An Oxidase-Permease-based Iron Transport System in *Schizosaccharomyces pombe* and Its Expression in *Saccharomyces cerevisiae*

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Genetic studies have demonstrated that high affinity ferrous transport in *Saccharomyces cerevisiae* requires an oxidase (Fet3p) and a permease (Ftr1p). Using an iron-independent galactose-based expression system, we show that expression of these two genes can mediate high affinity ferrous iron transport, indicating that these two genes are not only necessary, but sufficient for high affinity iron transport. *Schizosaccharomyces pombe* also employ an oxidase-permease system for high affinity iron transport. The *S. pombe* genes, *fio1* (ferrous iron oxidase) and *fip1* (ferrous permease), share significant similarity to *FET3* and *FTR1* from *S. cerevisiae*. Both *fio1* and *fip1* are transcriptionally regulated by iron need, and disruption of *fio1* results in a loss of high affinity iron transport. Expression of *fio1* alone in an *S. cerevisiae* fet3 disruption strain does not result in high affinity iron transport. This result indicates that the *S. pombe* ferroxidase, while functionally homologous to the *S. cerevisiae* ferroxidase, does not have enough similarity to interact with the *S. cerevisiae* permease. Simultaneous expression of both *S. pombe* genes, *fio1* and *fip1*, in *S. cerevisiae* can reconstitute high affinity iron transport. These results demonstrate that the oxidase and permease are all that is required to reconstitute high affinity iron transport and suggest that such transport systems are found in other eukaryotes.

*S. cerevisiae* has two plasma membrane transporters for elemental iron, a high affinity system (*K_m* = 0.15 μM) that transports only iron and a low affinity system (*K_m* = 30 μM) that is also capable of transporting other metals such as cobalt and cadmium (1, 2). Both systems rely on cell surface ferrireductases to convert extracellular ferric to ferrous iron. The low affinity transport system is encoded by the *FET4* gene (2). Two genes, *FET3* and *FTR1*, are directly required for high affinity iron transport (3, 4). The *FET3*-*FTR1* based transport system consists of an oxidase (Fet3p) and a permease (Ftr1p) that work together to facilitate transmembrane iron transport. The Fet3p is a type 1 plasma membrane protein in which the multicopper oxidase domain responsible for ferroxidase activity is present on the extracellular surface (5). Defects in copper transport result in reduced iron transport by affecting the production of active Fet3p (6, 7). The permease, Ftr1p, has multiple membrane spanning domains and contains a motif, REGLE, that may be involved in iron binding (4). Simultaneous expression of Fet3p and Ftr1p is required for proper localization of both proteins at the cell surface, suggesting that a complex is formed during movement of the proteins through the secretory pathway.

Genetic studies have demonstrated that *FET3* and *FTR1* are necessary for high affinity transport, but it has not been established that the combination of Fet3p and Ftr1p alone is sufficient to reconstitute transport activity. Normally *FET3* and *FTR1* are regulated transcriptionally by iron need and iron transport is not induced in high iron medium (1, 3, 4, 8). When incubated in low iron medium, cells with high-copy plasmids containing the *FET3* and *FTR1* genes show supernormal levels of iron transport activity (4). In this study, we demonstrate that expression of both genes from an iron-independent promoter results in high affinity iron transport even when the cells are grown in iron-replete conditions.

A transport system that relies on the permease-oxidase complex has been characterized only in the budding yeast *S. cerevisiae*. *Schizosaccharomyces pombe* also requires a cell surface ferrireductase to utilize ferric iron (9). This reductase has limited amino acid similarity to the *S. cerevisiae* ferrireductase *FRE1* and displays more similarity to the mammalian gp91phox subunit of the human NADPH phagocyte oxidoreductase. Examination of the DNA data bases revealed *S. pombe* genes very similar to *FET3* and *FTR1*. In this report, we describe studies that identify these genes as the molecular components responsible for the *S. pombe* high affinity inducible iron transport system. Expression of these two *S. pombe* genes in *S. cerevisiae* reconstitutes high affinity iron transport, indicating that the *S. pombe* genes, like the *S. cerevisiae* genes, are necessary and sufficient for iron transport.

