MOLECULAR AND CELLULAR BIOLOGY, May 1994, p. 3065–3073
0270-7306/94/$04.00+0

Vol. 14, No. 5

Two Distinctly Regulated Genes Are Required for Ferric Reduction, the First Step of Iron Uptake in *Saccharomyces cerevisiae*

ELENA GEORGATSOU[1] AND DESPINA ALEXANDRAKI[1,2]*

Foundation for Research and Technology-HELLAS, Institute of Molecular Biology and Biotechnology,[1] and Department of Biology, University of Crete,[2] Heraklion 711 10, Crete, Greece

Received 1 October 1993/Returned for modification 29 November 1993/Accepted 3 February 1994

Iron uptake in *Saccharomyces cerevisiae* involves at least two steps: reduction of ferric to ferrous ions extracellularly and transport of the reduced ions through the plasma membrane. We have cloned and molecularly characterized *FRE2*, a gene which is shown to account, together with *FRE1*, for the total membrane-associated ferric reductase activity of the cell. Although not similar at the nucleotide level, the two genes encode proteins with significantly similar primary structures and very similar hydrophobicity profiles. The FRE1 and FRE2 proteins are functionally related, having comparable properties as ferric reductases. *FRE2* expression, like *FRE1* expression, is induced by iron deprivation, and at least part of this control takes place at the transcriptional level, since 156 nucleotides upstream of the initiator AUG conferred iron-dependent regulation when fused to a heterologous gene. However, the two gene products have distinct temporal regulation of their activities during cell growth.

Iron is an indispensable element for living organisms. Oxygen storage and transport in plants and animals (leghemoglobins, hemoglobin, myoglobin, and hemerythrin), respiration, photosynthesis and electron transport (cytochromes), and nucleic acid metabolism (ribonucleotide reductase) are some examples of biological functions carried out principally by iron-containing proteins.

Two features render iron an arduous metal to be handled by living organisms. The first is its availability; although it is the second most abundant metal in the earth's crust, iron is found primarily in the ferric (Fe^{3+}) state, forming hydroxides or salts of very low solubility and thus biologically inaccessible by simple mechanisms. The second is its toxicity; iron, in conjunction with oxygen, is a generator of hydroxyl radicals, which have a variety of toxic effects on cells.

Living organisms as diverse as prokaryotes and mammals have developed a variety of mechanisms to overcome the problem of iron bioavailability as well as to regulate the iron concentration in biological fluids and cell compartments, in order to ensure its proper function as opposed to its toxic effects (reviewed in references 7 and 16). Two principal mechanisms have been described for iron uptake. One involves the use of iron chelators, either small molecules such as the siderophores secreted by bacteria, fungi, and plants or polypeptides such as transferrin and lactoferrin which are found in biological fluids of higher eukaryotes. All of these molecules keep ferric ions in a soluble form and deliver them to the cells mainly by receptor-mediated endocytosis. The second mechanism for iron uptake involves an initial reduction step in the external vicinity of the plasma membrane followed by internalization of the ferrous ions by means of ion transporters.

Membrane-associated ferric reductase activity has been detected in various biological systems. This activity is proposed to be involved in respiration of some bacterial species (21), in clearance of toxic molecules in higher eukaryotes (14), or in iron uptake in animal cells (37). *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are the only systems in which the existence of such an activity has been definitely associated with iron uptake by both biochemical and genetic evidence (8, 9, 10, 20, 30). Yeast cells do not excrete siderophores (24), although they seem to be able to internalize them under certain conditions (19). Instead, they possess ferric reductase activity on the plasma membrane which reduces the extracellular Fe^{3+} to Fe^{2+} (6, 8, 10, 20, 39). The transmembrane reductase apparently uses NADH as the intracellular electron donor (40). Following the reduction of iron, a transmembrane ferrous transporter system delivers the ferrous ions inside the cell. The two systems, the reductase and the transporter, have been shown to be distinctly but coordinately regulated by the extracellular iron concentration (8–10).

Recently, a yeast gene, *FRE1*, encoding a ferric reductase has been cloned and molecularly characterized (9). However, disruption of the *FRE1* gene in the genome did not completely abolish the ferric reductase activity of the cell, suggesting the existence of some additional gene(s) with similar function. In this report, we present the identification and molecular characterization of *FRE2*, a gene whose product, together with FRE1, accounts for the total plasma membrane associated ferric reductase activity in *S. cerevisiae*.

MATERIALS AND METHODS

Strains and growth media. All *S. cerevisiae* strains used were derivatives of S288C and had the following genotypes: *MAT***a** *ura3-52 leu2-1* and *MAT***a** *gcn4*:Δ:*URA3 ura3-52 leu2-1* (13). Yeast cells were grown in YPD medium (3) containing yeast extract, peptone, and glucose, minimal medium containing yeast nitrogen base and glucose (3), and iron-deficient minimum defined (MD) medium prepared as described by Dancis et al. (8) except that Chelex 100 was omitted and the basal medium Eagle vitamin solution was substituted with biotin (1

* Corresponding author. Mailing address: Foundation for Research and Technology-HELLAS, Institute of Molecular Biology and Biotechnology, P.O. Box 1527, Heraklion 711 10, Crete, Greece. Phone: (30) (81) 210 091. Fax: (30) (81) 230 469. Electronic mail address: alexandraki@nefeli.cc.uch.gr.

mg/liter), D-Ca pantothenate (1 mg/liter), myoinositol (2 mg/liter), nicotinamide (1 mg/liter), pyridoxal HCl (1 mg/liter), thiamine HCl (1 mg/liter), and riboflavin (100 μg/liter). A wild-type strain did not grow at all following a second dilution in MD medium (this medium did not provide enough external iron to the cells when the intracellular pools had been exhausted). For growth on solid medium, 2% agarose was added to the MD medium. Iron-containing media were rendered 2 mM with respect to $FeCl_3$ (Merck). For histidine limitation, the MD medium was supplemented with 5 mM 3-amino 1,2,4-triazole (3-AT). The bacterial strain used for plasmid propagation was *Escherichia coli* DH5α [*supE44* Δ*lac*U169 (ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] grown in LB medium supplemented with ampicillin (10 μg/ml) as required (3).

Genes and plasmids. The *FRE1* gene was isolated from a YCP50 yeast genomic library by using a synthetic oligonucleotide as the probe (Applied Biosystems oligonucleotide synthesizer; Department of Microchemistry, Heraklion, Institute of Molecular Biology and Biotechnology, Crete, Greece). The sequence of the oligonucleotide was 5′-GTGGAACCGTTA CACCATATGGGCCTTC-3′. The *FRE/GCN4* fusion plasmids were constructed starting from the low-copy-number, *URA3*-containing YCP50 vector carrying a *Bam*HI-*Sal*I fragment which contained the entire coding region of the *GCN4* gene except for the first 52 codons (36) (plasmid BS150). *FRE2* (−156 to +3) and *FRE1* (−374 to +3) promoter fragments were subcloned by the PCR method (3), using specific primers containing the *Bam*HI recognition site at their 5′ ends. Gene fusion took place between their AUG and codon 53 of *GCN4* to produce plasmids *FRE2/GCN4* and *FRE1/GCN4*. C101-1 is a single-copy plasmid carrying the entire *GCN4* gene (28).

