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AFT1: a mediator of iron regulated transcriptional control in *Saccharomyces cerevisiae*

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Using a scheme for selecting mutants of *Saccharomyces cerevisiae* with abnormalities of iron metabolism, we have identified a gene, *AFT1*, that mediates the control of iron uptake. *AFT1* encodes a 78 kDa protein with a highly basic amino terminal domain and a glutamine-rich C-terminal domain, reminiscent of transcriptional activators. The protein also contains an amino terminal and a C-terminal region with 10% His residues. A dominant mutant allele of this gene, termed *AFT1-1^{up}*, results in high levels of ferric reductase and ferrous iron uptake that are not repressed by exogenous iron. The increased iron uptake is associated with enhanced susceptibility to iron toxicity. These effects may be explained by the failure of iron to repress transcription of *FRE1, FRE2* and *FET3*. *FRE1* and *FRE2* encode plasma membrane ferric reductases, obligatory for ferric iron assimilation, and *FET3* encodes a copper-dependent membrane-associated oxidase required for ferrous iron uptake. Conversely, a strain with interruption of the *AFT1* gene manifests low ferric reductase and ferrous iron uptake and is susceptible to iron deprivation, because of deficient expression of *FRE1* and negligible expression of *FRE2* and *FET3*. Thus, *AFT1* functions to activate transcription of target genes in response to iron deprivation and thereby plays a central role in iron homeostasis.

Key words: ferric reductase/iron/transcription/transport

Introduction

Iron is required by virtually all organisms for essential functions, often involving oxidations and reductions. The capacity to participate in redox reactions probably underlies the toxicity of iron, which may interact with oxygen to generate free radicals able to damage vital cellular constituents. The uptake and metabolism of iron must therefore be highly regulated. In all organisms studied, the levels of iron uptake are tightly coupled to the levels of available iron and the needs of the cell for the metal. In complex eukaryotes, a large amount of work has focused on the controlled expression of genes involved in the uptake of iron from transferrin via the transferrin receptor, the storage and sequestration of iron in ferritin, and the utilization of iron in the production of heme. The critical target genes in these organisms are controlled post-transcriptionally, either by changes in the level of mRNA translation or the rate of mRNA degradation (reviewed in Klausner *et al.*, 1993).

Organisms as diverse as prokaryotes, simple eukaryotes and mammals all share a requirement for the regulation of iron metabolism. Bacteria and some fungi synthesize and secrete siderophores, small molecules capable of chelating and thereby solubilizing external ferric iron. Complexes of ferric iron bound to siderophores are then transported into the cell (Bagg and Neilands, 1987a). Siderophore-producing organisms regulate the rate of siderophore synthesis through the transcriptional control of iron-regulated genes. In *Escherichia coli*, this regulation is mediated by the Fur protein (Schaffer *et al.*, 1985), which acts as a simple repressor of transcription, binding to its target DNA sequences, referred to as the iron box, in the presence of the co-repressor, ferrous iron (Bagg and Neilands, 1987b). More recently, genetic studies of the fungus *Ustilago maydis* (Mei *et al.*, 1993) have led to the identification and characterization of the Urbs1 regulator, which represses transcription in the presence of iron, analogous to the bacterial Fur protein (Voisard *et al.*, 1993). Interestingly, the Urbs1 protein is quite similar to the transcription factor GATA-1, which functions as an activator of the transcription of erythroid specific genes in vertebrates (Tsai *et al.*, 1989).

Many eukaryotes, including budding and fission yeast, do not synthesize siderophores. Rather, a growing body of data points to a distinct strategy for iron uptake employed by these organisms. This strategy relies on the initial solubilization of ferric iron by an externally oriented ferric reductase followed by the transport of ferrous iron across the cell membrane via saturable, high affinity ferrous transporters. Ferric reductase activities have been described in bacteria (Lovley *et al.*, 1989), plants (Chaney *et al.*, 1972), budding (Lesuisse *et al.*, 1987) and fission yeasts (Roman *et al.*, 1993) and in animal cells (Raja *et al.*, 1992). In the gut of humans, a ferric reductase activity has been localized to the luminal membrane of enteroctyes responsible for absorption of iron into the body (Raja *et al.*, 1992). However, to date, genetic work on ferric reductases has been restricted to yeast (Dancis *et al.*, 1990, 1992; Roman *et al.*, 1993; Georgatsou and Alexandraki, 1994). In *Saccharomyces cerevisiae*, the expression of two unlinked genes, *FRE1* and *FRE2* is required for the external ferric reductase activity. The primary amino acid sequences of these genes suggests that they represent related members of plasma membrane flavocytochromes (Segal and Abo,1993). Expression of the ferric reductase genes is the rate limiting step in the assimilation of ferric iron, as shown by examination of interruption/deletion mutants. These mutants exhibit impaired growth on ferric iron-poor medium (Georgatsou

and Alexandraki, 1994) and low ferric iron uptake (Dancis *et al.*, 1992). Ferric reductase activity is regulated by iron availability, and this effect probably occurs at the level of transcription of *FRE1* and *FRE2*. In the case of the *FRE1* gene, the sequences of the 5′ flanking region were analyzed further and a segment which included a binding site for the general transcription factor RAP1 followed by a repeated element, TTTTTGCTCAYC, was identified which was capable of conferring iron-regulated expression to a reporter gene. Analysis of the data led to the conclusion that the iron regulation is probably mediated by an activator of transcription induced by iron deprivation (Dancis *et al.*, 1992).

Recently, it has been shown that copper uptake is required for high affinity ferrous transport into yeast cells (Dancis *et al.*, 1994a). Two genes, *CTR1* encoding a protein required for copper uptake, and *FET3*, encoding a copper-dependent oxidase, are both needed for iron assimilation (Askwith *et al.*, 1994). The mechanism by which the activity of the FET3 oxidase is coupled to ferrous transport is not clear, and the structural components of the ferrous transporter have not been identified at the molecular level. However, it has been shown that the ferrous transport step of iron assimilation is regulated by the availability of iron. This in part reflects the ability of iron to control the level of *FET3* transcription (Askwith *et al.*, 1994). In order to gain further insight into the regulation of this iron uptake system, we have utilized a genetic approach and identified an activator of ferrous transport, termed AFT1, which activates both the ferric reductases, *FET3* and ferrous transport in response to iron starvation through transcriptional control.

