Nfs1p is the yeast homolog of the bacterial proteins NifS and IscS, enzymes that release sulfur from cysteine for iron-sulfur cluster assembly. Here we show that the yeast mitochondrial protein Nfs1p regulates cellular and mitochondrial iron homeostasis. A strain of Saccharomyces cerevisiae, MA14, with a missense NFS1 allele (I191S) was isolated in a screen for altered iron-dependent gene regulation. This mutant exhibited constitutive up-regulation of the genes of the cellular iron uptake system, mediated through effects on the Aft1p iron-regulatory protein. Iron accumulating in the mutant cells was retained in the mitochondrial matrix while, at the same time, iron-sulfur proteins were deficient. In this work, the yeast protein was localized to mitochondria, and the gene was shown to be essential for viability. Furthermore, Nfs1p in the MA14 mutant was found to be markedly decreased, suggesting that this low protein level produced the observed regulatory effects. This hypothesis was confirmed by experiments in which expression of wild-type Nfs1p from a regulated galactose-induced promoter was turned off, leading to recapitulation of the iron regulatory phenotypes characteristic of the MA14 mutant. These phenotypes include decreases in iron-sulfur protein activities coordinated with increases in cellular iron uptake and iron distribution to mitochondria.

Iron-sulfur (Fe-S) clusters are cofactors of proteins involved in oxidation-reduction, electron transport, metabolic conversions, and regulatory functions (1). The iron and sulfur are assembled in fixed stoichiometries (e.g. 2Fe-2S, 4Fe-4S) characteristic of the particular protein and coordinated to critical cysteines in the primary peptide backbone (2). Within cells, iron availability for synthesis of iron-sulfur proteins and other biological functions must be tightly regulated, because excess iron is toxic (3). Excess iron leads to free radical reactions that damage membranes, proteins, and DNA (4). Here we describe a regulatory control mechanism that coordinates iron uptake, iron distribution, and the levels of iron-sulfur cluster proteins in the eukaryote Saccharomyces cerevisiae. The regulator responsible for these effects is Nfs1p.

Examination of the S. cerevisiae genome data base reveals that Nfs1p is the single yeast homolog of bacterial IscS (5, 6) and NifS (7). There is strong evidence, both biochemical and genetic, showing that the bacterial protein NifS mobilizes sulfur from cysteine and mediates Fe-S cluster assembly. Bacterial mutants of NifS were found to be deficient in the assembly of both Fe protein and MoFe protein subunits of nitrogenase (8, 9). NifS through its enzymatic activity was found to reactivate the apo form of nitrogenase in which the Fe-S cluster was removed by chelation (10). Elegant biochemical work has elucidated this catalytic process: NifS was shown to be a pyridoxal phosphate-containing homodimer that catalyzes the formation of elemental sulfur from L-cysteine (7). A conserved lysine residue in the bacterial NifS protein (equivalent to Lys-299 in the yeast protein) is the covalent attachment site for pyridoxal phosphate. A conserved cysteine residue (equivalent to Cys-421 in the yeast protein) forms a catalytic persulfide intermediate involved in sulfur abstraction from the l-cysteine substrate (11). In this work, we demonstrate that the yeast homolog, Nfs1p, is an essential mitochondrial protein. We show that Nfs1p is required not only for activities of Fe-S cluster proteins, but also for regulatory effects on cellular iron metabolism. Regulatory effects resulting from decreased levels of Nfs1p include up-regulation of iron uptake to the cell and distribution of cellular iron to mitochondria.

**EXPERIMENTAL PROCEDURES**

**Growth Media**—Methods for yeast manipulations and growth media have been described (12). For experiments with different concentrations of metals, standard defined medium was modified by addition of iron and copper. Iron was added as ferric ammonium sulfate and copper was added as copper sulfate. For some experiments, in order to avoid catechol repression, the yeast was grown in defined medium with 2% raffinose as the carbon source. For some experiments, in order to avoid catechol repression, the yeast was grown in defined medium supplemented with 10 μM copper sulfate and 20 μM ferric ammonium sulfate. Strains x316–1A-MA14 and x404-6D-MA14 were derived from the strain 81 after selection on defined medium supplemented with 10 μM copper sulfate and 20 μM ferric ammonium sulfate. Strains x316–1A-MA14 and x404-6D-MA14 were derived from MA14 by back-crossing with the wild-type strains CM3260 and YPH499, respectively. Heterozygote knock out of NPS1 gene was confirmed in strains CM3263 and YPH501 by transforming with the construct, nfs1KOUra. Sporulation yielded only uracil auxotrophs with 2 of 4 viable tetrad clones. Diploid wild-type strain D273-10B (ATCC 24657) was used for testing import into isolated mitochondria. AFT1 was interrupted in YPH499 and x404-6D-MA14 by transforming with 2 of 4 viable tetrad clones.
with the Xhol-Kpn1 fragment of pT20 (14) and selecting for tryptophan prototrophy, creating strains 499Δaaf1 and 6DΔaaf1.