Gene disruptions in the yeast genome. (i) *FRE2* disruption. A 850-bp *Xho*I fragment (codons 300 to 583) (2) was replaced by the 3.8-kb *Bam*HI-*Bgl*II fragment of pNKY51 (1) on a *FRE2* (−180 to +2850)/pUC18 recombinant plasmid. The 3.8-kb fragment contains the *S. cerevisiae URA3* gene flanked by two copies of the *Salmonella hisG* gene (*hisG-URA3-hisG*). The resulting insertion fragment in pUC18 was excised and used to transform a *ura3-52 leu2-1* strain. Uracil prototrophy was used for the selection of recombinant clones, which were subsequently shifted to uracil auxotrophy by selection on plates containing 5-fluoro-orotic acid (4). At this step, the *URA3* gene and one copy of the *hisG* gene were deleted from the genome, leaving only one copy of the 1.15-kb *hisG* fragment disrupting the *FRE2* gene (*fre2*Δ::*hisG*).

(ii) *FRE1* disruption. A 720-bp *Pst*I *FRE1* fragment (codons 169 to 409) (9) was replaced by the 1.1-kb *Hin*dIII fragment of the *S. cerevisiae URA3* gene on a *FRE1* (nucleotides −680 to +2280)/pUC18 recombinant plasmid. The resulted insertion fragment was excised from pUC18 and used to transform a *ura3-52 leu2-1* strain or a *fre2*Δ::*hisG ura3-52 leu2-1* strain. Uracil prototrophy was used for the selection of transformants.

DNA blot hybridization analysis. Yeast DNA was prepared as described by Philippsen et al. (29), digested with *Eco*RI and *Bcl*I, electrophoresed on 1% agarose gels, and transferred to GeneScreen membranes (NEN) (3). Membranes were hybridized sequentially with two different α-^{32}P-labeled probes: a 1,080-bp *Eco*RI-*Xho*I *FRE2* fragment and a 440-bp *Eco*RI-*Xho*I *FRE1* fragment (2, 9).

RNA blot hybridization analysis. Total RNA was isolated from cultures first grown to saturation in YPD medium and subsequently diluted and grown exponentially in the appropriate MD medium. Polyadenylated RNA samples were purified by oligo(dT)-cellulose (17), electrophoretically separated on formaldehyde-containing 1.2% agarose gels, and transferred to GeneScreen membranes (3). Three membrane filters containing identical samples were each hybridized with one of the following α-^{32}P-labeled DNAs: the 1.7-kb *Bam*HI fragment containing the *HIS3* and part of the adjacent *DED1* genes (35) and the *FRE1*/pUC18 and the *FRE2*/pUC18 recombinant plasmids described above.

Ferric reductase assays. Cells were grown to saturation in minimal medium. They were subsequently washed in MD medium and resuspended at a concentration of 4×10^6 to 5×10^6 cells per ml in MD medium or in iron-containing MD medium. When only one time point was taken, it was done after 8 to 10 h of growth. Cells were collected by centrifugation and resuspended in 1 ml of assay buffer consisting of 0.05 M sodium citrate (pH 6.5) with 5% glucose at a density of approximately 10^8 cells per ml. $FeCl_3$ and bathophenanthrolene disulfonate (BPS) were added to a final concentration of 1 mM (8). Following a 5- to 40-min incubation at 30°C, cells were removed by centrifugation (10 min, 12,000 × *g*), and the optical density at 520 nm (OD_{520}) of the supernatant was measured. The amount of ferrous ions produced was estimated by means of a calibration curve constructed from solutions of known ferrous ion concentrations. Parallel incubations of samples without cells were used to estimate the background values. Ferric reductase activity was expressed in nanomoles of Fe^{2+} produced under the assay conditions.

DNA and protein sequence analysis. The nucleotide sequence obtained during the course of the European Community project on yeast chromosome XI sequencing was analyzed by the DNA Strider software (22) to find potential open reading frames (ORFs) (2). The identified ORFs were compared against the GenBank, EMBL, SWISS-Prot, and NBRF data bases by using FastA (27) with the standard parameters of the Genetics Computer Group package software. Multiple alignment of similar protein sequences was performed by the CLUSTAL program (11). Hydrophobicity profiles of the protein sequences (18) were obtained by the DNA Strider software.

RESULTS

Identification of *FRE2*, a gene encoding a putative product significantly similar to the ferric reductase FRE1. Sequencing analysis of a 32.1-kb DNA fragment of yeast chromosome XI obtained during the course of the European Community project on yeast chromosome XI sequencing revealed an ORF of 711 codons (2) (EMBL accession number X75950) whose putative protein product exhibited significant similarity (24.5% identity in 693 overlapping amino acids) to the *S. cerevisiae* ferric reductase gene product, FRE1 (9).

The following structural features of the putative protein, named FRE2, indicated that *FRE2* encodes a protein related to ferric reductases. Amino acid sequence alignment revealed conservation of specific residues throughout the length of the FRE1 and FRE2 proteins. Moreover, multiple alignment of FRE1 and FRE2 amino acid sequences and the predicted amino acid sequence of the newly identified *frp1*$^+$ gene (30), which is required for iron uptake in *Schizosaccharomyces pombe*, showed conservation of most of the same amino acids (Fig. 1). Additionally, all three mentioned proteins exhibited significant similarities to the sequence of cytochrome b_{558} (the X-CGD protein), a critical component of the human phagocyte oxidoreductase (26). More specifically, they all contain at their carboxy-terminal regions sequence patterns that match the consensus of the NADPH oxidoreductase protein family for flavin adenine dinucleotide and NADPH binding (Fig. 1). These conserved regions have been shown by crystallographic