Results

Identification of an iron regulatory mutant

A selection scheme for mutants of *S.cerevisiae* with abnormalities of iron metabolism was designed using the properties of the *FRE1* promoter (Dancis *et al.*, 1994a). As previously demonstrated, the promoter region of the *FRE1* gene mediates a wide range of changes in transcription in response to changes in available iron (Dancis *et al.*, 1992), with iron excess resulting in repression and iron deprivation resulting in activation of transcription. The regulatory region of the *FRE1* gene was fused to the structural region for *HIS3*, an enzyme required for the biosynthesis of histidine. When this construct was expressed in a *his3*$^-$ strain, this strain exhibited iron-dependent auxotrophy for histidine, such that no growth occurred on iron-rich plates lacking histidine. As reported previously, 73 spontaneous mutants were isolated under these conditions (Dancis *et al.*, 1994a).

To determine whether these mutant phenotypes were dominant or recessive, each of the mutants was crossed with a wild type parental strain of the opposite mating type and the diploids were analyzed for histidine prototrophy on iron-rich plates. The mutants were also examined for high affinity ferrous iron uptake. Twelve of 73 independent mutants were classified as dominant, based on evaluation of the His$^+$ phenotype of the diploid. Ferrous uptake was measured in the 12 dominant mutants and three (M2, M26 and MA18) exhibited enhanced uptake. We considered that the histidine prototrophy in these strains might result

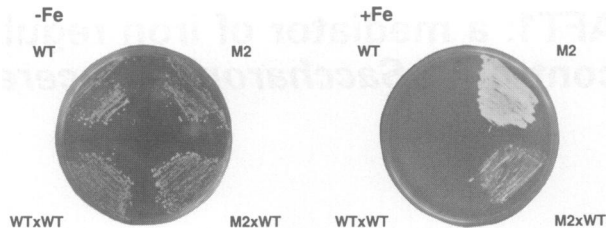


Fig. 1. Semidominance of the *AFT1-1*^{up} allele. Haploid strains 61 (WT) and the M2 (M2) and diploid strains Y4 (WT×WT) and Y17 (M2×WT) were evaluated for histidine auxotrophy on low iron (−Fe) or iron-rich (+Fe) plates. The solid medium consisted of modified defined medium with 50 mM MES buffer pH 6.0, copper sulfate 0.7 μM, iron sulfate 50 μM with 1 mM of the iron chelator ferrozine (−Fe) or without ferrozine (+Fe). The strains were transferred from a fresh YPD plate to a −Fe plate, grown for several days and then transferred to the test plates (−Fe, +Fe) and incubated for 4 days at 30°C prior to photographing.

from mutation of a regulatory protein affecting the ability of assimilated iron to repress the *FRE1* promoter, and so this group was selected for further study. All 12 tetrads obtained by crossing either the M2 or the M26 mutants with the MA18 mutant and then sporulating the resulting haploids showed prototrophy for histidine on iron plates (mutant phenotype), indicating that the mutations in M2, M26 and MA18 were probably allelic. The M2 mutant was chosen for further characterization. The features of the histidine prototrophy phenotype of the M2 mutant are shown in Figure 1. Four strains were evaluated for histidine prototrophy on iron limited (−Fe) and iron-rich (+Fe) plates: the haploid parental strain 61 (WT), the haploid mutant M2 (M2), the control diploid Y17 (WT×WT) and the diploid formed by crossing M2 with the related strain of the opposite mating type Y4 (M2×WT). As the mutant phenotype was dominant, the growth on the iron-rich plates was apparent for the M2 haploid and the Y4 (M2×WT) diploid. However, the mutant phenotype was more pronounced for the M2 haploid than the Y4 (M2×WT) diploid, indicating an effect of the wild type allele (i.e. semi-dominance of the mutant allele). The Y4 (M2×WT) diploid was sporulated. When the tetrads were evaluated, a $2^+:2^-$ segregation was observed, consistent with a single locus being responsible for the mutant phenotype. Ferrous uptake measured in the spore clones genetically co-segregated with the histidine prototrophy phenotype. Because this mutation resulted in high levels of ferrous iron transport, we referred to the responsible gene as *AFT1* for activator of ferrous transport.

Isolation of the AFT1 gene

Since the mutant phenotype of the M2 strain behaved in semi-dominant fashion, the gene responsible could not be cloned by complementation with a genomic library derived from a wild type strain. Instead, genomic DNA from the M2 mutant was isolated, and a library with an average insert size of 10 kb was created in the YCp50 shuttle vector. This library was used to transform a strain containing the indicator *FRE1−HIS3* fusion and ~10^5 transformants of this strain were evaluated for the mutant phenotype of histidine prototrophy on iron plates. A single transformant was identified that displayed the mutant phenotype and a plasmid with a 9.4 kb insert was rescued (Figure 2A). The responsible region of DNA within the cloned insert