**MATERIALS AND METHODS**

**Strains and Media**—The *S. cerevisiae* strains used in this study were the wild-type F113 (10) and the fet3 disruption strain 1397–6A (3). The *S. pombe* strains used were the wild-type FY254 (h+, can1–1, leu1–32, ade6-5210, ura4-D18) and the *fio1* disruption strain 4051. This strain was generated by creating a *fio1* disruption construct (fio1::URA4) by digesting the plasmid GALFIO1 (see below) with EcoRI, treating the vector with Klenow, and ligating the bluntened HindIII fragment of PT-ZUR4 containing the *ura4* gene (obtained from Dr. Susan Forsburg at the Salk Institute, San Diego, CA). The *fio1* disruption construct was cut with *PvuII* and *HindIII*, and the fragment containing the disrupted
The fio1 gene was purified and transformed into FY254 using the standard lithium acetate transformation protocol for *S. pombe* (11). Colonies able to grow on medium lacking uracil were examined by Southern analysis for the presence of the disrupted allele (data not shown). Strain 4051 was identified as containing the URA4 gene within *fio1*. The media used in this study included YPD (1.0% yeast extract, 0.2% peptone, 2.0% glucose) and CM-URA-LEU (0.69% yeast nitrogen base, 0.13% amino acid supplementation excluding uracil and leucine) with either 2.0% glucose or 2.0% galactose as the carbon source. Iron-limited medium was generated by adding bathophenanthrolinedisulfonic acid (BPS),3 disodium salt hydrate from Aldrich to a final concentration of 80 μM, and incubated into 10 ml CM-URA-LEU. In some instances, iron was added back to the media (from a 50 mM FeCl3 in 1 M HCl stock). For example, BPS(2.5) is BPS(0) media with 2.5 μM iron added back, and YPD(200) is YPD media with 200 μM iron added. All chemicals were obtained from Sigma unless otherwise noted. All restriction enzymes, ligase, and Klenow were obtained from New England Biolabs Inc. (Beverly, MA).

**Plasmids and Genes**—The *S. pombe* genome *fio1* and *fip1* were identified through BLAST searches of the NR data base using the FET3 and FTR1 amino acid sequences. The *fio1* gene is Swiss Prot accession number Q99920, open reading frame c1F7.08 from chromosome I, EBI accession number Z67995. The *fip1* gene is Swiss Prot accession number Q99919, open reading frame c1F7.07C from chromosome I, EBI accession number Z67996. The FET3 and the FTR1 sequence from *S. cerevisiae* have already been described (4, 5). Sequence comparisons were done with the Bestfit program from the GCG sequence analysis package (Genetics Computer Group, Madison, WI). Multicopper oxidase motifs and potential N-linked glycosylation sites were identified using the Motifs program from GCG.

To generate the GALFET3 plasmid, the plasmid pFET3 (3) containing the genomic FET3 region was digested with EcoO109I and ClaI and treated with Klenow to generate blunt ends. This fragment was purified using the Qiagen gel extraction kit, and ligated into the pYES2 galactose expression vector. The resulting plasmid pYFIO1 was digested and cloned into the YES2 vector. The resulting plasmid (GALFTR1 and GALFIP1) were transformed into 1397–6A, and colonies that contained plasmid were selected on rich medium containing 2.0% glucose.

**Northern Analysis—**Cells were grown in the indicated medium and harvested at optical densities between 0.5 and 2.0. Total RNA was isolated by conventional glass bead/phenol-chloroform extraction. RNA, 10 μg, was run on a 1% agarose/formaldehyde-MOPS gel, blotted onto nylon membrane, and UV-crosslinked. The blots were probed in Rapid-hyb buffer (Amersham) for 40 min. Probe was made from the restriction fragments and PCR products mentioned above for FET3, *fio1*, and *fip1*. The FTR1 probe was made from a PCR product corresponding to the first 600 base pairs of the FTR1 open reading frame using the primers FTR1–2 and FTR1–3 (5′-cttttc-3′). A BstBI restriction fragment containing the *S. cerevisiae* actin gene was gel purified. A 32P-labeled probe was made from the DNA fragments using the Prime-It II labelling kit and purified using the NuclTrap Probe purification columns from Stratagene (La Jolla, CA). FET3, FTR1, *fio1*1, and *fip1*1 probes were hybridized at 65°C for 3 h with 1 × 106 counts/ml of probe. For actin probe, hybridization was done at 55°C for 3 h. The blots were washed 4 × 15 min in 2 × SSC, 0.1% SDS, and exposed to film for 1 (FET3, *fio1*1, *fip1*1, FTR1, actin with *S. cerevisiae* RNA) to 3 h (S. Pombe RNA with actin).