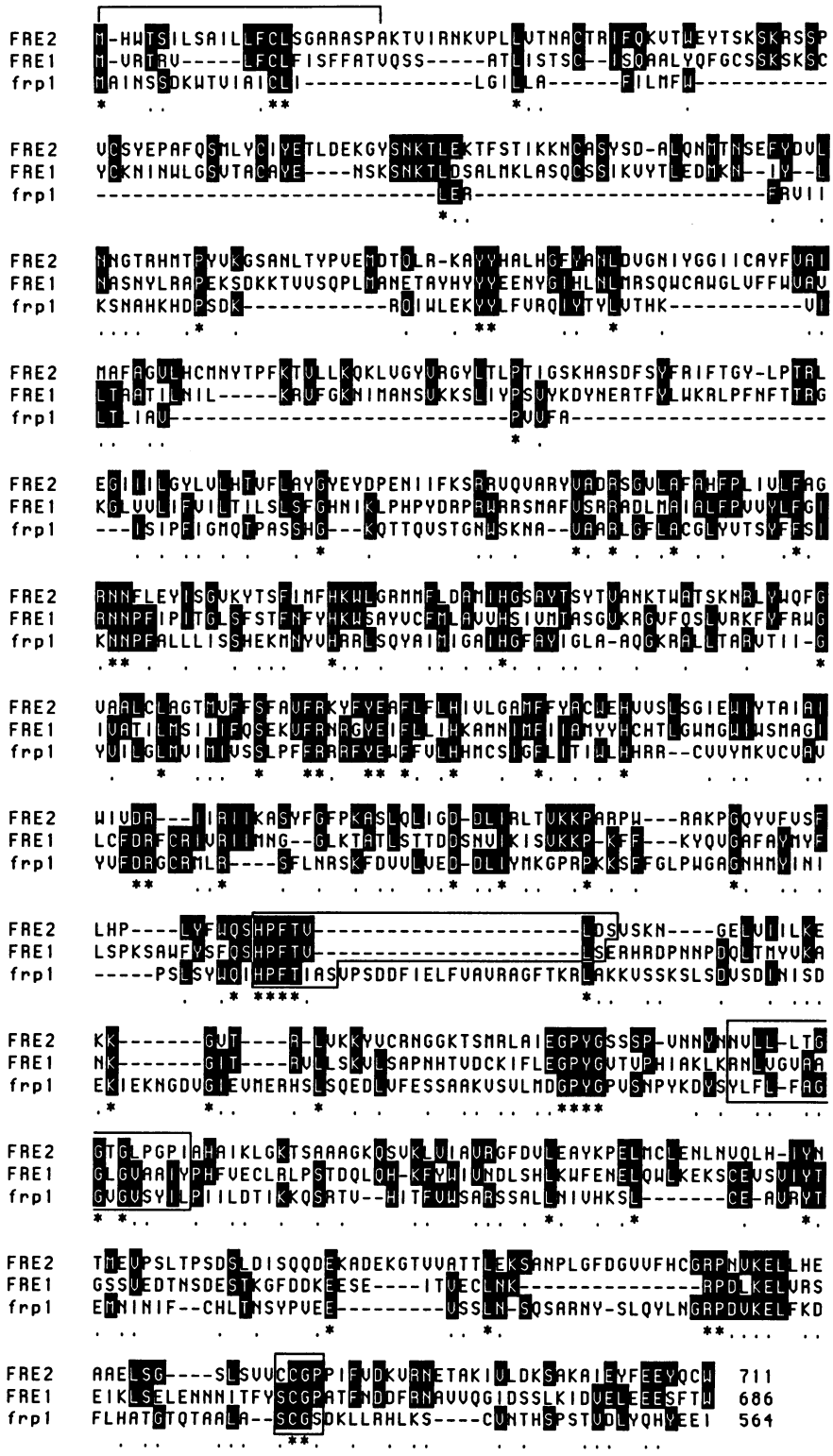

FIG. 1. Multiple sequence alignment of the FRE2, FRE1, and frp1 amino acid sequences, using the CLUSTAL program. Asterisks indicate residue identities in all three sequences, and dots indicate conserved substitutions. The blackened regions indicate residues identical between two of the three and in all three compared sequences. The potential leader peptide sequence of FRE2 is bracketed. The boxed areas include the sequence motifs which are significantly similar to those of NADPH ferredoxin reductase defined for binding of flavin adenine dinucleotide-isoalloxazine (HPFTXXS), NAD/P-ribose (glycine-rich motif), and NAD/P-adenine (cysteine-glycine motif) (15, 33, 34). The number given at the end of each sequence corresponds to the carboxy-terminal amino acid.

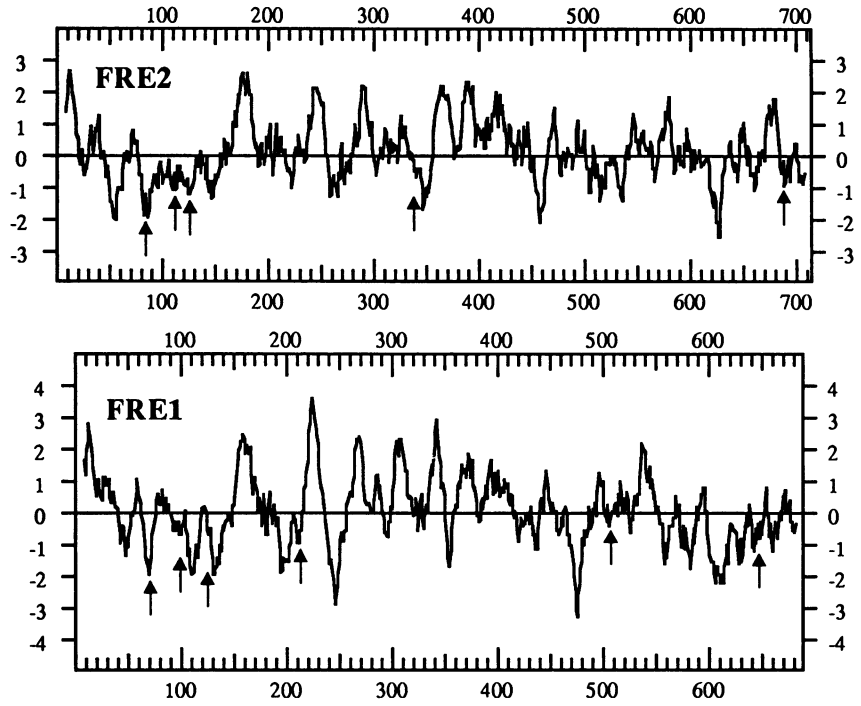

FIG. 2. Hydrophobicity profiles of the FRE2 and FRE1 proteins, determined by the method of Kyte and Doolittle (18). Positions of potential sites for N-linked sugars are indicated by arrows.

methods to be involved in the electron transport functions of this protein family (15, 34).

The FRE2 sequence also bears characteristics of membrane proteins. Its 23 amino-terminal residues conform to the von Heijne consensus for the leader peptide of membrane or secreted proteins (38). The hydrophobicity profile of the FRE2 ORF revealed a potential leader peptide followed by a hydrophilic amino-terminal region and at least seven hydrophobic regions, strong candidates for transmembrane domains. All of these features correlate well with the hydrophobicity profile of the FRE1 protein (Fig. 2). Finally, the FRE2 amino acid sequence contains six potential sites for the addition of N-linked sugars in positions analogous to those found in the FRE1 protein (Fig. 2). This structural evidence coupled with the fact that *FRE1* gene does not account for the total reductase activity of *S. cerevisiae* (9) led us to examine the putative ferric reductase function of the *FRE2* gene product.