Plasmids and DNA Constructions—pA55, pA96, and pA48 contained inserts of genomic DNA in the vector YCP50 and were selected by screening a library (15) for complementation of MA14. pB48Δ SaI was constructed by digesting pB48 with PvuII and HindIII and religating, thus removing the genomic EcoRI fragment. The genomic 3.4-kilobase EcoRI fragment contained the NFS1 ORF1 and 1133 base pairs of 5′-flanking region, including a portion of the adjacent YCL016c ORF. This fragment was subcloned into pRS416 creating plasmid pRS406-R1(NFS1) and into pRS416 creating plasmid pRS416(NFS1). Plasmid pRS406-R1(nfs1-fs) contained a frameshift introduced into the NFS1 open reading frame by digesting with AflIII, rendering the ends blunt with Klenow polymerase, and religating. Plasmid pRS416(NFS1) linearized with SpI and PjMI was used to recover the mutant allele (nfs1-14) from the strain x404-6D-MA14, as described in the gap repair method (16). The mutated base pair was identified by dyeoxy DNA sequencing of the NFS1 ORF. The NFS1 deletion/integrons constructs nfsKOUra and nfsKOHis were made by subcloning the NFS1 EcoRI fragment into pBluescript SK+ and inserting URA3 or HIS3 cassettes into the BglII sites within the coding region. For partial deletion and disruption of the gene, the modified genomic fragment between the SalI and EcoRI sites of pB48 was subcloned into pRS406-R1(NFS1) and into pRS416 creating plasmid pRS416(NFS1). Plasmid pRS406-R1(nfs1-fs) contained a frameshift introduced into the NFS1 open reading frame by digesting with AflIII, rendering the ends blunt with Klenow polymerase, and religating.

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1 The abbreviations used are: ORF, open reading frame; INT, p-iodonitrotetrazolium violet.

RPYD were harvested and RNA was isolated using hot acidic phenol (65 °C, pH 5.5). The RNA was recovered by formaldehyde-agarose gel, blotted to nitrocellulose, and hybridized with 32P-labeled probes. The probes were labeled by random prime synthesis using fragments derived from the coding regions of the genes to be studied. The blot was probed with the radiolabeled probe at 42 °C for 18 h (1% SDS) and exposed to a PhosphorImager screen. For immunoblotting, proteins were separated by electrophoresis on polyacrylamide gels and electrophoretically transferred to nitrocellulose. The primary antibodies were rabbit polyclonal antibodies and the signal was developed using a goat anti-rabbit IgG peroxidase conjugate and the ECL kit (Amersham).

Fractionation of Cells—Mitochondria were isolated from yeast (18). The cytosolic cell fraction was derived from a concentrated translation-competent yeast lysate. This lysate was centrifuged at 386,000 × g for 20 min and the supernatant was designated as cytosol.

Fractionation of Mitochondria—Intact mitochondria were suspended in 20 mM Hapes-KOH, pH 7.5, 0.6 M sorbitol. For releasing the soluble contents of the intermembrane space, the mitochondria were subjected to hypotonic shock by diluting the sorbitol to 0.1 M and incubating at 0 °C for 10 min (19). The mitoplasts were separated from the outer membrane and intermembrane space components by centrifuging at 12,000 × g for 10 min at 15,000 × g. For tracking the Fe label in mitochondria, aliquots of the fractions (mitochondria, intermembrane space, Triton X-100 soluble, and Triton X-100 insoluble mitoplast pellet) were suspended in scintillation mixture and counted in a Beckman scintillation counter.

Mitochondrial Import—The NFS1 ORF was amplified between NdeI (5′) and Xhol (3′) sites and cloned into pSP647 (20). Radiolabeled Nfs1p preprotein was generated by transcription followed by translation in the presence of a mixture of 35S-methionine and 35S-cysteine, and the radiolabeled product was used in a mitochondrial import study (21). Briefly, reactions containing 100 μg of mitochondria were initiated by adding the radiolabeled preprotein. Import reaction mixtures contained 4 mM ATP and 1 mM GTP. Following import at 20 °C for 15 min, reaction mixtures were treated with trypsin (0.1 mg/ml) for 30 min at 0 °C. The protease was inactivated and the samples were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

Assays—The assay for ferric reductase was a filter lift assay (22), modified by the addition of 50 μl copper sulfate and 10 μl ferric ammonium sulfate to YPD agar plates for growth of the colonies to be assayed. Under these conditions the wild-type exhibited repressed activity (Red−) and the MA14 strain was strongly positive (Red+). Assays for ferric reductase and high affinity ferrous iron uptake have been described in detail (14). Mitochondria were isolated from the MA14 strain by the replica plate method (23).