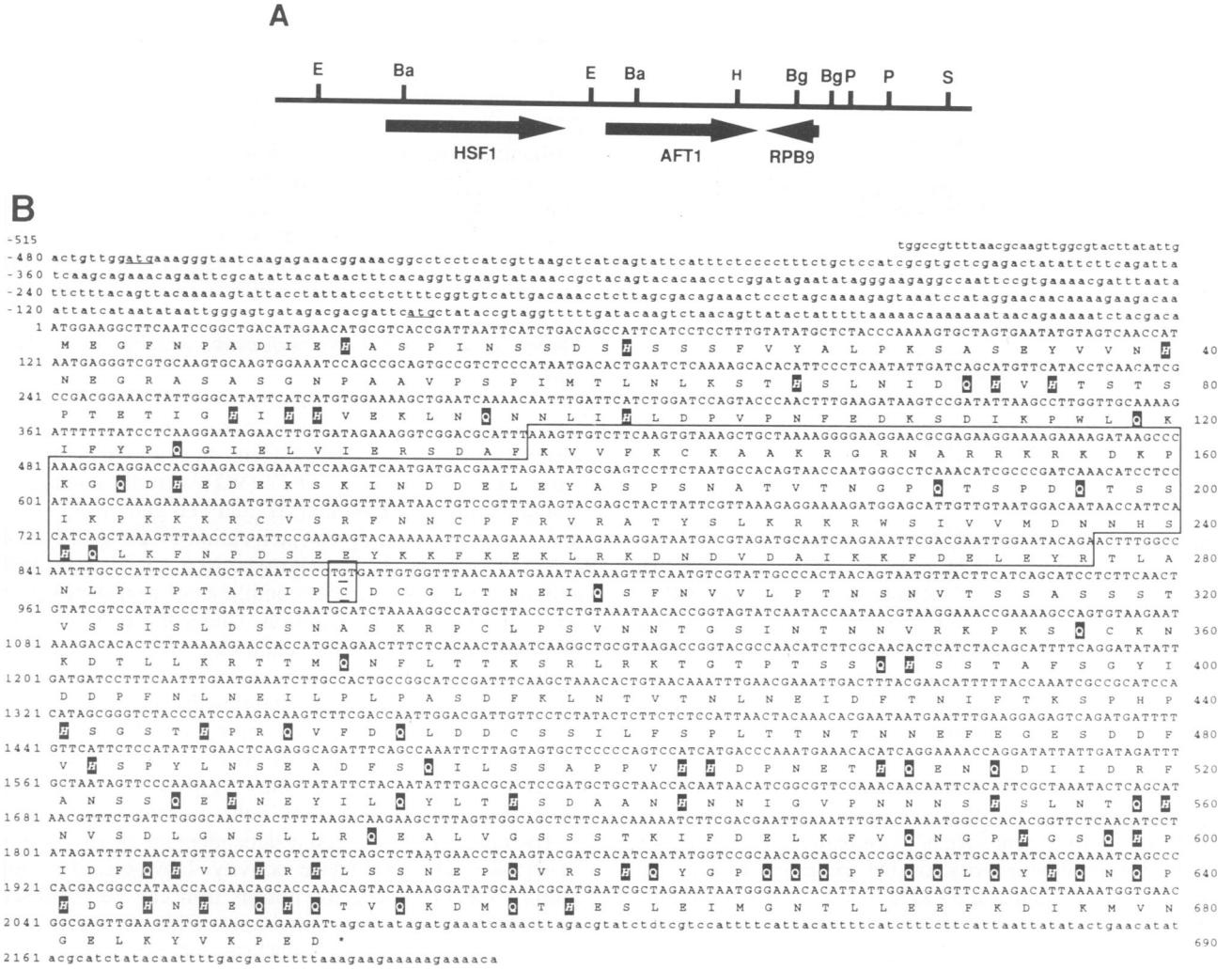


Fig. 2. Structure of the *AFT1* genomic locus and cDNA sequence. (**A**) Restriction enzyme map of the 9.4 kb mutant genomic insert from plasmid pT14. The map of a wild type genomic fragment was identical. The positions of the *AFT1* open reading frame and that of the adjacent genes (*HSF1* and *RPB9*) are indicated as arrows. The *Hin*dIII site shown as H was used to introduce a 4 bp insertional mutation. This *Hin*dIII site is not a unique site in this fragment. Ba, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I. (**B**) DNA sequence of the *AFT1* cDNA: a wild type cDNA library was screened and a complete cDNA clone was identified and sequenced. The untranslated regions are depicted in lower case and the ORF is depicted in upper case. The translated protein sequence is depicted by the single letter amino acid code. A single discrepancy between the wild type sequence and the mutant genomic sequence (G to T transversion) was found at position 872 as shown by underlining. The wild type sequence TGT encodes a cysteine at residue 291, and the mutant sequence TTT encodes a phenylalanine. The corresponding codon is boxed and the predicted amino acid is underlined. A basic residue cluster region is boxed, and histidine and glutamine residues are highlighted.

was determined by restriction fragment subcloning. A fragment of ~3 kb was found to confer the M2 phenotype and was subjected to sequencing. This revealed a 2070 base single open reading frame (ORF; Figure 2B). To establish that the ORF identified was responsible for the phenotype associated with expression of the gene in the tested cells, we introduced a frameshift mutation at codon 575 by a 4 bp insertion at a *Hin*dIII site in order to disrupt the reading frame. This abolished any phenotype of the introduced gene.

To identify the wild type *AFT1* gene, we used a fragment derived from within the ORF of the mutant genomic clone to probe both cDNA and wild type genomic libraries. A cDNA clone containing the entire predicted ORF and several wild type genomic clones were identified. The *AFT1* gene was fully sequenced from both cDNA and genomic clones and no discrepancies were noted. Furthermore these sequences completely matched the sequence of the ORF of the mutant genomic clone except for a single G to T missense mutation noted in the mutant sequence at base 892 (Figure 2B). The effect of this mutation would be to change the residue at position 291 from a cysteine to a phenylalanine. Transformation with the wild type genomic clone was incapable of conferring the mutant phenotype of histidine prototrophy on iron-rich plates, whereas transformation with the mutant clone which differed only by the single point mutation conferred the mutant phenotype. Therefore this point mutation was probably the cause of the mutant phenotype, and the allele was named *AFT1-1^{up}*.

Using the cDNA to probe for the *AFT1* transcript, a single mRNA species of ~2.8 kb was observed (Figure 3). This transcript was of sufficient length to encode the ORF predicted from the sequence of the genomic clones. The insert of the cDNA clone was of similar size and thus likely to represent a full length clone. The DNA

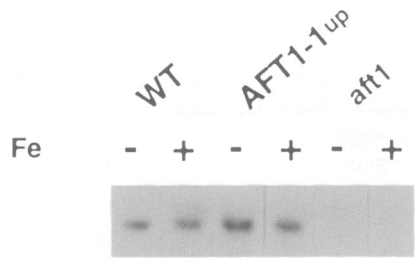


Fig. 3. Expression of *AFT1* mRNA. Total RNA was prepared from strains 61 (WT), M2 (*AFT1-1*^{up}) and Y18 (*aft1*) grown with (+) or without (−) added iron. The strains were grown to a high density in defined medium without iron or copper, and then diluted into modified defined medium and grown for an additional 4 h. This medium contained 50 mM MES pH 6.0, copper sulfate 40 μM, the iron chelator BPS 100 μM without (−) or with (+) 40 μM ferrous sulfate. Five μg of each RNA were analyzed by Northern blot hybridization. A 1.4 kb *Bam*HI−*Hin*dIII fragment from within the *AFT1* open reading frame was used as a probe.

sequence of this clone included a 515 nucleotide 5′ UTR, the entire ORF and a 134 base 3′ UTR preceding the poly(A) tail. The long 5′ UTR contained two small ORFs, suggesting the possibility that the expression of the AFT1 protein might be translationally regulated. The *AFT1* transcript was of moderate abundance, and its level was not affected by the iron status of the cell. Moreover, the M2 mutant expressed normal levels of full length *AFT1* transcript.