FRE2 conforms to its predicted function as a ferric reductase. To analyze the role of the *FRE2* gene, we first constructed a haploid strain in which the *FRE2* genomic sequence was deleted and replaced by the *Salmonella hisG* gene as described in Materials and Methods. DNA blot hybridization analysis confirmed the replacement of the deleted 0.85-kb fragment of *FRE2* (deleting codons 300 to 583) with the 1.15-kb *hisG* gene-containing fragment (Fig. 3 and 4). The disrupted gene was found to be unique in the yeast genome, as seen in the

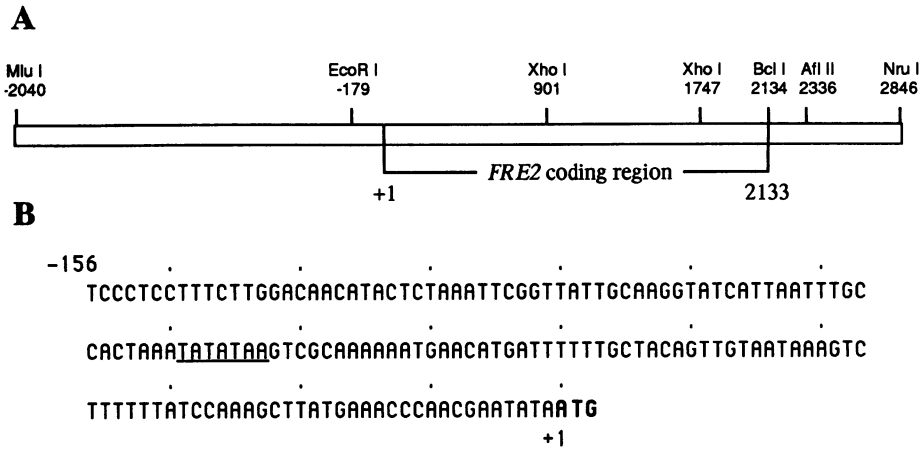

FIG. 3. (A) Restriction map for selected enzymes of the DNA region including the *FRE2* gene. (B) Nucleotide sequence of a region upstream of the initiator AUG (in boldface) of the *FRE2* gene. A potential TATA box is underlined.

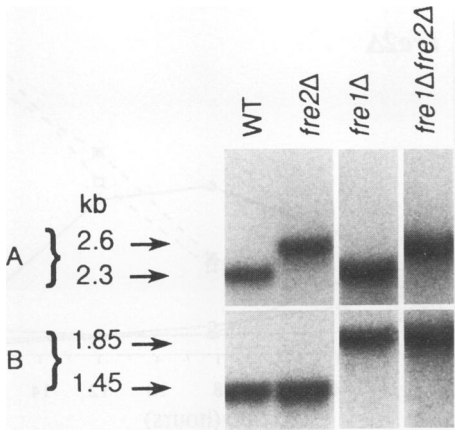

FIG. 4. DNA blot hybridization analysis of *FRE2* and *FRE1* gene-disrupted strains. Samples of genomic DNA isolated from haploid *MAT***a** *ura3-52 leu2-1* strains containing a disruption or deletion of *FRE1* (*fre1*Δ), *FRE2* (*fre2*Δ), or both (*fre1*Δ *fre2*Δ) as well as from the parental wild-type (WT) strain were analyzed as described in Materials and Methods. (A) *FRE2*-specific probe; (B) *FRE1*-specific probe.

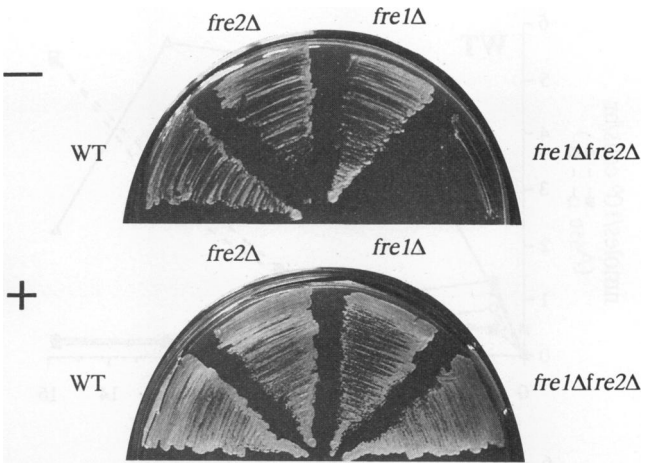

FIG. 5. Growth of the indicated wild-type (WT) and mutant *fre1*Δ, *fre2*Δ, and *fre1*Δ *fre2*Δ strains (derivatives of the *MAT***a** *ura3-52 leu2-1* strain) on MD agar plates (−) and on MD agar plates supplemented with 2 mM $FeCl_3$ (+).

same analysis. Growth of the disrupted haploid strain in minimal medium showed that the *FRE2* gene is not essential for cell viability.

Yeast ferric reductase activity has been shown to be regulated by the concentration of iron in the growth medium. Therefore, we assayed the ferric reductase activity in both wild-type and *fre2*Δ strains grown in either iron-deficient or iron-containing liquid medium. The wild-type strain grown in iron-deficient medium showed consistently a 10- to 30-fold increase of ferric reductase activity. The variable values were reproducible and dependent on whether the assayed cells were at the early or late exponential phase of growth. The activity of the *fre2*Δ strain in the same medium was found to be reproducibly lower, although to a highly variable extent (between 30 and 80% of the activity detected in the wild-type cells). The variability was also dependent on the growth phase of the tested cultures. When a high-copy-number plasmid containing the *FRE2* gene (nucleotides −2040 to +2336) was introduced into the wild-type or the *fre2*Δ cells, ferric reductase activity in iron-starved cells was three- to fourfold higher than in the nontransformed strains (data not shown).

These results indicated that the *FRE2* gene product has a function affecting the ferric reductase activity of the cell quantitatively and agreed with the findings of Dancis et al. (9), who demonstrated the persistence of an iron-regulated ferric reductase activity in *FRE1* gene-disrupted strains. To examine whether this residual activity was entirely attributable to the *FRE2* gene, we constructed a strain in which both *FRE1* and *FRE2* genes were deleted. For this purpose, we isolated the *FRE1* gene from a yeast genomic library (36), using an oligonucleotide probe designed according to the published sequence (9). Gene disruption and deletion of the *FRE1* gene in both wild-type and *fre2*Δ strains were performed as described in Materials and Methods. DNA blot hybridization analysis confirmed the 400-bp size increase of a *FRE1*-hybridizing DNA band (Fig. 4). The doubly disrupted-deleted strain exhibited a very low level of ferric reductase activity, 2 to 10% of the wild-type activity, which remained constant, independent of the presence of iron in the growth medium. This activity is consistent with that detected by Lesuisse et al. (20), which was excreted from the cells following incubation in the presence of glucose for a short period of time, and it was not regulated by the iron content of the growth medium. The ferric reductase activity in a *fre1*Δ strain was also reduced to a variable extent (20 to 70% of the wild-type activity), similarly to the *fre2*Δ strain, but not to the low levels reported by Dancis et al. (9).