RESULTS

New Complementation Group Identified Using FRE1-HIS3 Selection for Mutants of Iron Metabolism—A selection for mutants involved in cellular iron homeostasis was modified from our previous method (25). The FRE1 gene encodes the major
cell surface reductase and is required for iron acquisition from ferric iron chelates. FRE1 is active when cells are starved for iron, and inactive when cells are replete. To isolate mutants unable to respond to environmental iron, the FRE1 promoter was fused to the HIS3 coding region, and this construct was integrated into the chromosome of a Δhis3 strain. The strain carrying the integrated FRE1-HIS3 marker on the chromosome was cultured in rich (YPAD) medium and a dilute inoculum was spread on agar plates of the same composition, allowing colonies to arise from single cells. The colonies were replicated to selective plates consisting of defined medium lacking histidine and supplemented with iron (20 μM ferric ammonium sulfate). Under these conditions, uptake of the metal could proceed by genetically distinct high and low affinity uptake systems (26). Thus, mutations abrogating only one system were not selected, and instead, regulatory mutants and mutants affecting metal distribution were identified. Most colonies did not grow at all on the selection plates, but after 4 days, a small number of colonies emerged as papillae on a background of non-growing cells. The growing cells were transferred to rich (YPAD) plates. One of the mutants selected in this manner, MA14, was for further genetic analysis. The mutant exhibited non-repressed surface reductase, consistent with the selection scheme (Red+ for non-repressed reductase). Crossing with the parental strain of the opposite mating type revealed that the mutation was recessive, i.e., the diploid was Red−. Sporulation of this diploid revealed that a single locus was involved, because the reductase phenotype segregated as 2+:2− in 20 tetrads.

Several other mutants with similar phenotypes were selected, including numerous isolates of ssg1 mutants (27). Crossing of MA14 with 191-33C which carries a mutant allele of SSQ1, yielded diploid strains with completely normal iron metabolism. Iron uptake, surface reductase, and regulation of the above diploid strain were normal, indicating that the MA14 and SSQ1 mutant loci were not allelic. Similar analyses with mutants at other loci implicated in iron homeostasis were performed. Crosses with Δatm1 (28) and Δyfh1 (29) strains ruled out that MA14 was mutated at those loci.

Altered Cellular Iron Homeostasis in MA14 Mutant: Dependence on AFT1—We wondered if the dysregulation of the FRE1 promoter fusion in the MA14 mutant reflected a more general alteration in cellular iron homeostasis. In wild-type yeast, uptake of iron from ferric chelates in the medium requires the concerted action of several proteins (30). The surface reductases encoded by FRE1 and FRE2 act on external ferric iron chelates, releasing ferrous iron which can then be transported by an iron transport complex. The latter consists of the FTR1 permease and FET3 multicopper oxidase (31, 32). The genes for the proteins involved in iron uptake are coordinately regulated at the level of transcription by AFT1, an iron sensor-regulator (33). Iron deprivation induces AFT1-dependent transcription of the target genes. The mutant grown in standard rich (YPAD) medium was evaluated for up-regulation of the genes controlled by AFT1. As shown in Fig. 1A, the mRNA levels for surface reductases, FRE1 and FRE2, were increased in the MA14 mutant compared with the wild-type. FRE1 expression was present in the wild-type and increased roughly 2-fold in the mutant. FRE2 expression was undetectable in the wild-type and highly induced in the mutant. The different effects on FRE1 and FRE2 are probably due to the dual regulatory control of FRE1, which is dependent on both iron (14) and copper (34) levels, whereas FRE2 is dependent only on iron levels (35). The FET3 and FTR1 transcripts were also increased in the mutant. The extent of up-regulation of these components was similar to that observed in an AFT1−1rap strain, M2, in which the iron sensor-regulator remains constitutively induced due to a point mutation (Fig. 1A).

To investigate the role of AFT1 in the induction of the cellular iron uptake system in the MA14 mutant, the AFT1 gene was interrupted in the MA14 strain. Surface ferrous reductase activity was evaluated in the double mutant. The increment in ferric reductase in MA14 was found to be partially dependent on the presence of an intact copy of AFT1 (Fig. 1B). However,
increased activity was still observed in the double mutant compared with the wild-type (compare WT AFT1 with MA14 Δaft1 in Fig. 1B). This effect could be due to regulators other than AFT1 that are capable of inducing FRE1 or FRE2 expression. Regulation of FRE1 by the MAC1 regulator has been described (34, 36), and an AFT1 homologous gene present in the yeast genome (YPL202C) might also mediate the increased ferric reductase expression. By contrast, the increment in high affinity ferrous iron uptake observed in the MA14 mutant was completely dependent on AFT1 (Fig. 1B). The AFT1 sensor regulator is involved in homeostatic responses of the cell to environmental iron. Therefore, the response of the MA14 mutant to a more graded and controlled exposure to iron was tested. The wild-type and the mutant were grown in defined media with varying concentrations of iron, and high affinity ferrous iron uptake was measured. The results show that at every medium iron concentration tested, the iron uptake activity was higher in the MA14 than the wild-type (Fig. 1C). The wild-type exhibited a progressively decreasing iron uptake rate with increasing iron exposure, as would be predicted for a homeostatically regulated process. The magnitude of this regulation was about 5-fold between lowest and highest iron exposures. By contrast the magnitude of the iron uptake regulation by the MA14 mutant was only 2-fold. Together these data suggest that in the mutant, Aft1p was not responding appropriately to iron in the environment and in the cell. Possible explanations for this are that intracellular iron was sequestered away from Aft1p or that a factor required for the Aft1p iron response was lacking.