The 3′ portion of the ORF (372 of 690 codons) represented a sequence that was highly homologous to a recently reported gene, *RCS1*, originally cloned on the basis of immunoreactivity of the gene product to an antibody raised against a 33 kDa cell wall mannoprotein. This proved to be a fortuitous cross-reactivity. The authors of this study have named the gene *RCS1* because of the effects of mutation and disruption on cell size (Gil *et al.*, 1991). While there are many sequence differences between our ORF and the corresponding region of *RCS1*, we believe that these are due to polymorphisms and/or sequencing errors in the previous study. The genomic maps of *RCS1* and its surrounding DNA and our clone are identical and we believe that the reported *RCS1* gene is an incomplete fragment of the actual gene. Sequencing of the genomic insert on either side of the *AFT1* gene identified two known genes, locating the *AFT1* gene to chromosome VII. Located 5′ of *AFT1* is the gene encoding the heat shock transcription factor, *HSF1* (Wiederrecht *et al.*, 1988) while 3′ to *AFT1* is the gene encoding the RNA polymerase subunit *RPB9* (Woychik *et al.*, 1991; Figure 2A).

No other gene or protein sequence in the database revealed significant homology to *AFT1*. The reading frame predicts a protein with a molecular mass of 78 kDa. The protein is quite hydrophilic with no predicted membrane-spanning domains or hydrophobic leader sequence. There are, however, several remarkable details about the predicted protein sequence. The N-terminal region is quite basic with clusters of basic residues reminiscent of either nuclear localization signals or DNA binding proteins (or both). While the predicted pI of the entire protein is 6.88, the calculated pI of the region between residues 140 and 280 is 10.64. The C-terminal region is quite rich in glutamines which comprise >12% of the region between residues 500 and 690 and 25% of the region between residues 600 and 660. Glutamine-rich domains have been found in a number of transcriptional activators (Mitchell and Tjian, 1989). No previously defined metal binding or DNA binding motifs were identified. However, there is a distinctive distribution of histidines throughout the ORF and these might participate in metal binding. At the amino terminus, between residues 1 and 100, 10% of the amino acids are histidine. This drops to 1% over the next 300 amino acids. At the C terminus (residues 500–690), the histidine content rises again to 10.5%.

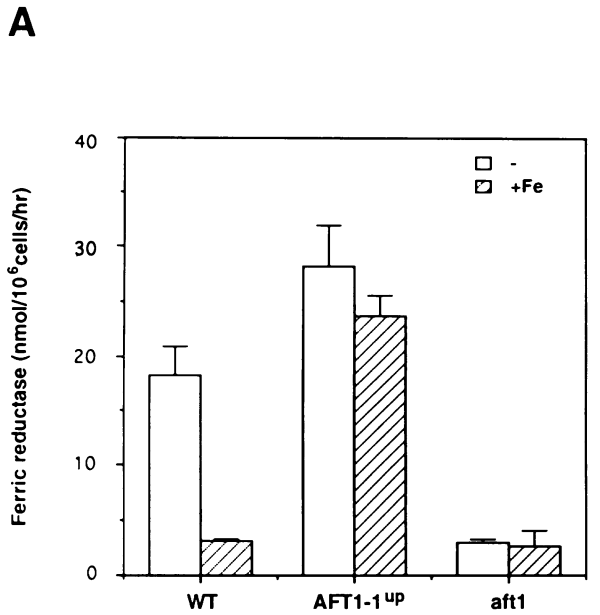
Effect of AFT1 on ferric reductase and ferrous iron uptake

In order to characterize the effects of AFT1 expression, we compared ferric reductase activity in haploid strains differing only at the *AFT1* locus that either contain a single copy of wild type *AFT1* (WT), a strain containing a single copy of the mutant M2 derived gene (*AFT1-1*^{up}) and a strain containing an interruption of the *AFT1* gene (*aft1*, strain Y18). In the wild type strain, ferric reductase activity was induced under conditions of iron deprivation and repressed by iron addition. Under the conditions of this experiment, the strain containing *AFT1-1*^{up} exhibited high reductase activity which was repressed to much less extent by addition of iron to the growth medium than seen in the WT strain. The partial repression observed likely reflects a leaky phenotype of *AFT1-1*^{up}. Finally, in the *aft1* strain little surface reductase activity was measured, regardless of the iron present in the medium (Figure 4A).

The uptake of ferric iron is dependent on the expression of surface ferric reductase activity. However, the high affinity specific uptake of ferrous iron can be regulated separately under some conditions (Eide *et al.*, 1992). Mutants affecting ferric reductase and not ferrous iron uptake (Dancis *et al.*, 1990) and vice versa (Askwith *et al.*, 1994) have been identified. The genetic co-segregation of the histidine prototrophy phenotype on iron-rich media with the elevated ferrous uptake phenotype in the meiotic products of the cross of the *AFT1-1*^{up} mutant with a parental strain suggested that both ferric reductase and ferrous uptake abnormalities would be affected by the *AFT1-1*^{up} mutant allele. This is shown in Figure 4B. Two additional points can be made here. First, not only was ferrous iron uptake enhanced in the *AFT1-1*^{up} strain, but the uptake was dysregulated. Growth in iron-rich medium failed appropriately to repress the level of ferrous iron uptake. Secondly, in the *aft1* strain there was little, if any, ferrous iron uptake. Thus, the expression of AFT1 was required for the expression of surface ferric reductase activity and ferrous iron transport, and the dominant mutant resulted in constitutively high reductase and ferrous transport.

Regulation of ferric reductase and FET3 gene expression by AFT1

FRE1 and *FRE2* encode proteins that are likely to be plasma membrane flavocytochromes responsible for the external ferric reductase activity of yeast. We therefore decided to test whether the effects of AFT1 on reductase activity could be mediated through effects on transcription of these genes. As shown in Figure 5, in the *AFT1*(WT) strain, transcript levels for *FRE1* and *FRE2* were repressed



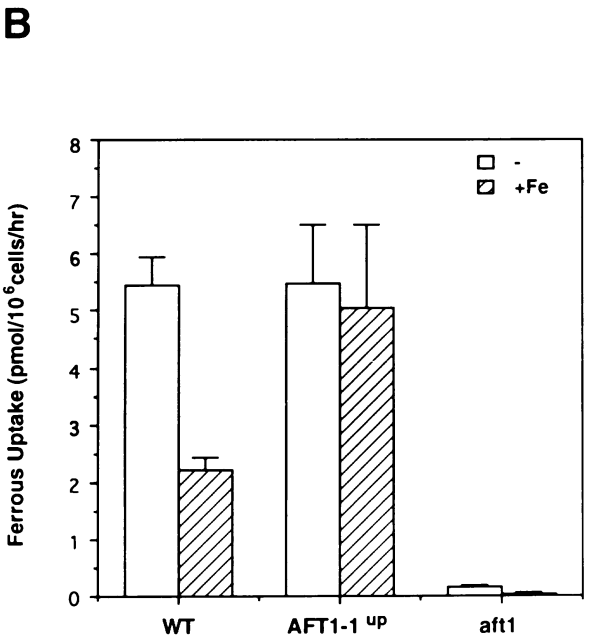


Fig. 4. Effect of AFT1 on ferric reductase activity and ferrous iron uptake. Strains 61 (WT), M2 (*AFT1-1*^{up}), and Y18 (*aft1*) were grown with (+Fe) or without (−Fe) iron, as for Figure 3, prior to assay of ferric reductase activity (**A**) or ferrous iron uptake (**B**). Standard deviation is indiated by error bars (n = 3).