When we tested the ability of the disrupted strains to grow in iron-depleted solid medium, we found that the reduced activity of ferric reductase in either the *fre1*Δ or *fre2*Δ strain was not sufficient to cause a strong growth phenotype of the colonies on agar MD plates. In contrast, the doubly disrupted-deleted strain (*fre1 fre2*Δ) was practically unable to grow on MD plates (Fig. 5).

Temporal analysis of FRE2 activity. To avoid the variability appearing in our described measurements and to examine more accurately the unique contribution of the FRE2 protein to iron reduction, we measured the ferric reductase activities in cultures of the wild-type, *fre1*Δ, *fre2*Δ, and *fre1 fre2*Δ strains in correlation with the phase of growth in both iron-depleted and iron-rich media. Cells were first grown to saturation in minimal medium. Under these conditions, their reductase activity was practically undetectable. Cells were subsequently washed and resuspended in MD or MD iron-supplemented medium at concentrations of 4×10^6 to 5×10^6 cells per ml, and activity was assayed at specific time points.

All four strains were capable of several rounds of divisions until their internal iron supplies were exhausted in MD medium. The cells were assayed as described in Materials and Methods. To distinguish between the activity of the transmembrane and the excreted reductases (20), we performed the experiment by including a step of preincubation of the cells in reductase assay buffer for 45 min and then tested the activities of the supernatant fractions and of the pelleted cells separately. The obtained ferric reductase activities and the growth profiles from a representative experiment are shown in Fig. 6. All four cultures exhibited the same profile of reductase activity in the supernatant fraction, independent of the presence of the *FRE1* and *FRE2* genes in the cells. This excreted activity increased during very early growth, in less than 1 h following passage of the cells in fresh medium, and dropped during further growth of the cells; it was not regulated by the

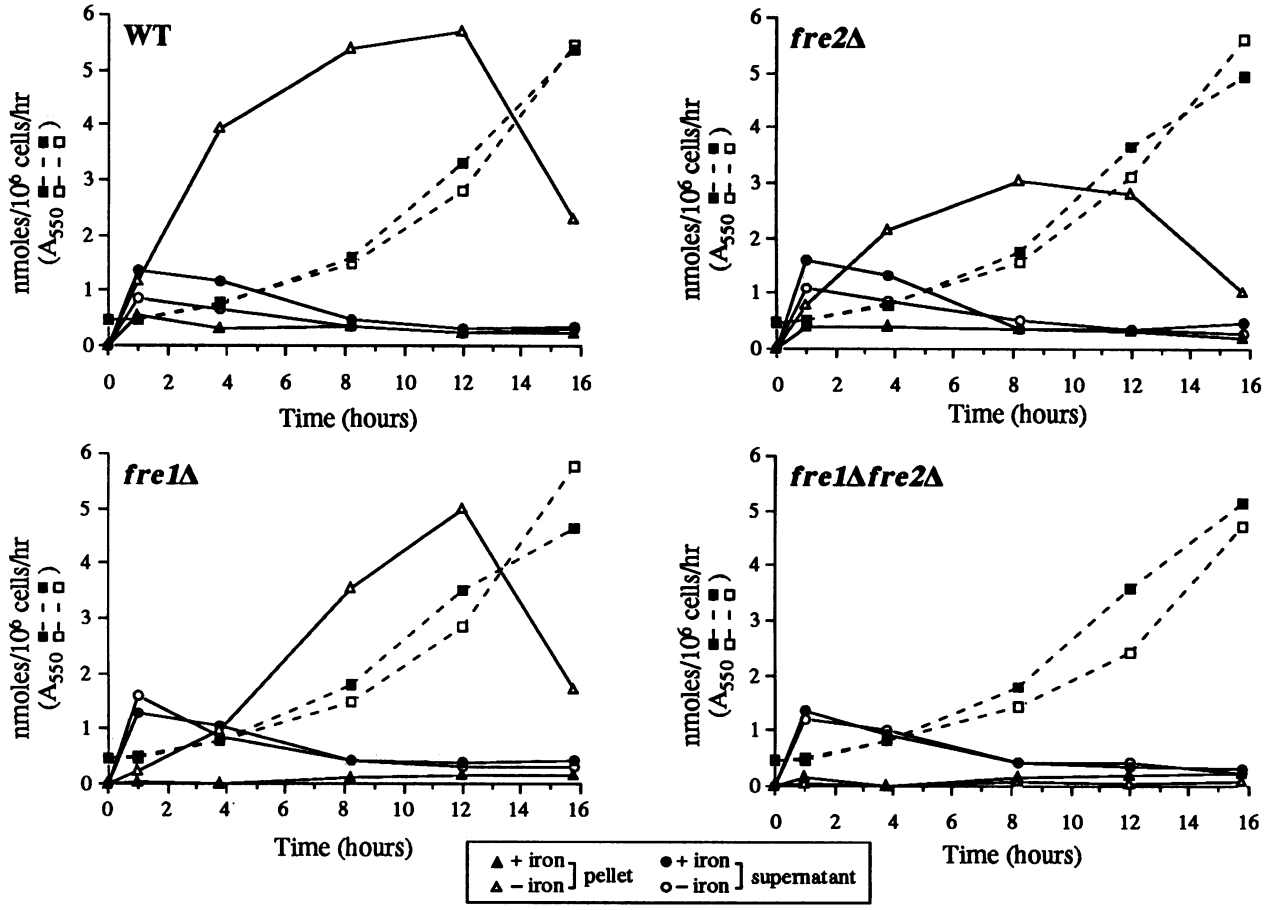

FIG. 6. Ferric reductase activity (nanomoles of ferrous ions produced per 10^6 cells per hour; solid lines) and A_{550} (dashed lines) of wild-type (WT) and *FRE* mutant (*fre1*Δ, *fre2*Δ, and *fre1*Δ *fre2*Δ derivatives of *MAT***a** *ura3-52 leu2-1*) strains in relation to the duration of growth. Numbers on the ordinate correspond to both nanomoles and A_{550}. Cells were grown to saturation in minimal medium, washed in MD medium, and resuspended in MD medium (open symbols) or in MD iron-supplemented medium (closed symbols) at a concentration of 4×10^6 to 5×10^6 cells per ml. The number of cells was estimated by counting on a Neubauer microscope slide. A linear relationship with the OD_{550} was established up to an OD of 0.6 (number of cells = $10.24 \times OD_{550} \times 10^6$). Ferric reductase activity values were obtained from pelleted cells (triangles) after preincubation in reductase buffer and from supernatant fractions (circles).

iron content of the medium. In contrast, clear differences were detected among the four cultures when activities of the pelleted cells were assayed. The *fre1*Δ *fre2*Δ strain showed practically no activity during the entire time course, whereas the wild-type strain exhibited an iron-regulated activity that reached maximum levels at 10 to 12 h with an approximately 25-fold difference between iron-rich and iron-deficient media. The *fre1*Δ strain showed a profile comparable to that of the wild type quantitatively except that the activity reached a peak somewhat later, at 12 h, and did so more abruptly. The *fre2*Δ strain reached maximum levels in approximately 8 h and, although it showed lower levels of activity than the wild-type and fre1Δ strains, it retained those levels for a longer period of time. When the cultures entered the stationary phase, the activity dropped even in the absence of iron.