Altered Mitochondrial Iron Homeostasis in MA14 Mutant: Mitochondrial Iron Sequestration—The observation that the rate of cellular iron uptake was increased in the MA14 mutant led us to wonder how the excess iron was distributed within the mutant cells. To address this question, the wild-type and MA14 strains were cultured in media containing different concentrations of iron to which Fe-55 was added as a tracer. After 16 h of growth during which the labeling of intracellular iron pools reached a steady state, mitochondria were isolated from the radiolaabeled cells. The iron levels in mitochondria isolated from wild-type and MA14 were indistinguishable at the lowest iron medium concentration of 0.1 μM (0.512 pmol/μg, Table I). This value is comparable to previously published values for yeast (37) and mammalian (38) mitochondrial iron levels. However, when the medium iron concentration was increased, MA14 mutant mitochondria accumulated dramatically more iron. At 1 μM the iron in the MA14 mitochondria was 18.6 pmol/μg, at 5 μM the level was 61.5, and at 50 μM the level was 75.3, whereas in the wild-type the maximum level achieved was 4.9 pmol/μg (Table I). The distribution of the iron within the mitochondria was also examined (Fig. 2). The iron content of the intermembrane space was evaluated after hypotonic shock that disrupts the outer membrane. No differences between wild-type and mutant were observed. The mitoplasts, consisting of intact inner membrane and matrix, were evaluated. Most of the iron in the MA14 mutant mitochondria was found in the mitoplasts. The mitoplasts were then lysed with 0.5% Triton X-100 and separated into a soluble supernatant fraction and an insoluble pellet fraction. Remarkably, for the mutant grown in iron concentrations of 1 μM or greater, most of the mitochondrial iron (53–60%) remained in the Triton X-100-insoluble pellet fraction. By contrast, only a small proportion of the iron (5–12%) was found in this fraction isolated from wild-type cells (Table I, Fig. 2). The mitochondrial fraction resistant to detergent solubilization would be expected to include membranes, large protein complexes and aggregates. The very marked increase of iron distributed to this fraction in the MA14 mutant suggests that the mutant phenotype alters iron trafficking to the mitochondria and iron solubility within that compartment.

Decreased Iron-Sulfur Protein Activities in the MA14 Mutant—Iron entering the mitochondria might have different final destinations: insertion into heme for use in heme proteins, insertion into Fe-S clusters for use in Fe-S cluster proteins, or retention in a matrix pool of unutilized iron. Mitochondria were isolated from the wild-type and the MA14 mutant after exposure to increasing media iron and were compared for the status of these iron pools. The results showed that as iron levels in the medium increased, the iron in the mitochondria of the MA14 mutant increased (see Fig. 2, iron ranging from 0.5 to 75 pmol of Fe/μg of mitochondrial protein), but heme proteins were largely unaffected. We evaluated cytochrome c levels as an indicator of the status of heme proteins in mitochondria. No changes of cytochrome c, evaluated by specific antibody, or heme, evaluated by pyridine hemochrome spectra (39), were noted in the MA14 mutant (not shown). By contrast, Fe-S protein activities were compromised at all media iron concentrations (Fig. 3). Aconitase is a soluble single subunit enzyme of the mitochondrial matrix and catalyzes the conversion of citrate to isocitrate. The enzymatic activity is mediated by substrate interaction with a 4Fe-4S complex on the active surface of the protein (40). In the MA14 mutant, aconitase activity was decreased to 17–33% of the wild-type level (Fig. 3). Succinate dehydrogenase activity was also measured. This enzyme consists of four nuclear encoded subunits assembled as a complex in the inner mitochondrial membrane with FAD and Fe-S cofactors (41). Succinate dehydrogenase activity was markedly decreased in the MA14 mutant, 18–53% of the wild-type level (Fig. 3). The increased mitochondrial iron levels in the MA14 mutant (Fig. 2) had very little, if any, effect on restoring Fe-S protein enzymatic activities. On the other hand, the increased mitochondrial iron did not exacerbate the deficiencies (Fig. 3).

