Transcription of the Yeast Iron Regulon Does Not Respond Directly to Iron but Rather to Iron-Sulfur Cluster Biosynthesis*

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Saccharomyces cerevisiae responds to iron deprivation by increased transcription of the iron regulon, including the high affinity cell-surface transport system encoded by FET3 and FTR1. Here we demonstrate that transcription of these genes does not respond directly to cytosolic iron but rather to the mitochondrial utilization of iron for the synthesis of iron-sulfur (Fe-S) clusters. We took advantage of a mutant form of an iron-dependent enzyme in the sterol pathway (Erg25-2p) to assess cytosolic iron levels. We showed that disruption of mitochondrial Fe-S biosynthesis, which results in excessive mitochondrial iron accumulation, leads to transcription of the iron transport system independent of the cytosolic iron level. There is an inverse correlation between the activity of the mitochondrial Fe-S-containing enzyme aconitase and the induction of FET3. Regulation of transcription by Fe-S biosynthesis represents a mechanism by which cellular iron acquisition is integrated with mitochondrial iron metabolism.

High affinity iron uptake in Saccharomyces cerevisiae is mediated by a transport system comprising the multicopper oxidase Fet3p and the transmembrane permease Ftr1p (1). Genes that encode these proteins are part of an iron regulon in which transcription is activated by Aft1p. Aft1p is cytosolic when iron is replete and translocates to the nucleus when iron is limited (2). It has been speculated that Aft1p responds to cytosolic levels of elemental iron, as conditions that lower cytosolic iron result in increased transcription of the iron regulon. Binding of iron to Aft1p, however, has not been demonstrated.

Mutations that affect mitochondrial Fe-S biosynthesis or export result in increased cellular and mitochondrial iron as a result of decreased mitochondrial iron efflux (3). It has been thought that the increase in mitochondrial iron occurs at the expense of cytosolic iron, and the decrease in cytosolic iron is responsible for the increase in Aft1p-mediated transcription of the iron regulon (3, 4). Herein, we demonstrate that activation of Aft1p does not directly respond to cytosolic iron. Using a novel measure of cytosolic iron, we showed that activation of the iron regulon can occur when cytosolic iron levels are high. We further demonstrated that activation of the iron regulon is controlled by the synthesis of Fe-S clusters, which in yeast is localized within mitochondria. Our studies revealed that cellular iron acquisition is coordinated with the mitochondrial use of iron.

MATERIALS AND METHODS

Yeast Strains—All yeast strains used in this study were derived from a W303 background strain. The following yeast strains have been described previously: DY150 (Mata ade2-1 his3-11 leu2-3,112 trp1-1 ura3-52 can1-100(oc)), METYF1H1 (Mata ade2-1 his3-11 yph1::HIS3 leu2-3,112 trp1-1 ura3-52 can1-100(oc)), pMETYF1H1 [URA3] (5), METNFS1 (Mata ade2-1 his3-11 yph1::HIS3 leu2-3,112 trp1-1 ura3-52 can1-100(oc)), pMETNFS1 [URA3] (3), and erg25-2 (Mata erg25-2 ade2-1 his3-11 leu2-3,112 trp1-1 ura3-52 can1-100(oc)) (6). DNA transformations of Escherichia coli and S. cerevisiae were performed by standard procedures (7). An erg25-2 METCCC1 strain was generated by transforming pMETCCC1 (5) into the erg25-2 strain. An erg25-2 METYF1H1 strain was generated by crossing the METYF1H1 strain with an erg25-2 strain. The erg25-2 METNFS1 strain was generated by crossing the METNFS1 strain with an erg25-2 strain. Sporulation and dissection of diploids generated the haploid strains erg25-2 METYF1H1 and erg25-2 METNFS1. To generate methionine-regulated genes, the promoter region of MET3 was fused in-frame to the indicated open reading frame (3). Gene expression was achieved by incubating the strain that contained the construct in methionine-free medium. To turn the methionine-regulated gene off, 10-fold normal medium methionine was supplemented to the medium (final concentration 0.56 mg/ml). To generate strains carrying a FET3-LacZ reporter construct, a plasmid containing the FET3-LacZ reporter was integrated at the HO locus (8). Strains carrying a PGK1-regulated FET3/FTR1 were constructed by integrating a plasmid (pFET3/FTR1) at the ade2-1 locus (9).

Media—Yeast strains were grown in rich medium (YPD; 1% yeast extract, 2% peptone, 2% dextrose) or in synthetic complete medium (SC; 0.7% yeast nitrogen base without amino acids, 2% dextrose, and 0.12% amino acid drop-out mix). The media were made iron-limited by the addition of 80 μM bathophenanthroline disulfonate (BPS)† to YPD and 40 μM BPS to the CM. To vary the concentration of medium iron, FeSO4 (50 mM stock in 0.1 M HCl) was added back to the media at the indicated concentrations.

Experimental Protocols—Cells were iron-loaded by the addition of 50 μM FeSO4 in the presence of 1 mM ascorbate for 30 min. Ascorbate-reduced iron is the substrate for Fet3p/Ftr1p and bypasses the rate-limiting cell-surface reductases. β-Galactosidase was assayed as described by Li et al. (10), sterols were extracted and analyzed by gas chromatography as described by Kennedy and Bard (11), and aconitase was assayed as described by Chen et al. (3).