**RESULTS**

Iron-independent Expression of FET3 and FTR1 in *S. cerevisiae—*Studies indicate that high affinity iron transport in *S. cerevisiae* is regulated at the level of transcription of FET3 and FTR1 (8). If these two molecules alone are sufficient for iron transport, then their iron-independent expression should permit high affinity iron transport. To examine this possibility, FET3 was placed under the control of a galactose promoter and transformed into a fet3 disruption strain. Under low iron conditions, the presence of galactose allowed the expression of FET3 and restored iron transport (Fig. 1). Under high iron conditions, little iron transport was seen although Fet3p was expressed to levels approximately equal to that seen in cells expressing the genomic FET3 region.
induced in low iron medium (data not shown). Stearmann et al. (4) showed that Ftr1p and Fet3p must be synthesized simultaneously for high affinity iron transport activity. We confirmed this result by placing FTR1 under galactose control as well. The fet3 disruption strain was transformed with plasmids expressing FET3 and FTR1 under the control of the galactose promoter. When these cells were grown in galactose high iron medium, no significant increase in iron uptake was observed unless ascorbate was added to the assay to bypass the need for ferrireductase activity (Fig. 1). In the presence of ascorbate, cells grown in high iron medium expressing both FET3 and FTR1 displayed an increased rate of iron transport compared with cells expressing FET3 alone. When grown in low iron galactose containing medium, cells expressing both FET3 and FTR1 show even higher levels of ferrous iron transport. This transport activity probably reflects induction of both endogenous and galactose driven FTR1 transcription. The observation that iron-independent expression of FET3 and FTR1 induces high affinity iron transport activity demonstrates that the expression of these two proteins is sufficient for high affinity iron transport activity.

Characterization of S. pombe Ferrous Iron Transport—To determine the characteristics of iron transport in S. pombe, we measured iron accumulation in the S. pombe wild-type strain FY254. S. pombe and S. cerevisiae wild-type (F113) cells were grown overnight in YPD and inoculated into iron-free medium. At specified times, cells were harvested and ferrous transport was assayed (Fig. 2A). Incubation in low iron medium resulted in an increased rate of iron transport. The kinetics and magnitude of the induction of S. pombe iron transport were similar to that of S. cerevisiae.

To define the concentration dependence of S. pombe iron transport, FY254 and F113 cells were inoculated into low or high iron medium, grown for 9.5 h, and assayed for iron transport using different concentrations of iron (Fig. 2B). At low concentrations of iron, S. pombe displays a concentration dependence of iron transport that is similar to S. cerevisiae. The apparent Km for transport in S. pombe (0.2 μM) is nearly the same as the Km for S. cerevisiae (0.15 μM). At higher concentrations of iron, however, the rate of iron transport decreases in S. pombe, whereas it remains high in S. cerevisiae.

Identification of FET3 and FTR1 Homologues in S. pombe—Data base analysis revealed genes in S. pombe similar to FET3 and FTR1. The putative S. pombe homologue of FET3, fio1 (ferrous iron oxidase) shows 38% identity and 60% similarity on the amino acid level (Fig. 3A). The S. pombe Fio1p shows hydrophobic regions at the N terminus and near the C terminus, similar to those found within the Fet3p (5). As in Fet3p, Fio1p also has two multicopper oxidase motifs and possesses all canonical ligands necessary for copper binding (3, 12). Fio1p, like Fet3p, has 13 potential N-linked glycosylation sites, and 8 of these sites are present in the same location as sites within the Fet3p.

The putative FTR1 homologue is fip1 (ferriferous permease), which is 46% identical and 70% similar to Ftr1p on the amino acid level (Fig. 3B). The S. pombe Fip1p has hydrophobic regions similar to Ftr1p and contains the REGLE domain, which may be important for iron binding (4). Both fip1 and fio1 are located on chromosome I and are oriented next to each other separated by a common upstream region, suggesting that they may be controlled by a common promoter.

Northern Analysis of fio1 and fip1—Both S. cerevisiae genes, FET3 and FTR1, are regulated by iron deprivation; transcription is increased during conditions of iron limitation (3, 4, 8). Northern blot analysis on iron-deprived cells revealed that transcription of the S. pombe genes fio1 and fip1 was regulated by iron need (Fig. 4). Both fio1 and fip1 transcripts were induced when cells were grown in iron-limited conditions (BPS(0)). Alternatively, when cells were grown in iron-replete conditions, the level of fio1 and fip1 transcripts was reduced. Though the S. cerevisiae and S. pombe genes are similar, cross hybridization between FET3 and fio1 or FTR1 and fip1 was not detected under the hybridization conditions used in this study.