by growth in iron-replete medium. In the *AFT1-1*^{up} strain, mRNA levels were higher and incompletely repressed by iron and in the *aft1* loss-of-function mutant there was little detectable message for these genes. Growth of the aft1 interruption mutant in iron replete or iron limited media had no effect on this result, although copper deprivation was capable of inducing *FRE1* expression (not shown) in this strain. Actin mRNA levels were not affected by the iron manipulations or by the different *AFT1* alleles (not shown). The results of these assays were qualitatively similar to the reductase assays (compare Figures 4A and 5), consistent with the hypothesis that the reductase

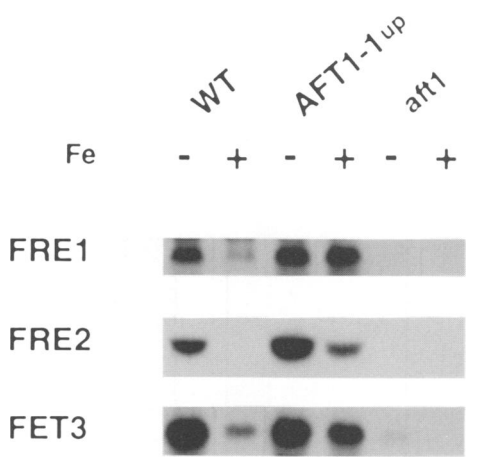


Fig. 5. Effect of AFT1 on *FRE1, FRE2* and *FET3* mRNA: strains 61 (WT), M2 (*AFT1-1*^{up}) and Y18 (*aft1*) were grown as in Figure 3 in iron depleted (−) or iron-rich (+) media prior isolation of total RNA. RNAs from each sample were analyzed by Northern blot, using probes for FET3 [2 kb *Hin*dIII−*Bam*HI fragment (Askwith *et al.*, 1994)], FRE1 [1.5 kb *Eco*RI−*Bst*EII fragment (Dancis *et al.*, 1992)], and FRE2 [0.5 kb fragment obtained by PCR amplification of nt 1–500 of the FRE2 sequence (Georgatsou and Alexandraki, 1994) from genomic DNA].

phenotypes resulted from effects of AFT1 on the transcription of *FRE1* and *FRE2*.

The *FET3* gene encodes a multi-copper oxidase that is required for ferrous iron uptake and its expression is iron regulated (Askwith *et al.*, 1994). We therefore asked whether the effects of AFT1 on ferrous uptake would be reflected in changes of *FET3* transcript levels. The pattern of regulation of *FET3* expression resembled the pattern of ferrous uptake regulation and was similar to the pattern of *FRE1* and *FRE2* expression: high transcript levels with incomplete iron repression were seen in the *AFT1-1*^{up} strain and negligible transcript levels were found in the aft1 strain.

To establish that AFT1 influences the expression of these target genes at the level of transcription, the 5′ flanking region of the *FET3* gene was fused to the reporter β-galactosidase gene and this construct was introduced into the strains carrying different *AFT1* alleles (Figure 6A). As expected, the *AFT1*(WT) strain displayed iron-regulated β-galactosidase activity. In the *Aft1-1*^{up} strain, enzyme activity was induced to high levels and was not repressed by iron, while in the *aft1* strain, β-galactosidase activity was low and could not be induced by iron deprivation. We also examined the β-galactosidase activity using a construct containing the β-galactosidase gene driven by heterologous promoter, *CYC1*, with the upstream iron responsive region of the *FRE1* gene (Dancis *et al.*, 1992) and confirmed that AFT1 acts via transcriptional regulation (Figure 6B).

Consequences of AFT1 mediated dysregulation of iron uptake

If AFT1 plays a central role in the homeostatic control of iron uptake, we predicted that abnormal AFT1 activity should make cells vulnerable to either iron starvation or iron toxicity. To evaluate this, we compared the growth of several strains as a function of iron levels in the medium

