferric reductase activity. Cell density was estimated by reading the optical density at 600 nm. Cell number was determined by counting in a calibrated hemacytometer, and a linear relationship was established with the optical density at 600 nm for values up to 0.7. Reductase activity was measured as described in Materials and Methods. (A) Concentration of cells at different times. The growth rate is the same in all the cultures ($t_{1/2}$ = 2.4 ± 0.1 h). As described in the text, several generations are required in iron-deficient medium before the growth rate is reduced. (B) Ferric reductase activity for different times and for different iron concentrations in the growth media. The enzymatic activity at 4 h reaches a maximum which depends on the iron concentration in the media. The maximum is maintained until growth reaches the stationary phase, and then the activity disappears.

RESULTS

Yeast mutant lacking both ferric reductase and ferric uptake activities. In *S. cerevisiae*, the need for reduction of ferric to ferrous iron in the uptake of this metal has been postulated (14). The presence of a reductase activity in the plasma membrane of these cells that is able to reduce ferric ions is well documented (5, 15). This activity is regulated by the amount of iron in the growth medium and by the growth state of the cells (Fig. 1). The enzymatic activity is maximal when the cells are in the logarithmic growth phase in low-iron media. Increasing concentrations of iron in the growth medium produce a progressive decrease in activity. On the other hand, when the cells enter the stationary phase, the activity rapidly disappears. This disappearance is not prevented by iron deprivation.

Well-defined iron uptake systems present in the plasma membrane of a variety of cells are regulated by iron. Thus, deprivation of iron results in increased synthesis of siderophore receptors in *E. coli* and of transferrin receptors in mammalian cells (2, 11, 24). Similar regulation of this plasma membrane reductase activity in *S. cerevisiae* suggests an analogous function for this enzyme. To test the potential role of the reductase in iron uptake, we sought to isolate mutants lacking this activity. We screened roughly 2,000 colonies of the mutagenized haploid strain F113 for lack of activity and identified one such mutant. We refer to this strain as W103 and designate the mutation responsible for the reductase deficiency (Fre^-) as *fre1-1*. Figure 2 illustrates the lack of detectable membrane reductase activity in the mutant.

We compared the effect of iron deprivation on the growth of the mutant and wild-type strains. When either F113 or W103 cells were transferred from iron-rich to iron-deficient medium, their growth rate progressively decreased, and after four to five cell doublings, growth stopped. The initial growth in iron-deficient medium was probably due to small amounts of the metal transferred with the cells. This iron may be bound to the exterior of the cells or contained in a storage compartment such as the vacuole (23). When iron was added back to the arrested cells, growth was restored for both mutant and wild type (Fig. 3). However, wild-type cells achieved their maximal growth rate at 20 μM iron, whereas mutant cells did not resume growth until 400 μM iron was present in the medium. Moreover, the mutant cells did not attain the growth rate of the wild type even at 2 mM iron.

The ability of iron in the medium to partially correct the growth abnormality of the mutant suggests that the mutation affects the iron uptake system. This point was demonstrated directly by analysis of the uptake of ferric iron. The wild type exhibited significant uptake of ferric iron, whereas uptake in the mutant was nearly indistinguishable from background (Fig. 4). Thus, the presence of reductase activity is correlated with ferric iron uptake. It is interesting that in wild-type cells, the iron uptake was regulated by the level of previous exposure to iron in the same way as the reductase activity: ferric iron uptake was maximal when the cells were previously deprived of exogenous iron.

Ferric reductase and ferric uptake deficiencies result from a single mutation. Strains W103 (*MATa ura3-52 ino1-1 fre1-1*) and F113 (*MATa ura3-52 ino1-1 FRE1*) were crossed with wild-type strain H746 (*MATα leu2-3 leu2-112 FRE1*). The ferric reductase activity in the *FRE1/fre1-1* heterozygote was