Cloning the Wild-type Allele for the Mutant Gene in MA14: Identification as NFS1—A wild-type genomic library (15) was screened for the ability to complement the non-repressed ferric reductase phenotype of the MA14 mutant. Three independently isolated plasmids with complementing activity were isolated from the radiolabeled cells. After 16 h of growth during which the labeling of intracellular iron pools reached a steady state, mitochondria were isolated from the radiolaabeled cells. The iron levels in mitochondria isolated from wild-type and MA14 were indistinguishable at the lowest iron medium concentration of 0.1 μM (0.512 versus 0.541 pmol/μg, Table I). This value is comparable to previously published values for yeast (37) and mammalian (38) mitochondrial iron levels. However, when the medium iron concentration was increased, MA14 mutant mitochondria accumulated dramatically more iron. At 1 μM the iron in the MA14 mitochondria was 18.6 pmol/μg, at 5 μM the level was 61.5, and at 50 μM the level was 75.3, whereas in the wild-type the maximum level achieved was 4.9 pmol/μg (Table I). The distribution of the iron within the mitochondria was also examined (Fig. 2). The iron content of the intermembrane space was evaluated after hypotonic shock that disrupts the outer membr...
cross were Red– and Ura+ (wild-type phenotype) or Red+ and Ura– (mutant phenotype) (Fig. 4). The absence of recombination between the marked wild-type NFS1 and the MA14 mutant phenotype suggests that they are allelic. Thus, complementation data and meiotic mapping both supported that the mutation in MA14 was in NFS1. We next proceeded to rescue the mutant allele, called nfs1–14, by gap repair and to determine its DNA sequence. A single nucleotide change was found within the coding region compared with the wild-type sequence. Nucleotide 572 was changed from T to G, altering codon 191 from ATC (isoleucine) to AGC (serine). The mutation resulted in changing a conserved residue (Fig. 5). The altered amino acid was conserved in the bacterial IscS sequence (Fig. 5) and predicted to be present in the mature protein rather than in the mitochondrial leader. The location of the mutated amino acid in the primary sequence was distant from the pyridoxal phosphate-binding domain and the active site cysteine involved in sulfur transfer. DNA sequencing of the mutant and wild-type NFS1 alleles revealed another deviation from the database sequence. Nucleotide 448 was T in the database and C in the sequence of both the wild-type and mutant alleles rescued from strain 81 and the congenic MA14. The sequence of the wild-type allele cloned from the library also had this position. Thus the sequence difference with the database may represent a polymorphism due to strain variation or a sequence error. The effect of the nucleotide change would be to replace tyrosine at position 150 of the ORF in the database sequence with histidine. Neither amino acid resembles the bacterial IscS sequence, which has a glutamine in this position (Fig. 5).

Identification of NFS1 as an Essential Gene and Plasmid Shuffle to Evaluate NFS1 Mutant Alleles—The effects of deleting NFS1 from the haploid genome were evaluated in three different ways, and in each case, the gene was found to be required for viability. An interruption-deletion construct was designed that removes the central portion of the NFS1 ORF including the putative pyridoxal phosphate-binding site and replaces it with a HIS3 marker (Fig. 5). Transformation of two different haploid strains (CM3260, YPH499) and selection for histidine prototrophy yielded no viable transformants. Diploid transformants in both backgrounds (CM3263, YPH501) were sporulated, and the tetrads showed a 2:2 pattern for viability with all the viable tetrads being auxotrophic for histidine. The tetrad clones carrying the interruption/deletion were presumed to be non-viable or unable to germinate. Finally, a plasmid shuffle strategy (see below) confirmed the essentiality of the NFS1 gene.

Methodology for studying essential genes using plasmid shuffling has been described (17). Briefly, a shuffle strain was constructed. The NFS1 gene was interrupted with HIS3 in a cycloheximide-resistant diploid strain YPH501 (cyh2/cyh2). Plasmid pRS318, carrying the NFS1 wild-type allele, a LEU2 marker gene, and the CYH2 gene for counterselection, was transformed into the nfs1::HIS3/NFS1 heterozygous diploid,
and the diploid transformant was sporulated. A spore clone was identified by its His+ mutation. A frameshift was introduced at a unique EcoRI site, creating a frameshift mutation. When these strains were exposed to cycloheximide, the covering plasmid was ejected leaving the empty vector pRS406 and the diploid transformant was viable (Fig. 6, row 5). We expected that since the only copy of NFS1 in the strain after cycloheximide treatment was the nfs1–14 mutant allele, this strain would recapitulate the phenotype of the MA14 strain. Indeed, high affinity ferrous iron uptake in this strain was significantly increased (24 pmol/106 cells/h for nfs1–14 versus 2.5 pmol/106 cells/h in the complemented strain). The increment was not as marked as in the original MA14 mutant, but this could be due to genetic background differences and different culture conditions. Point mutations were constructed in the ORF for NFS1 (depicted in Fig. 5). A frameshift was introduced at a unique ApaI site, creating nfs1-fs. The pyridoxal phosphate-conjugating lysine was changed to alanine (K299A) by site-directed mutagenesis. In a separate construct, the critical active site lysine was changed to alanine (K299A) by site-directed mutagenesis. For meiotic mapping, the pRS406-RI(NFS1) plasmid was integrated at MscI in the genome of a wild-type strain, YPH500, placing the URA3 marker close to this site. This marked strain was crossed with the MA14 mutant and sporulated. Sixteen tetrads or 64 spore clones were analyzed for ferric reductase activity (Red+) and uracil prototrophy (Ura+). Shown are the numbers of spore clones in each category.
on the presence of a membrane potential and was completely inhibited by valinomycin (Fig. 7A, compare lanes 2 and 3). Judging from the approximate size of the precursor and mature forms, the leader sequence removed by the processing cleavage was ~3 kDa. This would be consistent with removal of a 33-amino acid leader sequence with arginine residues in -2 and -3 positions with respect to the processing cleavage site (Fig. 5, putative cleavage site is underlined). The mature processed protein (m) formed after import was protected from externally added protease (Fig. 7A, lane 4).