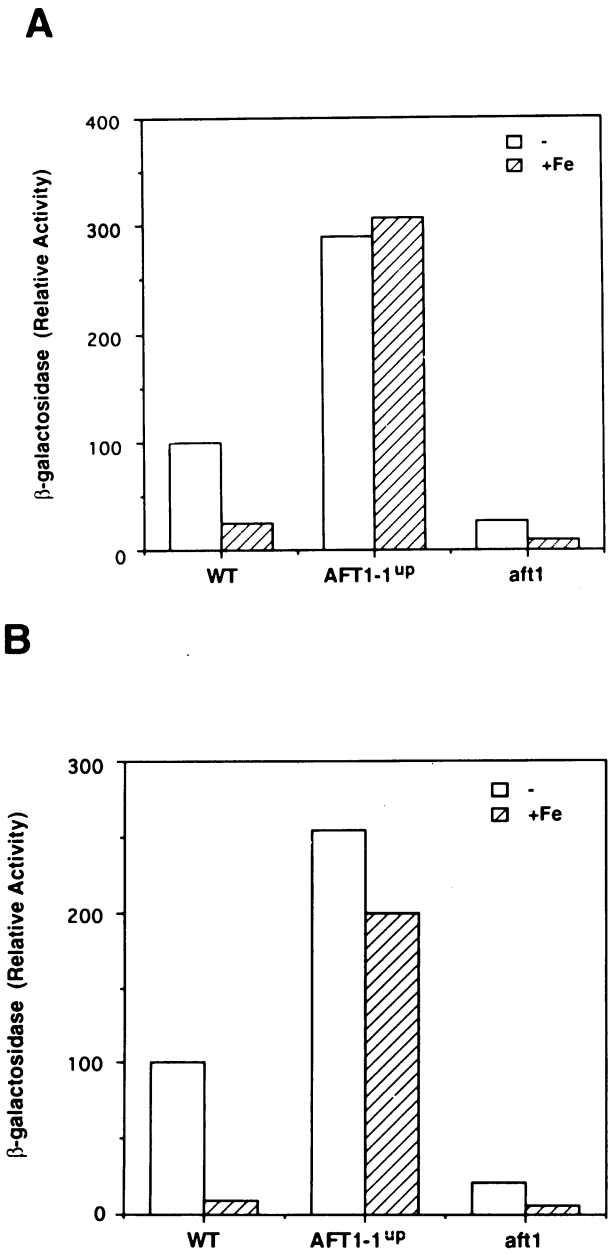


Fig. 6. Effect of AFT1 on the β-galactosidase activity of *FET3–lacZ* (**A**) and *FRE1–lacZ* (**B**). Strains 61 (WT), M2 (*AFT1-1*^{up}) and Y18 (*aft1*) were transformed with plasmid pT22 containing a fusion of the 5′ flanking region of *FET3* to a β-galactosidase reporter (A) or with plasmid pGC6-9 (Dancis *et al.*, 1992) containing the upstream region of *FRE1* with a β-galactosidase reporter driven by *CYC1* promoter (B). The transformants were grown on plates designed to maintain selection for the plasmid and to vary the available iron concentration. The plates consisted of agarose as the solid support and modified defined medium including MES buffer 50 mM pH 6.0, complete media supplements −uracil, copper sulfate 0.7 μM, and no added iron (−) or iron 10 μM (+). The transformants were incubated for 12 h on (−) or (+) iron plates prior to growth in liquid medium of the same composition for an additional 6 h. β-galactosidase activity measured on whole cells is shown for the culture with iron (+Fe) or without iron (−Fe).

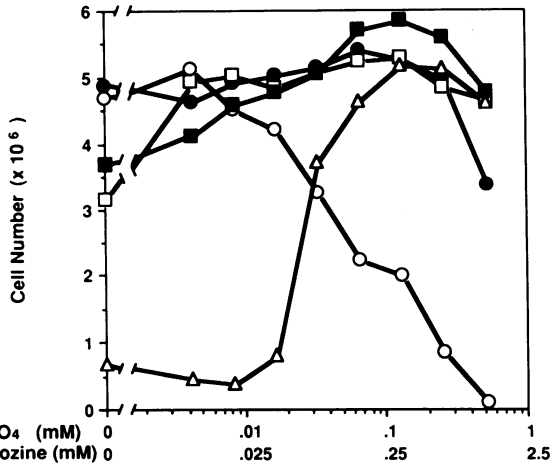


Fig. 7. Iron-dependent growth. Strains 61 (WT; ●), M2 (*AFT1-1*^{up}; ○), Y18 (*aft1*; △), Y19 (*fet3*; □), and Y20 (*AFT1-1*^{up}, *fet3*; ■) were inoculated into modified defined medium at a density at OD_{600} of 0.005. Various concentrations of iron were added as a complex of ferrous iron and ferrozine in order to keep the iron in solution. The strains were cultured in a microtiter plate dish for 14 h prior to measurement of the OD_{600}. The cell number was calculated from a calibration curve.

(Figure 7). To maintain iron in solution over a wide range of concentrations, a ferrozine–iron chelate was added at different concentrations to low iron medium (Stookey, 1970). In the medium without added iron, the wild type *AFT1* strain grew at the maximum rate. The rate of growth of the *fet3* mutant (Y19) was slightly retarded, despite the fact that the measured high affinity ferrous uptake was neglible in this strain (Askwith *et al.*, 1994). The ability of cells to grow under these conditions depends on the carryover of iron from the medium in which the strains were maintained prior to the start of the experiment. By contrast, when subjected to the same mild degree of iron deprivation, the *aft1* mutant failed to grow. For both of these mutants, the growth defect could be completely corrected by iron supplementation. Despite the comparable levels of measured ferrous uptake, the sensitivity to iron deprivation was more profound in the aft1 strain. This suggests the possible existence of additional AFT1 target genes affecting iron utilization or low affinity iron uptake.

While iron excess is known to have toxic effects on cells, in practice we have not previously been able to achieve such effects in wild-type strains of *S.cerevisiae*, even when high concentrations of iron were added to the growth medium. In the *AFT1-1*^{up} strain, however, growth was inhibited by high concentrations of iron in the medium. We considered that this effect was most likely due to bypass of the normal homeostatic mechanisms controlling cellular iron uptake and/or detoxification. Another explanation for the iron toxic phenotype was considered. Could the elevated and non-repressed ferric reductase activity be functioning to increase toxic free radical formation at the cell surface? To distinguish between these possibilities, a *fet3* interruption was introduced in the *AFT1-1*^{up} strain and the sensitivity to iron toxicity evaluated. The double *AFT1-1*^{up}, *fet3* mutant (Y20) still exhibited constitutive ferric reductase activity but high affinity ferrous uptake was absent (not shown). As can be seen in Figure 7, the iron toxicity attributed to the *AFT1-1*^{up} mutation was abrogated in this strain by the second site mutation in *FET3*. Therefore, the toxicity of iron for the *AFT1-1*^{up} strain probably relates to uncontrolled cellular iron uptake.

Discussion

In this paper, we report the identification of the *AFT1* gene of *S.cerevisiae*. *AFT1* encodes a mediator of iron-dependent gene expression in this organism and is the first such protein to be described. Effects of iron on gene expression in this organism have been shown for three genes: *FRE1* (Dancis *et al.*, 1992), *FRE2* (Georgatsou and Alexandraki, 1994) and *FET3* (Askwith *et al.*, 1994). The expression of each of these genes is induced by starvation for iron, and this control has been shown to occur through changes in gene transcription. Consistent with this type of regulation, the three iron-controlled genes are involved in the uptake of extracellular iron. FRE1 and FRE2 constitute the structural elements of the surface reductases that are required to solubilize extracellular ferric chelates in order to provide the ferrous iron that the cell actually assimilates. The FET3 protein is a membrane-associated copper-dependent oxidase that is required for high affinity ferrous uptake, and therefore acts downstream of the ferric reductases in the process of iron assimilation. In this study we analyzed the effects of the expression of a dominant mutant allele of *AFT1* and of interruption of the *AFT1* gene on expression of these iron-regulated genes of *S.cerevisiae*. The *AFT1-1*^{up} mutant was identified because of failure of the *FRE1* promoter to respond to high levels of exogenous iron. Likewise, in this mutant, the *FET3* promoter is constitutively active, and cannot be repressed by iron addition to the growth medium. These effects could be due to loss-of-function mutation of an iron-dependent transcriptional repressor or gain-of-function mutation of an iron-inhibited transcriptional activator. Examination of the phenotypes of the *aft1* interruption strain leads us to favor the latter interpretation. In the absence of a functional *AFT1* gene, the transcripts for *FRE1, FRE2* and *FET3* are not detected. The effects of AFT1 on its target genes are probably mediated through the promoters of these genes. This was shown by the study of chimeric constructs fusing the *FRE1* promoter to the *HIS3* reporter and the *FET3* promoter to the β-galactosidase reporter. An interpretation consistent with our results is that the AFT1 protein is functioning as an essential transcriptional activator for the target genes. Iron regulation is accomplished by iron inhibition of the activating function of AFT1.