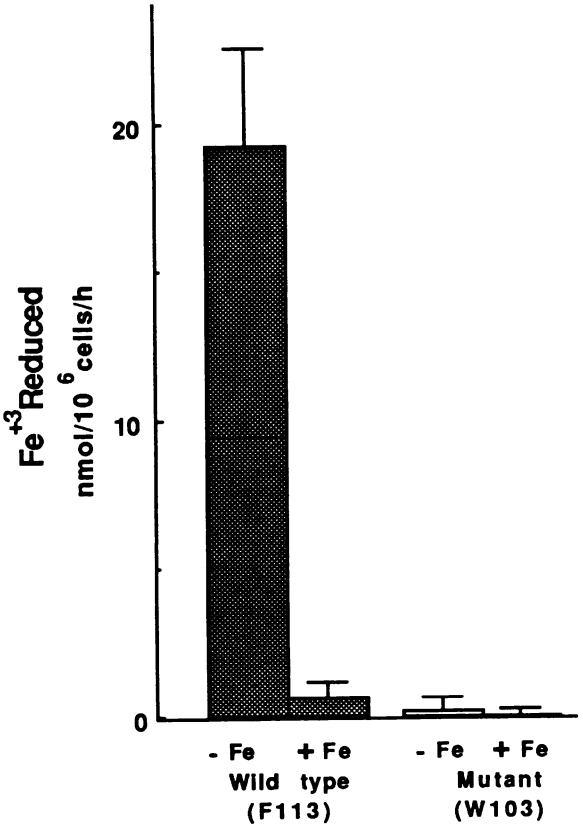

FIG. 2. Lack of reductase activity in *fre1-1* mutant. Wild-type strain F113 and *fre1-1* mutant strain W103 were grown in YPD and when growing exponentially were transferred to MD medium with no iron (−Fe) or with 2 mM ferric chloride (+Fe). Under these conditions, an iron supplement is not needed for normal growth of the mutant. Four hours later, when the cells were in the logarithmic growth phase, reductase activity was measured as described in the legend to Fig. 1. The activity of the mutant cells was at the limit of resolution of the assay. The cells may have no activity or a residual activity less than 2% of wild type.

roughly 50% of that measured for the *FRE1/FRE1* homozygote, and ferric uptake was comparable in both diploids. These results suggest that *fre1-1* represents a loss of function allele.

The *FRE1/fre1-1* heterozygote was sporulated, and the products of 12 tetrads showing 2+:2− segregation for uracil and leucine auxotrophy were analyzed for reductase and ferric iron uptake. A 2+:2− segregation for reductase activity was observed, and in each tetrad the Fre^- segregants had impaired ferric iron uptake. This result indicated that both deficiencies arise from either a single mutation or two mutations separated by a distance of 4 centimorgans or less (~12 kb) (18). Additional evidence supporting the single-mutation hypothesis comes from reversion analysis. Spontaneous revertants of W103 were selected for the ability to grow on low-iron-containing solid medium. We identified nine strains with greatly increased iron uptake capacity compared with W103. All nine revertants also showed a significant increase in reductase activity. The frequency of coreversion (10^{-7}) is most consistent with a single mutation being the cause for both mutant phenotypes. To obtain definitive evidence that a single mutation is responsible for the lack of both reductase and ferric uptake activities in the mutant cells, we sought to clone the *FRE1* gene.

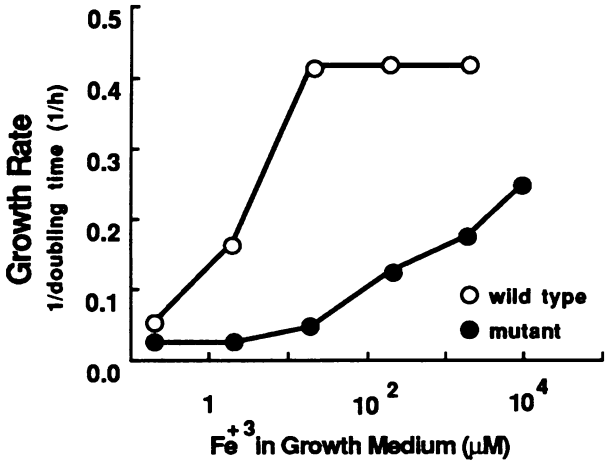

FIG. 3. Iron-dependent growth of wild-type and Fre^- mutant cells. Mutant strain W103 and its wild-type parent F113 were grown to the logarithmic phase in YPD and then transferred to defined medium lacking iron at a density of roughly 10^6 cells per ml. After 10 h in these conditions, $FeCl_3$ was added to samples of iron-starved cells to give the concentrations indicated in the figure. Cell density was determined periodically by reading the optical density at 600 nm. The iron concentrations required to produce an increase in growth rate of wild-type cells are 2 orders of magnitude lower than the concentrations required to produce an increase in growth rate of mutant cells.