The predicted mitochondrial localization of the mature Nfs1p was evaluated directly using a monospecific polyclonal antibody generated against the bacterially expressed protein. Enriched cytoplasmic and mitochondrial fractions were separated on polyacrylamide gel, blotted to nitrocellulose, and examined by immunoblotting with marker antibodies for subcellular compartments or with the anti-Nfs1p antibody (Fig. 7B). The marker antibody studies confirmed the effectiveness of the fractionation procedure. Anti-Pgk1p, directed against a mitochondrial matrix marker protein, reacted only with the mitochondrial fraction. Anti-Pgk1p (anti-phosphoglycerate kinase), a mouse monoclonal antibody used at 1:5000 dilution; anti-Map2p, a rabbit polyclonal antibody used at 1:1000; and anti-Nfs1p, a rabbit polyclonal antibody used at 1:1000. The secondary antibodies used were goat anti-mouse or goat anti-rabbit peroxidase, and the signal was developed with ECL (Amersham Pharmacia Biotech). Molecular mass markers in kDa are shown to the left and right of the panel. The mobilities of the major bands were 45 kDa for PGK, 61 kDa and 66 kDa for anti-Nfs1p, 54 kDa for anti-Nfs1p, and 50 kDa for anti-Map2p.

The size of the mature Nfs1p by immunoblotting was consistent with the predicted size for the processed mitochondrial form (51 kDa) and migrated more rapidly than the purified full-length preprotein expressed in bacteria (not shown).

The mutant Nfs1p in MA14 was not processed (Fig. 7C, anti-Nfs1p (1'). Only upon overexposure of the film could we discern a weak signal, approximately 1/50 of the intensity of the wild-type (Fig. 7C, anti-Nfs1p (30')) in the mutant (Fig. 7A, lane 4). This weak signal in the mutant migrated at the same position as the wild-type protein at roughly 51 kDa, suggesting that the minimal residual Nfs1p in the mutant was processed to the mature form (Fig. 7B).
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7C). A second more weakly reactive band migrating just above the 45-kDa marker likely represents a Nfs1p degradation product. Porin, a mitochondrial outer membrane protein, was not affected in the mutant (Fig. 7C).

Repressing Expression of Nfs1p from the GAL1 Promoter Recapitulates the MA14 Phenotypes—The NFS1 ORF was placed under control of the inducible GAL1 promoter, and the wild-type allele was replaced with this regulated construct. The strain was grown to mid-logarithmic phase under inducing conditions in 1.5 liter of YPR 0.5% galactose. The cells were pelleted by centrifugation and resuspended in YPR 0.2% glucose, which does not induce the GAL1 promoter. At 0 time (before the shift) and at 2.5, 5, 7.5, and 10 h, 750 ml of culture volume was removed and replaced with fresh media. High affinity ferrous iron uptake rate (picomole/10^6 cells/h) was measured on purified mitochondria.

The activities of iron-sulfur proteins decreased rapidly as Nfs1p levels declined. The kinetics of this decrease was remarkably similar for aconitase, a mitochondrial matrix enzyme with one subunit, and for succinate dehydrogenase, a multi-subunit complex of the mitochondrial inner membrane (Fig. 8). A control experiment was performed in which the wild-type strain, YPH499, was subjected to identical growth conditions. Negligible changes (less than 10%) in Nfs1p levels, iron-sulfur protein activities, cellular iron uptake, or mitochondrial iron levels were observed (not shown). Thus, the decrease in the quantity of wild-type Nfs1p produces iron regulatory effects similar to those observed for the MA14 mutant. The iron regulatory phenotypes of the MA14 mutant are very likely due to the decreased amount of Nfs1p, although other allele specific effects are not completely excluded by these experiments.