Supporting the function of Aft1p as a transcription factor are the sequence similarities to transcriptional activators, including a basic region, perhaps involved in DNA recognition (Frankel and Kim, 1991), and glutamine-rich domains which have been shown to function in transciptional activation in a number of protein (Mitchell and Tjian, 1989). Unfortunately, Aft1p does not share enough similarity to any known transcriptional activator to allow us confidence in predicting more about its function. Alternatively, it may function as part of a signal transduction system that senses iron and perhaps other environmental stimuli and acts upstream of the target promoters.

Iron may interact directly with Aft1p and inhibit its ability to activate transcription. Two features of the amino acid sequence are of interest here. The histidine-rich domains, comprising up to 10% of residues in two discrete regions of the predicted protein, might coordinate iron (Arnold and Haymore, 1991). The Fur protein of bacteria (Schaffer *et al.*, 1985) and Urbs1 protein of *U.maydis* (Voisard *et al.*, 1993) both contain histidine-rich domains that are thought to interact with iron. These proteins function as repressors of siderophore biosynthesis in the presence of iron.

The point mutation present in the dominant *AFT1-1*^{up} mutant allele is intriguing because the residue that is altered is located in a Cys-X-Cys triplet that might be postulated to interact with iron. Cys-X-Cys motifs may ligate iron as part of an Fe−S cluster. A mechanism of gene regulation involving an Fe−S cluster has been well characterized in complex eukaryotes. The iron sensor protein IRP1 contains a reversibly assembled [4Fe−4S] cluster ligated to the protein via three cysteinyl residues (Kennedy *et al.*, 1992; Haile *et al.*, 1992). In the presence of iron, the assembled cluster stabilizes a protein conformation that prevents the protein from interacting with sequences in the target RNAs. In this way, the IRP1 exerts post-transcriptional control on the expression of genes involved in iron uptake, sequestration and utilization. Mutations of the Fe−S cluster ligating cysteine in human IRP1 result in a constitutively active RNA binding protein, no longer sensitive to iron levels (Phillpot *et al.*, 1994). An analogous mechanism for the AFT1-mediated iron-dependent transcriptional regulation can be proposed, according to which, in the presence of available iron, the Aft1p might bind iron or assemble an Fe−S cluster, which inhibits its ability to act as a transcriptional activator. In the absence of iron, the metal or the cluster is not present and the protein is able to function in activating transcription of the target genes. The Cys to Phe mutation resulting in the *Aft1-1*^{up} allele, by preventing the ligation of the metal or the Fe−S cluster, might mimic the effects of iron deprivation, accounting for the constitutive activation seen in this mutant.

The AFT1 iron regulator has numerous parallels with the recently reported MAC1 regulator of copper controlled gene transcription (Jungmann *et al.*, 1993). This gene also appears to encode a metal-inhibited transcriptional activator, in this case specific for copper. It exhibits sequence homology to *ACE1* and *AMT1*, genes coding for copper fist proteins that are transcriptional activators of metallothionein genes (Furst *et al.*, 1988; Zou and Thiele,1991). In addition to providing parallel examples of such metal inhibited activators, AFT1 and MAC1 share at least one target gene, *FRE1*. Thus, iron via AFT1 and copper via MAC1 regulate overlapping but non-identical sets of genes. It will be particularly interesting to identify the precise DNA targets of these two factors and to thereby explain the dual control of *FRE1*.

After iron has been taken up by a eukaryotic cell it must be incorporated into proteins that serve a wide array of functions, including oxygen transport, electron transfer and oxidation/reduction. Not much is known regarding the intracellular handling of iron, but it is likely that specific gene products are involved. For example, in order for heme synthesis to be completed, iron in the ferrous form must be delivered to the ferrochelatase active sites in the mitochondrial interior. The mechanism by which this process occurs and the proteins involved have not been defined. A protein such as AFT1 which mediates the homeostatic control of cellular iron uptake might also

Table I. Yeast strains

Strain name	Short genotype	Complete genotype
Haploid		
61[a]	*AFT1 FET3*	*MATα trp1-63 leu2-3,112 gcn4–101 his3-609 FRE1-HIS3::URA3*
66[b]	*AFT1 FET3*	*MATα trp1-63 leu2-3,112 gcn4-101 his3-609 FRE1-HIS3::LEU2*
81[a]	*AFT1 FET3*	*MATa ino1-13 leu2-3,112 gcn4-101 his3-609 FRE1-HIS3::URA3*
M2	*AFT1-1^{up} FET3*	*MATα trp1-63 leu2-3,112 gcn4-101 his3-609 FRE1-HIS3::URA3 AFT1-1^{up}*
Y18	*aft1 FET3*	*MATα trp1-63 leu2-3,112 gcn4-101 his3-609 FRE1-HIS3::URA3 aft1::TRP1*
Y19	*AFT1 fet3*	*MATα trp1-63 leu2-3,112 gcn4-101 his3-609 fet3::URA3*
Y20	*AFT1-1^{up} fet3*	*MATα trp1-63 leu2-3,112 gcn4-101 his3-609 AFT1-1^{up} fet3::URA3*
Diploid		
Y4 (M2×81)	*AFT1-1^{up}/AFT1*	*MATa/α leu2-3,112 gcn4-101 his3-609 FRE1-HIS3:URA3 AFT1-1^{up}/AFT1*
Y17 (61×81)	*AFT1/AFT1*	*MATa/α leu2-3,112 gcn4-101 his3-609 FRE1-HIS3:URA3 AFT1/AFT1*

[a]Dancis *et al.* (1994a); [b]Dancis *et al.* (1994b); all other strains are from this study

control intracellular iron transport. The targets of AFT1 regulation might include proteins involved in the poorly characterized processes of iron movement within the cell and iron delivery to enzymatic pools. An observation that supports this possibility is the comparison of isogenic mutant yeast strains with loss of function mutation of *FET3*, and loss of function mutation of *AFT1*. In the former case, high affinity ferrous uptake is completely and specifically abrogated, leading to a growth defect of this strain in media lacking iron. However, the aft1 mutant strain manifests an external iron requirement that exceeds the requirement of the *fet3* mutant. Perhaps the delivery of iron to intracellular locations for the incorporation into proteins is impaired in the aft1 mutant, and the high medium iron concentration overcomes this defect by recruiting a non-specific intracellular transport system. In any case, the possibility that the AFT1 regulator affects intracellular iron utilization makes the identification of additional targets of this gene of particular interest.