Cloned *FRE1* gene restores ferric reductase and ferric uptake activities in Fre^- mutant. We screened a wild-type yeast genomic library constructed in a low-copy-number yeast plasmid for the ability to restore reductase activity in the W103 strain. Three different plasmids were isolated that complement the Fre^- phenotype of W103. Figure 5 shows the effect of transforming W103 with one of these plasmids (pWDC9). The three plasmids restored 50% of the wild-type reductase activity, as was observed for the *FRE1/fre1-1*

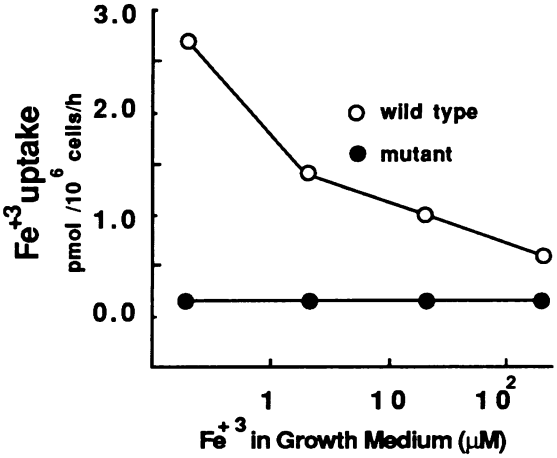

FIG. 4. Ferric iron uptake by wild-type and Fre^- mutant. F113 (wild-type) and W103 (*fre1-1*) cells were grown in defined medium with different concentrations of iron as described in the legend to Fig. 1. Samples were taken from exponentially growing cultures, the cells were collected and washed two times in iron-free defined medium, and the incorporation of radioactivity from $^{55}Fe^{3+}$ into the cells was measured as described in Materials and Methods. Wild-type cells take up ferric iron and regulate the uptake, whereas mutant cells do not take up ferric iron.