The observed quantitative and qualitative differences (in time and in height and width of activity curves) between the *fre1*Δ and *fre2*Δ strains were seen reproducibly in five independent experiments. In two additional experiments, performed by diluting exponentially grown cells (instead of saturated cultures) in MD or MD iron-supplemented medium, we obtained the same results as those shown in Fig. 6 except for shorter lag times (data not shown). Therefore, the *FRE1* and *FRE2* genes exhibit different ferric reductase activity profiles during culture growth, the sum of which constitutes the membrane-bound activity profile of the wild-type strain.

The *FRE2* transcript is highly inducible by iron deprivation. It is clear from the findings presented that *FRE2* gene activity is regulated according to the iron content of the environment. To examine whether this regulation is operative at the level of mRNA accumulation, we performed RNA blot hybridization analysis. For this analysis, we used polyadenylated RNA because of the low abundance of the *FRE2* message in total cellular RNA. *FRE2* mRNA was easily detected in RNA from exponentially grown cells under iron starvation conditions, whereas it was barely detectable in cells grown in the presence of iron (Fig. 7). Longer exposures of the X-ray films permitted a gross estimate of this difference, which is of the order of 50-fold or more. A *FRE1* gene-specific probe and a *HIS3-DED1*-gene-specific probe were also used on duplicate samples of the filter hybridized with the *FRE2* gene-specific probe for quantitative comparisons. The *HIS3-DED1* DNA probe hybridized to mRNAs which are not regulated by iron and can be considered internal controls for the amount of RNA loaded

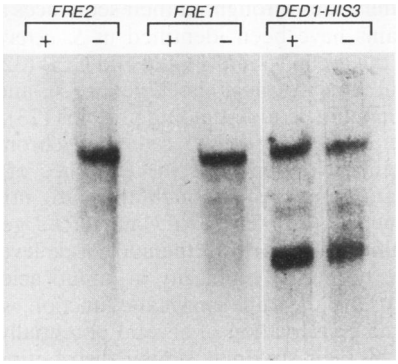

FIG. 7. Regulation of *FRE2* mRNA accumulation by iron determined by blot hybridization analysis of polyadenylated RNA samples isolated from cells grown exponentially for 12 h in MD medium (−) or in MD iron-supplemented medium (+). Triplicate samples of the same quantities were analyzed and hybridized to the indicated DNA probes.

on the gels. The *FRE1* DNA probe reproduced the results of Dancis et al. (9). Considering that similar quantities of polyadenylated RNAs were loaded on the gels and the two probes had similar specific activities, we can conclude that *FRE1* and *FRE2* mRNAs accumulated at comparable levels under the growth conditions used.

The *FRE2* promoter accounts for part of the iron-dependent regulation of *FRE2* expression. To examine whether the iron-regulated accumulation of *FRE2* mRNA occurs at the level of RNA synthesis, we investigated the ability of the *FRE2* promoter to direct regulated transcription in a heterologous coding region. For that purpose, we used the *GCN4* gene as a reporter.

GCN4 is an *S. cerevisiae* transcriptional activator necessary for the induction of amino acid biosynthetic genes under amino acid starvation conditions. A *gcn4* strain does not grow in the presence of 3-AT, a competitive inhibitor of the *HIS3* gene product (imidazoleglycerol-P dehydratase), whereas a wild-type strain survives that stress by inducing the *HIS3* transcription levels via GCN4 (12). A *FRE2* DNA fragment containing 156 bp upstream of the AUG (Fig. 3) was fused to the *S. cerevisiae GCN4* gene as described in Materials and Methods. We also constructed a *FRE1/GCN4* fusion gene as a positive control, using a promoter fragment reported to be sufficient for directing efficient and iron-regulated transcription of a reporter gene (9). Both chimeric constructs were used to transform a *gcn4* strain. As shown in Fig. 8, the transformed strains failed to grow on plates containing both 3-AT and iron, whereas they grew on iron-depleted 3-AT plates. The *FRE2* 156-bp promoter fragment was the minimum tested region (data not shown) able to direct the transcription of *GCN4* mRNA in levels sufficient for the survival of the cells under histidine starvation conditions but only in the iron-depleted medium.

DISCUSSION

Ferric reductase is necessary for the iron supply of yeast cells, which are capable of transporting only ferrous ions through the plasma membrane. This study reveals all elements which account for the total ferric reductase activity involved in iron uptake in *S. cerevisiae*. These include two membrane-bound ferric reductases and an excreted reductase activity.

We have cloned and molecularly characterized *FRE2*, a gene responsible for a significant part of the ferric reductase activity carried out by yeast cells, as shown by measurements of the activity of the *FRE2*-disrupted strain. Analysis of the predicted polypeptide sequence and comparisons with the sequences of other ferric reductases indicated that FRE2 is an externally directed transmembrane protein harboring domains which function in electron transport. It is similar in structural and functional characteristics to FRE1, the first ferric reductase to be characterized in *S. cerevisiae* (9).

Deletion of both the *FRE1* and *FRE2* genes from the yeast genome completely abolished the membrane-associated ferric reductase activity, rendering the cell incapable of growing for an extended period of time in iron-deficient media. However, deletion of the *FRE1* or *FRE2* gene separately did not change the levels of reductase activity drastically, nor did it significantly alter the growth rate of the cells in iron-deficient liquid media. Moreover, the growth phenotype of both the *fre1*Δ and *fre2*Δ strains was similar to that of the wild-type strain in iron-deficient solid media, indicating that each gene product could substitute for the other at least qualitatively under the experimental conditions used.