In the *AFT1-1*^{up} mutant on the other hand, the homeostatic control of iron uptake is bypassed, and iron uptake continues in excess of cellular needs. As a result, this mutant manifests toxic effects when grown in iron-rich medium. The study of this mutant should shed light on the mechanisms by which excess iron damages cells and the mechanisms activated to defend against this toxicity. In summary, the identification of a sensor/regulator of iron mediated transcriptional control should provide the opportunity to study the mechanisms whereby this eukaryote maintains the homeostasis of this essential but toxic element.

Materials and methods

Cloning of the AFT1-1^{up} and AFT1 genes

A library was constructed from genomic DNA prepared from the M2 strain. The DNA was digested with *Sau*3AI, and DNA fragments >5 kb were isolated by sucrose density gradient fractionation and ligated into the *Bam*HI site of the centromere-based YCp50 vector. Strain 66 was transformed with the M2 genomic library and transformants were initially selected for uracil prototrophy. The Ura$^+$ transformants were then transferred by velveteen replica to iron-rich plates without histidine and a colony appearing after 3 days was selected. Plasmid DNA (pT14) was rescued from the selected colony and analyzed further. A *Bam*HI–*Hin*dIII fragment from within the *AFT1* ORF was used to probe wild type, genomic (Rose *et al.*, 1987) and cDNA (Liu *et al.*, 1992) libraries according to standard colony hybridization procedures (Sambrook *et al.*, 1989).

Plasmid constructions

For disruption of *AFT1*, the plasmid pT20 was made as follows: the *Bam*HI–*Pst*I genomic fragment of pT14 was subcloned into the vector BluescriptII SK$^+$ (Stratagene). A 0.9 kb fragment containing the *TRP1* gene was obtained by digesting pRS414 with *Bsp*MII and *Ssp*I and inserted in to the unique *Hin*dIII site of *AFT1*.

For disruption of *FET3*, the plasmid pT21 was made as follows. A genomic fragment including the *FET3* gene and flanked by *Xba*I and *Hin*dIII from plasmid pDS8 (Askwith *et al.*, 1994) was subcloned into BluescriptII SK$^+$. The *URA3* gene flanked by *Bam*HI sites was inserted into the corresponding unique *Bam*HI site of *FET3* as described (Askwith *et al.*, 1994). The modified genomic fragment was linearized and used to transform yeast to uracil prototrophy.

For *FET3–lacZ* fusion, the plasmid pT22 was made by fusing 0.9 kb of the *FET3* 5′ flanking region to the coding region of the bacterial β-galactosidase gene in the high copy number vector YEGal4 (Dancis *et al.*, 1994b). The sequence of the fusion at the *Bam*HI site between the two genes was directly determined to be tgaccaacggaatgaacatGGATCCCGTC, where the *FET3* sequence is shown in lower case, the *lacZ* sequence is shown in upper case and the *Bam*HI site is underlined.

Yeast strains and growth conditions

The strains of *S.cerevisiae* used in this study are listed in Table I. Strain Y18 was created by transforming strain 61 with the *Bam*HI–*Pst*I fragment of pT20. The tryptophan prototrophs were selected and checked for the presence of the interruption of the *AFT1* locus by Southern blot. Strain Y19 was made by growing strain 61 on plates containing 5-fluoroorotic acid (FOA, Fluka) to select for loss of the *FRE1–HIS3* fusion gene which was integrated at the *URA3* locus. The uracil auxotrophic strain was then transformed with pT21. A similar method was used to create strain Y20. M2 was treated with FOA and transformed with the fragment from pT21. Interruption of the *FET3* gene in Y19 and Y20 was confirmed by Southern blot. The diploid Y4 was made by crossing M2 and 81, and the diploid Y17 was made by crossing 61 and 81.

The defined media were designed to study the effect of varying iron concentration. This medium consisted of 2% glucose, and MES buffer 50 mM pH 6.0. These components were treated with Chelex-100 resin to remove metal contamination. Yeast nitrogen base was added to the medium as described (Dancis *et al.*, 1994a) omitting iron and copper. Copper was added back in concentrations sufficient to allow maximal growth, and iron availability was varied. This was accomplished by the addition of various concentrations of iron or by the use of the iron (II) chelators ferrozine (Stookey, 1970) or bathophenanthrolene disulfonate (BPS; Bell *et al.*, 1991).

Strain construction, mating, sporulation and tetrad analysis followed standard protocols (Sherman *et al.*, 1989).

Assays

Radioactive iron uptake and ferric reductase assays were carried out essentially as described (Dancis *et al.*, 1990). Cells in log phase growth were harvested and resuspended in ice-cold assay buffer (5% glucose, 50 mM sodium citrate, pH 6.5) For ferrous uptake, cells were incubated with radioactive iron (^{55}Fe; 37–50 mCi/mg total iron; Amersham) in the presence of 1 mM ascorbate at 30°C, and filtered through a glass fiber filter, and the retained radioactivity was measured by liquid scintillation

counting. Ferric reductase activity was assayed with BPS and ferric chloride. Absorbance at 520 nm was measured after pelleting the cells.

β-galactosidase was assayed in transformants of plasmid pT22 by permeabilizing whole cells with chloroform−SDS and incubating with 0.4 mg/ml of *o*-nitrophenyl-β-galactopyranoside (ONPG) in 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol in 100 mM sodium phosphate buffer pH 7.0 at 30°C as described previously (Guarente, 1983). The reaction was terminated by adding sodium carbonate and the absorbance of the supernatant was measured at 420 nm. Activity was normalized to cell density (OD_{600}).

DNA procedures and RNA procedures

Plasmid DNA preparation, recombinant DNA methods, DNA sequencing, Southern blot analysis, and fragment amplification by PCR were performed by standard techniques (Sambrook *et al.*, 1989). Sequence analysis and homology searches were accomplished by using the GCG software package (Devereux *et al.*, 1984).

Total RNA from each strain was isolated by the hot phenol method (Kohrer and Domdey, 1991), and subjected to electrophoresis on a 1% agarose gel containing formaldehyde, transferred to nylon membranes, and hybridized with ^{32}P-labeled probe at 42°C in Hybrisol II (Oncor). Hybridized membranes were washed twice with 2× SSC and 0.1% SDS at 42°C, then twice with 0.2× SSC and 0.1% SDS at 65°C and exposed to X-ray film (Kodak).

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