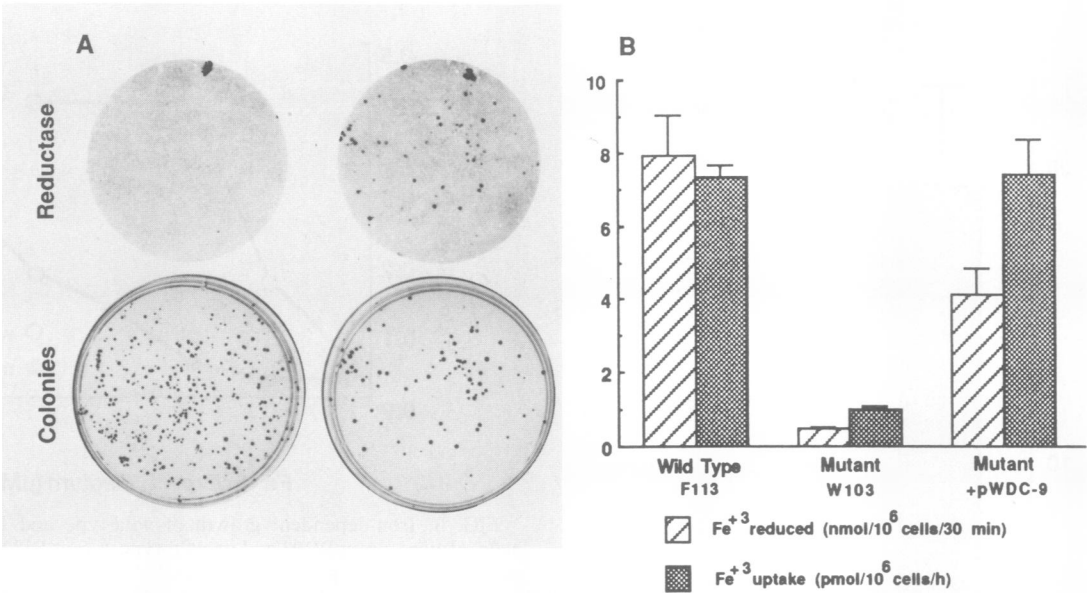

FIG. 5. Functional complementation of Fre$^-$ cells with genomic DNA. W103 cells were transformed by the lithium acetate method with a wild-type yeast genomic library constructed in the low-copy-number plasmid YCp50 containing the yeast *URA3* gene as a selectable marker. After 5 days of growth on solid medium lacking uracil, Ura$^+$ transformants were visible. Roughly 10^4 colonies were screened for reductase activity, and 4 Fre$^+$ clones were identified. The plasmids were rescued from these four clones by transformation of *E. coli* to ampicillin resistance and were shown to retransform W103 cells from Fre$^-$ to Fre$^+$. (A) Left, Colonies of the *fre1-1* mutant W103 transformed with the library that are all negative for reductase activity. Right, Colonies of W103 transformed with one of the four rescued plasmids (pWDC9) that confers a Fre$^+$ phenotype. (B) Transformation of the *fre1-1* mutant with this plasmid restores roughly 50% of wild-type reductase activity and 100% of wild-type ferric ion uptake.

diploid heterozygote. They also conferred a wild-type capacity for iron uptake. The restriction map of the genomic fragment (C17) contained in the pWDC9 plasmid is shown in Fig. 6. The corresponding maps for the other two cloned fragments indicate that they overlap with C17 (data not shown). Deletion analysis of C17 showed that the 3.5-kb *Sac*II-*Sac*I fragment is the minimal sequence able to restore reductase activity to W103, locating the complementing gene within these two restriction sites. A DNA probe derived from this fragment was used to analyze DNA prepared from wild-type and *fre1-1* mutant cells. Digestion of genomic DNA from both types of cells with the restriction enzyme *Eco*RI, *Hin*dIII, or *Bam*HI gave in each case a single

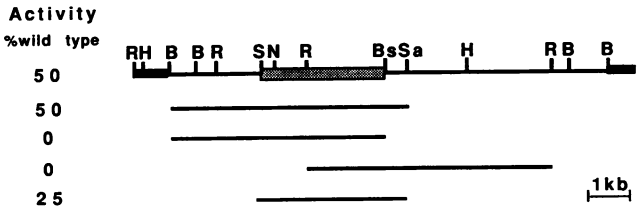

FIG. 6. Restriction map of yeast genomic DNA fragment C17. The plasmid pWDC9 containing the DNA fragment C17 was digested with the restriction enzymes *Eco*RI (R), *Hin*dIII (H), *Bam*HI (B), *Sac*II (S), *Nru*I (N), *Bst*EII (Bs), and *Sac*I (Sa), and the fragments produced were analyzed by electrophoresis in 1% agarose gels. The map shows the positions of the restriction sites in the C17 genomic insert (thin line) and in the flanking vector sequences (thick line). The shaded box illustrates the minimal fragment able to complement the reductase deficiency in W103. Lines below the map represent four fragments subcloned in YCp50. The four resulting plasmids (pWDC10 to -13, respectively) were used to transform W103. To the left of each line is indicated the reductase activity found in the transformed cells compared with the wild-type levels.

fragment that hybridizes with the probe (data not shown). This indicates that the cloned sequences in pWDC9 are present in single copy in the *S. cerevisiae* genome.

We then sought to demonstrate that the cloned fragment in pWDC9 contains the wild-type allele of *FRE1*. To do so, a marker gene (*URA3*) was inserted into the yeast genome by recombination between sequences flanking the complementing gene present on the cloned fragment and homologous sequences in genomic DNA (see Materials and Methods). Integration of *URA3* at the genomic site homologous to the cloned sequences was confirmed by DNA blot hybridization analysis (data not shown). The distance between the inserted marker and the *FRE1* locus was estimated from the frequency of recombination between the two in 58 tetrads and was found to be 2.8 centimorgans, consistent with the assertion that the complementing fragment contains the *FRE1* gene.