However, our detailed analysis of reductase activity in growing yeast cultures revealed that the two genes are dis-

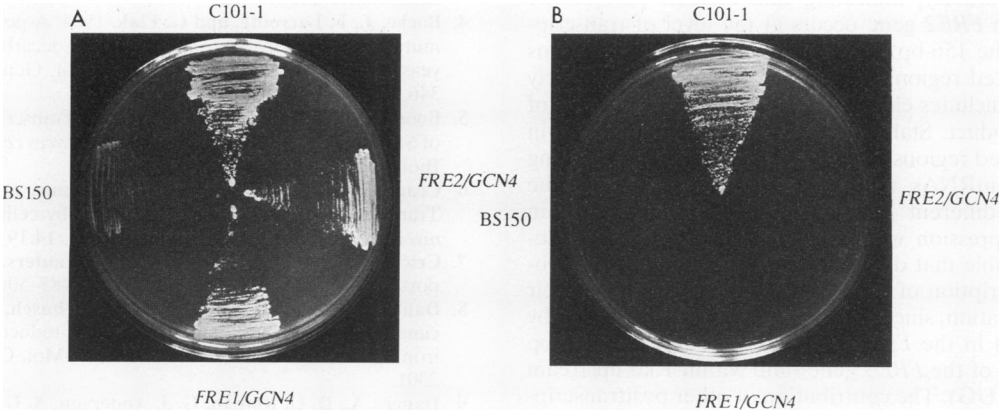

FIG. 8. Growth of *MAT***a** *gcn4*:Δ:*URA3 ura3-52 leu2-1* cells transformed with the indicated plasmids (described in Materials and Methods) on 3-AT-containing MD plates in the presence (A) or absence (B) of iron. The plates were incubated at 30°C for 3 and 7 days, respectively, in order to have a comparable growth phenotype of the C101-1 plasmid-containing strain.

tinctly regulated. Each one accounts for more than 50% of the total ferric reductase activity of the cells but at distinct time points during exponential growth. For example, *FRE2* accounts for at least 80% of the activity after 12 h of growth in iron-deficient media, whereas *FRE1* is responsible for more than 65% of the wild-type activity after 3 to 4 h of growth. Our results suggest a strong participation of the *FRE2* gene in the total reductase activity of the cell. Other reports attributed 80 to 98% of the total activity to the *FRE1* gene (8, 9). However, these authors measured ferric reductase activity either early or very late following the passage of the cells to fresh media. According to our results, under these conditions, low FRE2 activity should have been detected. Our data indicate that another part of this discordance can be attributed to the fact that we have made the distinction and measured separately the activities of the membrane-associated and the excreted ferric reductase forms. This latter activity is not at all negligible up to at least 6 to 8 h of growth, accounting for most of the total activity in the first 2 h of growth. It clearly interferes with the total activity measured, and most importantly, it interferes to a variable extent depending on the duration of incubation of the cells for the reductase assay, thus rendering the correlation between the ferric reductase activity and the time of incubation not linear.

Taken together, our results give a more complete picture of how yeast cells ensure the proper and external condition-adjusted reduction of environmental ferric ions to ferrous ions in order to be taken up intercellularly. We assume that the excreted ferric reducing activity, which was found to be considerable in early exponential growth, accounts for a significant part of the total ferric reducing activity of the cell, necessary for quick iron uptake when cells enter this early phase of growth. This activity could satisfy the cell's needs for iron in newly synthesized proteins or for storage, the membrane-associated reducing activities being still repressed at that stage. On the other hand, two different genes account for the membrane-associated ferric reductase activities of the cell which are inducible and active after the onset of exponential growth.

Our present data do not reveal the elements that regulate the temporally different expression of the two *FRE* genes. Our RNA blot hybridization analysis of exponentially grown cells showed that expression of the *FRE2* gene is iron regulated at the level of mRNA accumulation similarly to *FRE1* gene expression. Our preliminary promoter analysis of the *FRE2* gene defined a minimal region upstream of the AUG that is capable of sustaining iron-regulated expression of a heterologous gene qualitatively, indicating that at least part of the regulation of the *FRE2* gene occurs at the level of transcription. Although the 156-bp tested promoter fragment contains the 5′ untranslated region of the *FRE2* mRNA, it is unlikely that this region includes elements that regulate the stability of the chimeric product. Stability elements are mostly found in the 3′ untranslated regions and, more rarely, within the coding regions of the mRNAs (31). Quantitative analysis of the contribution of different regions of the *FRE2* promoter in heterologous expression will identify specific regulatory elements. It is possible that different *cis*-acting elements contribute to the transcription of the two genes, accounting for their differential regulation, since none of the elements identified by Dancis et al. (9) in the *FRE1* promoter exists in the 156-bp promoter region of the *FRE2* gene (and within 1 kb upstream of the initiator AUG). The contribution of other posttranscriptionally regulated steps to the expression of each gene remains to be uncovered.

Several examples of functionally related proteins which exhibit few similarities throughout their sequences and more at specific domains have been identified in *S. cerevisiae*. Some examples are the cyclin subunits CLN and CLB (32), members of the *SSA* subfamily of heat shock genes (5), and the RHO family of guanine nucleotide-binding proteins (23). Moreover, the complete sequencing of *S. cerevisiae* chromosome III provided a general example for the existence of genes that have redundant functions although they are not obviously similar in structure (25). The *FRE1* and *FRE2* genes do not show any significant similarity at the nucleotide level, and their products have borderline similarity in amino acid sequence. However, they have the same enzymatic function, which is also reflected by the conservation of several potentially important domains and by their strikingly similar distribution of hydrophobic and hydrophilic regions. The two genes have probably derived from a common ancestor gene following extensive nucleotide divergence. The only preserved sequences in their products are those that ensure their enzymatic function and membrane localization. Moreover, our results demonstrated that the activities attributed to the two products are not simply additive but contribute to a more complex functional relationship. Although each one seemingly substitutes for the other, the two have different roles at different phases of cell growth. It is of great interest to understand the elements responsible for the regulation of the two *FRE* genes in concordance with the cellular needs for iron at different stages of growth.

ACKNOWLEDGMENTS

We thank George Thireos for helpful discussions and critical reading of the manuscript, Rudi J. Planta for helpful suggestions, Georgia Houlaki for assistance with artwork, and Lila Kalogeraki for assistance with photography.

This work was supported by the Greek Ministry of Industry, Energy and Technology and by the Commission of the European Communities under the BRIDGE program of the Division of Biotechnology.

REFERENCES

1. **Alani, E., L. Cao, and N. Kleckner.** 1987. A method for gene disruption that allows repeated use of *URA3* selection in the construction of multiply disrupted yeast strains. Genetics **116:**541–545.
2. **Alexandraki, D., and M. Tzermia.** Sequencing of a 13.2 kb segment next to the left telomere of yeast chromosome XI revealed five open reading frames and recent recombination events with the right arms of chromosomes III and V. Yeast, in press.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1987. Current protocols in molecular biology. Greene Publishing Associates and Wiley-Interscience, New York.
4. **Boeke, J., F. Lacroute, and G. Fink.** 1984. A positive selection for mutants lacking orotidine 5′ phosphate decarboxylase activity in yeast: 5-fluoroorotic acid resistance. Mol. Gen. Genet. **197:**345–346.
5. **Boorstein, W. R., and E. A. Craig.** 1990. Transcriptional regulation of *SSA3*, an HSP70 gene from *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:**3262–3267.
6. **Crane, F. L., H. Roberts, A. W. Linnane, and H. Low.** 1982. Transmembrane ferricyanide reduction by cells of the *Saccharomyces cerevisiae*. J. Bioenerg. Biomembr. **14:**191–205.
7. **Crichton, R. R., and M. Charloteaux-Wauters.** 1987. Iron transport and storage. Eur. J. Biochem. **164:**485–506.
8. **Dancis, A., R. D. Klausner, A. G. Hinnebusch, and J. G. Barriocanal.** 1990. Genetic evidence that ferric reductase is required for iron uptake in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:**2294–2301.
9. **Dancis, A., D. G. Roman, G. J. Anderson, A. G. Hinnebusch, and R. Klausner.** 1992. Ferric reductase of *Saccharomyces cerevisiae*: molecular characterization, role in iron uptake, and transcriptional control by iron. Proc. Natl. Acad. Sci. USA **89:**3869–3873.