DISCUSSION

Nfs1p is the yeast homolog of the bacterial proteins NifS and IcsS. The bacterial proteins possess cysteine desulfurase activities that provide elemental sulfur from L-cysteine for Fe-S cluster synthesis (5, 7). We have shown here in two ways that yeast Nfs1p is required for activity of Fe-S proteins. First, the mutant strain carrying the nfs1–14 allele was found to have a reduced amount of Nfs1p, and both aconitase and succinate dehydrogenase activities were deficient in this strain. These enzymes are Fe-S proteins of the mitochondrial matrix and inner membrane, respectively. Second, repression of Nfs1p expression using the regulated GAL1 promoter led to rapid decline of both aconitase and succinate dehydrogenase activities.

In this work, the NFS1-encoded protein was localized to mitochondria, and the gene was found to be essential for viability. Point mutations in the conserved pyridoxal phosphate lysine (Lys-299) or in the conserved active site cysteine (Cys-421), both predicted to disrupt cysteine desulfurase activity (5), resulted in cell inviability. Therefore, a critical Fe-S protein is likely to be required for viability. The specific target of this effect is not easily discerned, because most of the Fe-S proteins of yeast cells are non-essential. These include Fe-S proteins within mitochondria (Rieske iron-sulfur protein (42), succinate dehydrogenase (42), aconitase (40), homoaconitase (43, 44), or outside mitochondria (3-isopropylmalate isomerase (45)). Other proteins that reside outside mitochondria and are likely to contain Fe-S clusters include an endonuclease III-like protein (46) and RNase L inhibitor ortholog (47). The localization of Nfs1p to mitochondria and its apparent absence from the cytoplasm raises an intriguing question. Is mitochondrial Nfs1p required for synthesis or maintenance of cytoplasmic iron-sulfur proteins? Leu1p is a cytoplasmic protein (45) with 3-isopropylmalate isomerase activity (48) that is likely to contain an iron-sulfur cluster. Although MA14 is not auxotrophic for leucine, further work will be required to evaluate if the activities of cytoplasmic iron-sulfur proteins are diminished in the MA14 mutant. The human homolog of Nfs1p has been localized to both cytoplasmic as well as mitochondrial cellular compartments (49), but we find no evidence of the yeast Nfs1p in the cytoplasm.

A new finding of this work is that Nfs1p regulates iron homeostasis. Thus, Nfs1p regulates both the activities of Fe-S cluster proteins and cellular iron uptake and distribution. The
decreased level of Nfs1p, due to the nfs1–14 point mutation (changing Ile to Ser at amino acid 191) or due to repressed expression from a galactose-regulated promotor, led to up-regulation of cellular iron uptake. The surface reductases, FRE1 and FRE2, were expressed at higher levels. The FET3 and FTR1 transcripts were present at higher levels, consistent with induction of the high affinity ferrous transport system. The cellular iron uptake system remained active under iron conditions that normally repress expression in a wild-type strain. These effects on cellular iron uptake were dependent upon Aft1p, the iron sensor-regulator. Nfs1p levels also affected iron distribution within the cell. The iron assimilated by the nfs1–14 mutant cells was retained in mitochondria and seques-
tered in a fraction that was easily sedimented and resistant to solubilization with Triton X-100. The biological function, if any, of this iron pool is not clear. By contrast, the cytoplasm was relatively depleted of iron as compared with the wild-type (data not shown). The time course following repressed expression of Nfs1p was remarkable. Reduction in Fe-S enzyme activities occurred prior to mitochondrial iron overload, implying that the primary changes affect Fe-S enzymes. Mitochondrial iron accumulation is thus likely to be a secondary effect. This inter-
pretation is in conflict with the hypothesis proposed to explain Fe-S protein deficiencies in clinical iron overload states such as Friedreich ataxia. According to this hypothesis, primary iron overload causes oxidative stress by iron-catalyzed Fenton chemistry. Fe-S proteins represent critical targets for oxygen-free radicals (50) and lose activity as a consequence of the oxidative stress (51).

The mitochondrial location of Nfs1p suggests that a signal-
ning pathway exists that couples mitochondrial events with cellular iron uptake. According to this speculative pathway, Nfs1p enhances the bioavailability of iron and its delivery to Fe-S proteins within mitochondria. The soluble iron, perhaps “sensed” through a specific regulatory Fe-S protein, then re-
presses iron accumulation in the mitochondria. Signals must also emanate from the mitochondria to the plasma membrane iron uptake system, likely mediated via effects on Aft1p.