***FRE1* transcript is induced by iron deprivation.** A DNA probe derived from the cloned *FRE1* gene was used to analyze RNA extracted from *fre1-1* mutant and wild-type cells grown in different conditions. The probe hybridized with an RNA of about 3 kb in both types of cells (Fig. 7). The same hybridizing species was enriched in poly(A)$^+$ RNA, indicating that it corresponds to an mRNA (data not shown). The levels of the mRNA in wild-type cells paralleled those of the reductase activity (compare Fig. 1 and Fig. 7). Enzymatic activity and mRNA levels were high in cells grown in defined medium lacking iron and were much lower in cells grown in the same medium containing high concentrations of iron. Similar low amounts of transcript were also observed when the cells reached the stationary phase (data not shown). Interestingly, an mRNA with the same mobility was detected in the *fre1-1* mutant cells, although at reduced levels. The amount of this mRNA can be regulated by manipulations of iron in the growth media, while enzyme

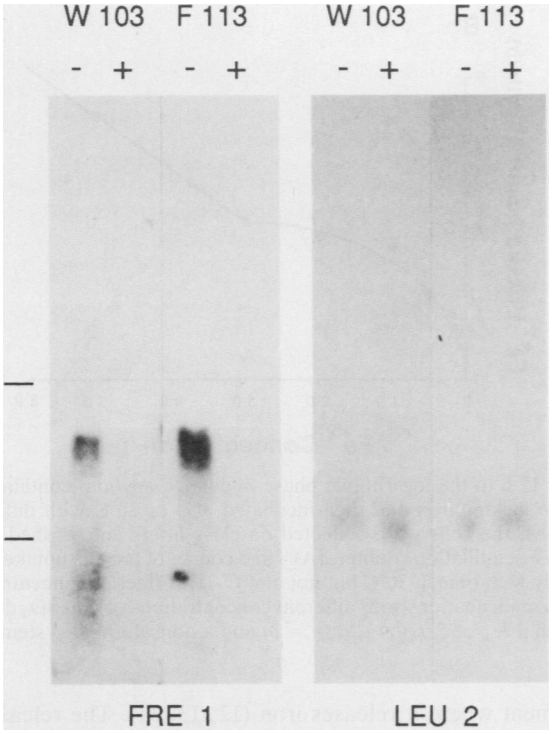

FIG. 7. RNA blot hybridization analysis of *FRE1* and *fre1-1*. Total RNA was extracted from F113 and W103 cells grown exponentially for 12 h in defined medium lacking iron (−) or containing 2 mM $FeCl_3$ (+). A 15-μg sample of each RNA preparation was separated by electrophoresis in a 1% agarose gel and transferred to a nylon membrane. The membrane was hybridized with a DNA probe made with the *Eco*RI-*Sac*I fragment of C17 (Fig. 6). The membrane was subsequently stripped of this probe and rehybridized with a control probe prepared from the yeast *LEU2* gene. Shown are autoradiograms of the membrane after hybridization with each probe. The lines to the left indicate the positions of the 18S and 28S rRNA. The left panels, labeled *FRE1*, show a labeled RNA of approximately 3 kb that represents the *FRE1* transcript. This RNA is expressed in both wild-type (F113) and mutant (W103) cells grown in iron-deficient medium (− lanes) and cannot be detected in cells grown in medium containing 2 mM $FeCl_3$ (+ lanes). The right panels, labeled *LEU2*, show the *LEU2* mRNA. In contrast to *FRE1*, the levels of this mRNA are not significantly affected by the concentration of iron in the growth medium.

activity remains undetectable under the different iron conditions.

Ferrous uptake system is present in wild-type and *fre1-1* cells. The above results suggest that the ferric reductase activity of the plasma membrane is very likely to be essential for ferric iron uptake; however, they do not explain the transport of iron across the plasma membrane. We looked for evidence of a translocator or channel which could direct the movement of ferrous ions from the external environment to the cytoplasm. Time course experiments demonstrated an uptake of ferrous ions at 30°C in both wild-type (data not shown) and mutant (Fig. 8A) cells. This uptake was linear with time for up to 30 min and was inhibited at 4°C. Analysis of uptake at different ferrous concentrations showed a saturable system with an apparent K_m of 5 μM (Fig. 8B). In addition to this saturable system, we also observed nonsaturable uptake at concentrations of ferrous iron higher than 30 μM. The presence of saturable and nonsaturable iron uptake systems has also been reported for gut mucosa (7). The nonsaturable uptake may represent the movement of ferrous iron across a cation channel, driven by the high extracellular concentrations used in these measurements. The concentrations of ferrous iron attainable under regular aerobic conditions are much lower than these. Therefore, it is very unlikely that the nonsaturable system plays any role in iron uptake in aerobic conditions. The presence of a functional ferrous uptake system in the Fre^- mutant can explain the viability of these cells in iron-rich media (Fig. 3): reduction of some ferric iron either by chemical means or by residual reductase activity may supply enough ferrous iron to allow for their survival and growth.