Iron Transport of a fio1 Disruption Strain—To determine whether fio1 played a role in high affinity iron transport, a fio1 disruption strain, 4051, was generated, and iron transport activity of this strain was assayed (Fig. 5). The cells were grown overnight in YPD, inoculated into iron-limited or iron-replete media, grown for 6 h, and assayed for iron transport. The fio1 disruption strain grown in low or high iron conditions showed no iron transport activity. Within the same experiment, wild-type (FY254) cells showed normal levels of transport. The inability of 4051 to transport low concentrations of iron indicates a direct role for the fio1 gene in high affinity iron transport. Examination of the concentration curve for wild-type S. pombe iron transport again suggests that there is a significant inhibition of the S. pombe high affinity transport system at high iron concentrations even with incubations as short as 10 min.
An Oxidase-Permease-based Iron Transport System in S. pombe

Expression of fio1+ and fip1+ in S. cerevisiae fet3 Disruption Strain—To further define the role of the S. pombe gene fio1+ in iron transport, this gene was cloned into a S. cerevisiae strain lacking FET3. Comparison between the amino acid sequence of FET3 and fio1+ was done using the amino acid sequence of FET3 and fip1+. Sequences in bold represent the REGLE domain. Underlined sequences represent potential N-linked glycosylation sites.

B. S. cerevisiae Ftr1p compared to S. pombe Fip1p

fio1+ and fip1+ in the S. cerevisiae fet3 Disruption Strain—To further define the role of the S. pombe gene fio1+ in iron transport, this gene was cloned into a S. cerevisiae strain lacking FET3. Comparison between the amino acid sequence of FET3 and fio1+ were done using the Bestfit program from the GCG sequence analysis package. Underlined sequences correspond to potential N-linked glycosylation sites. Sequences in bold correspond to multicopper oxidase motifs. Underlined sequences represent potential N-linked glycosylation sites.
over hours not minutes (1). The rapidity of the change in transport rate in *S. pombe* suggests a post-translational effect. The reduction in transport activity due to high iron is not seen in *S. cerevisiae* expressing the *S. pombe* genes (data not shown), suggesting that any regulatory effect is not intrinsic to the transporter proteins. Thus, *S. pombe* iron transport may be regulated by genes that are not present in *S. cerevisiae*.

The *S. pombe* genes *fio1* and *fip1* show amino acid similarity, analogous motifs, and hydrophobic regions corresponding to their *S. cerevisiae* counterparts. Both *fio1* and *fip1* gene transcripts are also induced when cells are grown in iron-depleted conditions. Disruption of *fio1* in *S. pombe* results in a loss of high affinity iron transport activity. Expression of both *fio1* and *fip1* in *S. cerevisiae* mediates high affinity iron transport. Together these results strongly support the conclusion that these genes constitute a high affinity ferrous iron transport system. Expression of the *S. pombe* gene *fio1* alone in an *S. cerevisiae* *fet3* disruption strain did not result in complementation of the *fet3* defect. Although *fio1* and *FET3* are homologous in function, Fio1p is not able to interact with Ftr1p and mediate iron transport.

The *FET3*-FTR1 based system of transmembrane iron transport in *S. cerevisiae* is complex. This system was unexpected as most other transition metal transport systems require only a transmembrane transporter. For example, in *S. cerevisiae*, transport of iron by the low affinity transport system (*FET4*) seems to be simpler, involving the activity of just one gene (2). We have suggested that the oxidase-permease complex is required for high affinity iron transport, rather than a simple transmembrane transporter, because the ferroxidase imbues selectivity on the transport system (5). High affinity iron transport results from a complex set of reactions requiring reduction of iron followed by its subsequent re-oxidation by the cell surface ferroxidase (Fet3p) and transport of Fe(III) by the transmembrane permease (*Ftr1p*) (3, 4, 5, 10, 14). Identification of an oxidase-permease transport system in the evolutionary distinct fission yeast *S. pombe* demonstrates that this complex system is not restricted solely to *S. cerevisiae* and may have a broad distribution in eukaryotes.

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