The involvement of Fe-S proteins in regulatory functions has been described recently (52). Fe-S clusters are well suited for functions involving uptake and donation of electrons, and in some cases changes in oxidation state may transduce regula-
tory signals. Oxidation of the E. coli SoxR protein by O$_2^-$ induces reversible oxidation of the [2Fe-2S]$^2^+$ clusters, and that in turn renders the DNA-bound protein competent to activate tran-
scription. In this way, an environmental input alters gene expression (53). In another type of regulation, Fe-S clusters may be alternately destroyed and rebuilt as part of regulatory switching in response to environmental signals. The FNR pro-
tein in bacteria contains a [4Fe-4S]$^{2+}$ cluster that is required for transcriptional activity. Upon exposure to oxygen, the cluster is initially converted to a more oxygen stable [2Fe-2S]$^{2+}$ form and then becomes completely disassembled into the apo form. The latter forms of FNR are inactive as transcription factors (54). Perhaps the most relevant example of a regulatory iron-sulfur protein is the cytosolic aconitase/iron-regulatory pro-
tein iron sensor. In iron-deprived mammalian cells, the apo form of this protein (IRP1), binds to the iron response element in mRNA, inducing expression of the target genes. These in-
clude genes of the cellular iron uptake system such as the transferrin receptor. In iron replete cells, the Fe-S cluster of the regulator is rebuilt and inserted into the apo protein, inacti-
vating the mRNA-binding function of the protein and activat-
ing the aconitase function. Thus, this iron-dependent regula-
tory switch depends on rebuilding the iron-sulfur cluster (55).

A protein homolog of IRP1 other than the mitochondrial acon-

tase (Aco1p) is lacking from the yeast genome, nor has cyto-
solic aconitase activity been detected in yeast. However, the role for Nfs1p in yeast described here suggests that rebuilding iron-sulfur clusters in an iron regulatory protein (other than IRP1) may be involved in the control of cellular and mitochon-
drial iron homeostasis.

A key to further defining how this regulation works will be the identification of the proteins involved. An operon encoding proteins dedicated to the in vivo synthesis of Fe-S clusters has been characterized in prokaryotes. In addition to the IscS pro-
tein, homologous to Nfs1p, other proteins of this operon include HscA and HscB, IscU, and ferredoxin (5). Each of these bacte-
rial proteins has a yeast counterpart containing an NH$_2$-termi-
nal extension with features of a mitochondrial leader sequence (6). Mutants of the yeast homologs of IscS (NFS1), HscA (SSQ1), and HscB (JAK1) were identified in a screen for sup-
pressors of the lysine auxotrophy of &Delta;od1 mutants (6). The mechanism by which mutations in these putative mitochon-
drial proteins suppress defects in the cytoplasmic copper-zinc superoxide dismutase is not entirely clear. Furthermore, an iron regulatory function that recalls the NFS1 effects has been ascribed to SSQ1. Yeast mutants lacking Ssq1p, like mutants lacking Nfs1p, were selected using the FRE1-HIS3 selection scheme. These loss-of-function mutants exhibited up-regula-
tion of cellular iron uptake activity and diversion of iron to mitochondria reminiscent of the nfs1–14 mutant (27). Ssq1p belongs to the Hsp70 class of chaperones with functions in protein translocation, protein folding, and proteolysis. The spe-
cific substrates of Ssq1p that mediate control of iron metabo-
lism and Fe-S cluster protein synthesis are unknown.

One possible substrate for Ssq1p with a role in iron metabol-
ism is Yfh1p, the yeast frataxin homolog (29, 56). Yfh1p is synthesized on cytoplasmic ribosomes and imported into mitochon-
dria, where the preprotein is processed in two steps. The second processing step is impaired in ssg1 mutants, suggesting a functional association between these two proteins (27). Yfh1p is thought to mediate iron export from the mitochondria (57). The human homolog of Yfh1p, called FRDA or frataxin, has been implicated in the neurodegenerative disease Friedreich ataxia (58). Another protein with a role in mitochondrial iron homeostasis is Atm1p. Atm1p is a transporter of the inner mitochondrial membrane oriented with a probable ATP-bind-
ing site in the matrix, so that it could function to export sub-
strates from the matrix to the cytoplasm (59). The human gene has been implicated in a rare disorder involving ataxia and sideroblastic anemia (60). Loss-of-function mutations in yeast lead to iron accumulation in the mitochondria (61) and defi-
ciency of heme proteins (59, 61). The substrates for this puta-
tive mitochondrial export pump are unknown.

The specific roles of Nfs1p and interacting proteins in medi-
ating iron homeostasis remain to be defined. There must be mechanisms for transport of iron into and out of mitochondria, for delivery of iron in functional form within the mitochondrial matrix, for assembly of the Fe-S clusters in mitochondria and in the cytoplasm, for unfolding of proteins for insertion of Fe-S clusters and for refolding these proteins. Finally, all these processes must be coordinated so that iron is present in suffi-
cient amounts but not in toxic excess. Our work suggests that Nfs1p plays a role in this coordination. The conservation be-
tween yeast and humans of the proteins involved in normal iron homeostasis (Nfs1p, Yfh1p, and Atm1p) makes it likely that mechanisms observed in yeast will be relevant to human biology and disease.

Note Added in Proof—After this manuscript was submitted, an arti-
cle by Lill and coworkers (Kispal, G., Csere, P., Prohl, C., and Lill, R. (1999) EMBO J. 18, 3981–3989) was published indicating that Nfs1 is
an essential protein of mitochondria with a role in maintaining or synthesizing cytosolic Fe-S proteins.

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