DISCUSSION

The aim of this study was to initiate a molecular analysis of iron uptake in eucaryotes, using *S. cerevisiae* as a model system. A mechanism proposed for some eucaryotic cells involves reduction of ferric to ferrous iron and subsequent transport of the latter to the cytoplasm. However, until now there has been no direct evidence for the involvement of ferric reductase activity in the process of iron uptake, nor any molecular characterization of the components of transmembrane uptake of iron in eucaryotes. We decided to test the reductase model in *S. cerevisiae*, for which a plasma membrane reductase activity has been described (5, 15). To this end, we isolated a mutant strain lacking ferric reductase activity and found that it is also unable to take up ferric iron from the medium. Genetic analysis showed that a single mutation, *fre1-1*, is responsible for both defects. This result strongly suggests that the ferric reductase activity lacking in the Fre^- mutant is involved in iron uptake.

However, the possibility of a pleiotropic mutation affecting two or more independent functions should also be considered. For instance, a single mutation blocking the transport of proteins to the plasma membrane could conceivably lead to reductase and iron uptake deficiencies without implying that the two functions are related. To address this possibility, we analyzed several plasma membrane activities in *fre1-1* cells. Uptake of several sugars and amino acids was normal (data not shown), as was uptake of ferrous iron (Fig. 8). Therefore, the *fre1-1* mutation does not appear to have a nonspecific effect on the expression of proteins in the plasma membrane.

Other pleiotropic defects are still possible; however, the fact that *FRE1* encodes a transcript whose abundance is regulated by iron availability suggests that the gene product is directly involved in iron metabolism. *FRE1* may encode a subunit of the plasma membrane reductase. Alternatively, the protein could be a transcriptional activator required for expression of one or more reductase components.

The activity measured in the *FRE1/fre1-1* heterozygote was 50% that of the *FRE1/FRE1* homozygote. A possible molecular explanation for this observation is that ferric reductase may be a multimolecular complex into which the FRE1 polypeptide assembles. The *fre1-1* allele could code for a polypeptide that is still able to assemble into this complex, but with a defect rendering the complex inactive as a ferric reductase. The presence in the heterozygote of both normal and defective polypeptides competing for assembly could explain the decrease of function in this setting. The structural characterization of ferric reductase should define whether it is a complex and what the role of the FRE1 gene product is in that complex.

The involvement of an external ferric reductase activity in iron uptake implies the existence of a mechanism for trans-