10. **Eide, D., S. Davis-Kaplan, I. Jordan, D. Sipe, and J. Kaplan.** 1992. Regulation of iron uptake in *Saccharomyces cerevisiae*. J. Biol. Chem. **267**:20774–20781.
11. **Higgins, D. G., and P. M. Sharp.** 1988. CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. Gene **73**:237–244.
12. **Hinnebush, A. G.** 1988. Mechanisms of gene regulation in the general control of amino acid biosynthesis in *Saccharomyces cerevisiae*. Microbiol. Rev. **52**:248–273.
13. **Hope, I. A., and K. Struhl.** 1986. Functional dissection of a eukaryotic transcriptional activation protein, GCN4 of yeast. Cell **46**:885–894.
14. **Kaplan, J., I. Jordan, and A. Sturrock.** 1991. Regulation of the transferrin-independent iron transport system in cultured cells. J. Biol. Chem. **266**:2997–3004.
15. **Karplus, A. P., M. J. Daniels, and J. R. Herriott.** 1991. Atomic structure of ferredoxin-NADP$^+$ reductase: prototype for a structurally novel flavoenzyme family. Science **251**:60–66.
16. **Klausner, R. D., T. A. Rouault, and J. B. Hartford.** 1993. Regulating the fate of mRNA: the control of cellular iron metabolism. Cell **72**:19–28.
17. **Kohrer, K., and H. Domdey.** 1991. Preparation of high molecular weight RNA. Methods Enzymol. **194**:398–405.
18. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydrophobic character of a protein. J. Mol. Biol. **157**:105–132.
19. **Lesuisse, E., and P. Labbe.** 1989. Reductive and non reductive mechanisms of iron assimilation by the yeast *Saccharomyces cerevisiae*. J. Gen. Microbiol. **135**:257–263.
20. **Lesuisse, E., F. Raguzzi, and R. R. Chrichton.** 1987. Iron uptake by the yeast *Saccharomyces cerevisiae*: involvement of a reduction step. J. Gen. Microbiol. **133**:3229–3236.
21. **Lovley, D. R., M. J. Baedecker, D. J. Lonergan, I. M. Cozzarelli, E. J. P. Phillips, and D. I. Siegel.** 1989. Oxidation of aromatic contaminants coupled to microbial iron reduction. Nature (London) **339**:297–299.
22. **Marck, C.** 1988. 'DNA Strider': a 'C' program for the fast analysis of DNA and protein sequences on the Apple Macintosh family of computer. Nucleic Acids Res. **16**:829–1836.
23. **Matsui, Y., and A. Toh-e.** 1992. Isolation and characterization of two novel *ras* superfamily genes in *Saccharomyces cerevisiae*. Gene **114**:43–49.
24. **Neilands, J. B., K. Konopka, B. Schwyn, M. Coy, R. I. Francis, B. H. Paw, and A. Bagg.** 1987. Comparative biochemistry of microbial iron assimilation, p. 3–34. *In* G. Winkelmann, D. van der Helm, and J. B. Neilands (ed.), Iron transport in microbes, plants and animals. VCH, Weinheim, Germany.
25. **Oliver, S. G., et al.** 1992. The complete DNA sequence of yeast chromosome III. Nature (London) **357**:38–46.
26. **Orkin, S. H.** 1989. Molecular genetics of chronic granulomatous disease. Annu. Rev. Immunol. **7**:277–307.
27. **Pearson, V. R., and D. J. Lipman.** 1988. Improved tools for biological sequence analysis. Proc. Natl. Acad. Sci. USA **85**:2444–2448.
28. **Penn, D. M., G. Thireos, and H. Greer.** 1983. Temporal analysis of general control of amino acid biosynthesis in *Saccharomyces cerevisiae*: role of positive regulatory genes in initiation and maintenance of mRNA derepression. Mol. Cell. Biol. **4**:520–528.
29. **Philippsen, P., A. Stotz, and C. Scherf.** 1991. DNA of *Saccharomyces cerevisiae*. Methods Enzymol. **194**:169–182.
30. **Roman, D. G., A. Dancis, G. J. Anderson, and R. D. Klausner.** 1993. The fission yeast ferric reductase gene *frp1*$^+$ is required for ferric iron uptake and encodes a protein that is homologous to the gp91-*phox* subunit of the human NADPH phagocyte oxidoreductase. Mol. Cell. Biol. **13**:4342–4350.
31. **Sachs, A. B.** 1993. Messenger RNA degradation in eukaryotes. Cell **74**:413–421.
32. **Schwob, E., and K. Nasmyth.** 1993. *CLB5* and *CLB6*, a new pair of B cyclins involved in DNA replication in *Saccharomyces cerevisiae*. Genes Dev. **7**:1160–1175.
33. **Segal, A. W., and A. Abo.** 1993. The biochemical basis of the NADPH oxidase of phagocytes. Trends Biochem. Sci. **18**:43–47.
34. **Segal, A. W., I. West, F. Wientjes, J. H. A. Nugent, A. J. Chavan, B. Haley, R. C. Garcia, H. Rosen, and G. Scrace.** 1992. Cytochrome b_{245} is a flavocytochrome containing FAD and the NADPH-binding site of the microbial oxidase of phagocytes. Biochem. J. **284**:781–788.
35. **Struhl, K.** 1985. Naturally occurring poly (dA-dT) sequences are upstream promoter elements for constitutive transcription in yeast. Proc. Natl. Acad. Sci. USA **82**:8419–8423.
36. **Thireos, G., D. M. Penn, and H. Greer.** 1984. 5′ untranslated sequences are required for the translational control of a yeast regulatory gene. Proc. Natl. Acad. Sci. USA **81**:5096–5100.
37. **Thorstensen, K., and I. Romslo.** 1988. Uptake of iron from transferrin by isolated rat hepatocytes. A redox mediated plasma membrane process? J. Biol. Chem. **263**:8844–8850.
38. **von Heijne, G.** 1983. Patterns of amino acids near signal-sequence cleavage sites. Eur. J. Biochem. **133**:17–21.
39. **Yamashoji, S., and G. Kajimoto.** 1986. Catalytic action of vitamin K3 on ferricyanide reduction by yeast cells. Biochim. Biophys. Acta **849**:223–228.
40. **Yamashoji, S., and G. Kajimoto.** 1986. Decrease of NADH in yeast cells by external ferricyanide reduction. Biochim. Biophys. Acta **852**:25–29.