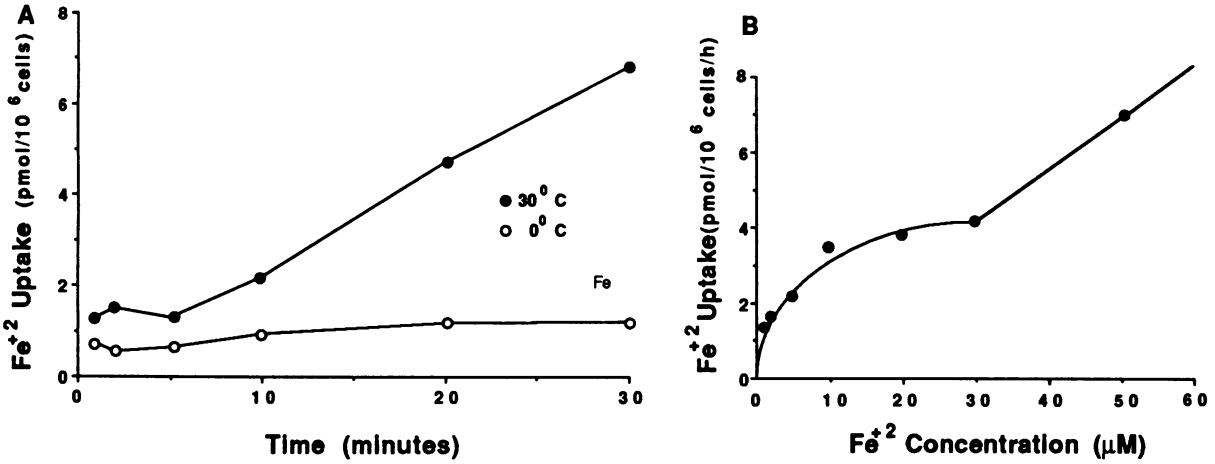

FIG. 8. Ferrous uptake by *fre1-1* mutant cells. W103 cells were grown for 12 h to the logarithmic phase in defined medium containing 2 mM $FeCl_3$. The cells were collected and washed twice with defined medium lacking iron and then incubated at 4 or 30°C with different concentrations of $^{55}FeSO_4$ for different periods. At the end of the incubations, the cells were collected on glass filters and washed. The cell-associated iron was measured by counting the radioactivity of the filters in a scintillation counter. (A) Time course of ferrous uptake with 30 μM $^{55}FeSO_4$. The amount of iron associated with the cells increases linearly with time at 30°C but not at 4°C. (B) Effect of concentration on the rate of iron uptake. Shown is the rate of uptake of ferrous iron measured in incubations with different concentrations of $^{55}FeSO_4$ during 30 min at 30°C. Two uptake systems can be observed, a saturable system with a K_m of 5 ± 0.4 μM (n = 3) and a nonsaturable system that operates at ferrous ion concentrations in excess of 30 μM.

porting ferrous ions into the cell. There are reports in the literature describing the uptake of ferrous iron by gut mucosa, hepatic, and erythroid cells (7, 8, 34). However, ferrous ions in solution are unstable, and it is possible that during the iron uptake assays some ferrous iron is oxidized to the ferric form, making it difficult to discriminate which ionic form is being transported. In this study, we observed the transport of ferrous iron even in mutant cells incapable of ferric uptake (Fig. 4 and 8). This fact demonstrates the existence of a ferrous iron uptake system in the plasma membrane of *S. cerevisiae*. We did not observe any iron-mediated regulation of this uptake activity such as that seen for reductase. However, in the presence of a reduced level of reductase, as observed for the *FRE1/fre1-1* heterozygote, the measured ferric uptake was unchanged. This result may indicate that there is a control of cellular iron uptake other than by modulation of reductase activity.

On the basis of our results, we propose that iron assimilation in *S. cerevisiae* begins with reduction of insoluble ferric ions to soluble but unstable ferrous ions. These ions are then transported to the interior of the cell. Once transported, the ferrous form of iron will be stable in the reducing environment of the cytoplasm and can either be used by the cell or be stored. This mechanism may exist in other organisms with plasma membrane ferric reductase activity.

In humans, as in other mammals, iron is absorbed through the gut mucosa and transported to the blood, where it is bound to transferrin and distributed throughout the organism. The gut mucosal uptake is the only regulated interaction with the environment for iron homeostasis, since there is no excretory mechanism (6, 27). This regulation is of crucial importance, as illustrated in patients with hereditary hemochromatosis, in which the regulation of this process appears to be faulty. Since iron in food is primarily present as ferric ion, unless it is in heme, a reduction step is likely to be involved at the point of initial iron uptake. Transferrin, as a specific ferric chelator, can be thought of as a mammalian siderophore designed to distribute iron to all cells of the organism. It binds to a specific, high-affinity cell surface receptor and is internalized into an acidic endosomal compartment where it releases iron (12, 13, 31). The release of iron from this compartment and transport into the cytoplasm is another possible location for a reduction step and ferrous transporter. Indeed, this is thought to be the site of the genetic defect in the Belgrade rat (4). In hepatocytes, an iron uptake system independent of transferrin has been described which may have reductase and ferrous uptake components (34). It is tempting to speculate that the iron uptake process in the simple eucaryote *S. cerevisiae*, described in this report, provides a model for the missing link in mammalian cellular iron metabolism. A reductase-ferrous transport system may well explain iron uptake from the gut, from transferrin in the acidic endosome, and from nontransferrin carriers.

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