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RESULTS

Decreased Cytosolic Iron Levels Induce the Iron Regulon—We developed a system in which we could determine the effect of cytosolic iron on induction of the iron transport system. To increase cellular iron, we utilized an integrating plasmid that contains FET3/FTR1 under the control of a constitutive iron-independent promoter (PGK1) (9). For these and other experiments, ascorbate-reduced iron was employed to bypass the need for a ferrireductase to provide reduced iron, which is the substrate for the Fet3p/Ftr1p transport system. Increased expression of FET3/FTR1 led to a 10-fold increase in cellular iron uptake above that seen in cells where the endogenous FET3/FTR1 is maximally induced (8). To determine whether the increase in iron due to the integrated FET3/FTR1 plasmid was detrimental to cell survival (i.e. increased oxidative damage), we examined cell growth in response to iron loading. Cells containing the integrated FET3/FTR1 plasmid that had been incubated with iron grew as well as cells without the plasmid or cells with the plasmid not exposed to iron (data not shown). To determine the effect of iron loading on induction of the iron regulon, cells containing a FET3-LacZ reporter construct both with and without the integrated FET3/FTR1 plasmid were exposed to ascorbate-reduced iron for 10 min and then incubated in media without bioavailable iron. As might be expected from the increased cytosolic iron, the cells that were exposed to iron and expressing higher levels of FET3/FTR1 showed a lag in the induction of the FET3-LacZ reporter construct (Fig. 1A).

Overexpression of the vacuolar iron transporter Ccc1p leads to the increased expression of FET3 mRNA, even in cells grown in iron-replete media (5, 10). We examined whether increased cytosolic iron could attenuate induction of a FET3 reporter construct in cells with increased vacuolar iron storage. Increased expression of the vacuolar iron storage transporter Ccc1p was accomplished through the use of a methionine-regulated plasmid. Strains were engineered that contained a methionine-regulated CCC1 plasmid and a FET3-LacZ reporter construct but differed in that one of the two contained the integrated FET3/FTR1 plasmid. Cells with and without the integrated FET3/FTR1 plasmid were exposed to ascorbate-reduced iron 30 min before induction of CCC1. Overexpression of CCC1 in the strain lacking the integrated FET3/FTR1 construct resulted in immediate activation of FET3-LacZ (Fig. 1B). However, there was a delay in the induction of FET3-LacZ when CCC1 was overexpressed in cells containing the integrated FET3/FTR1 construct. Western analysis showed that Ccc1p was expressed with similar kinetics in both strains (data not shown). Control experiments have shown that the addition or subtraction of methionine does not affect the induction of the FET3-LacZ reporter construct in response to low iron. These results indicate that increased cytosolic iron can affect the rate of induction of the FET3-LacZ reporter construct when cytosolic iron is depleted by storage in the vacuole.

Expression of the iron transport system has often been used as a measure of cytosolic iron. We developed an alternative method of measuring cytosolic iron. To examine the relationship between iron and the expression of iron transport genes, we utilized strains that contained a mutant allele of ERG25, erg25-2, which is responsive to cytosolic iron (6). ERG25 encodes C-4 methyl sterol oxidase, an enzyme essential for ergosterol biosynthesis. Erg25p has been shown to localize to the endoplasmic reticulum and contains histidine motifs thought to coordinate oxo-diiron as a catalytic group (6). ERG25-2 has a point mutation that appears to result in an enzyme with a lower affinity for iron. When cytosolic iron levels are low, iron is released from the mutant enzyme abolishing its catalytic activity. Thus, enzyme activity is a reflection of cytosolic iron concentration. Under low iron conditions, the erg25-2 strain does not synthesize ergosterol and accumulates the ergosterol intermediate 4,4-dimethylzymosterol (4,4-DMZ). Initially, we constructed a standard curve where we assayed both FET3-LacZ induction and the sterol profile in erg25-2 cells with respect to varying concentrations of media iron. Decreased media iron resulted in an induction in β-galactosidase activity, a decrease in ergosterol biosynthesis, and an increase in 4,4-DMZ (Fig. 2). This result confirms previous work (6) showing that ergosterol synthesis in cells with the erg25-2 allele responds to cytosolic iron. Consequently, the levels of the sterol intermediate 4,4-DMZ can be used as a measure of cytosolic iron, as the concentration of 4,4-DMZ varies inversely with cytosolic iron.

We utilized the erg25-2 strain to confirm that constitutive expression of FET3/FTR1 results in increased cytosolic iron and that induction of Ccc1p can deplete cytosolic iron. We constructed two strains that contained the erg25-2 allele and a regulated CCC1 plasmid. One of the two strains also contained...
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The integrated FET3/FTR1 construct. The two strains were incubated with ascorbate iron for 30 min before the induction of CCC1. At the indicated times, the cells were harvested, and the sterol profile was determined. As expected, the induction of CCC1 in cells containing the erg25-2 allele resulted in an increase in 4,4-DMZ (Fig. 3). No increase in 4,4-DMZ was seen in cells with a wild-type ERG25 allele (data not shown). In cells containing the integrated FET3/FTR1 plasmid, there was approximately a 2-h delay before the level of 4,4-DMZ increased. These results support the data utilizing the FET3-LacZ reporter construct and demonstrate that reduction in cytosolic iron, either through decreased external iron or increased vacuolar iron transport, induces the iron regulon.

Decreased Fe-S Cluster Synthesis Increases the Iron Regulon in Iron-replete Cells—To determine whether increased cytosolic iron would affect expression of the FET3-LacZ reporter construct when Fe-S biosynthesis is disrupted, a yeast strain was constructed that permitted the regulated expression of YFH1, the yeast orthologue of the mammalian gene frataxin (responsible for the human disease Friedreich ataxia (12)). Loss of YFH1 expression results in decreased Fe-S biosynthesis and increased cellular and mitochondrial iron accumulation (3). We examined whether increased cytosolic iron would affect the kinetics of the expression of a FET3 reporter construct in cells that did not express YFH1. Cells containing a chromosomal deletion of YFH1 (a plasmid containing a methionine-regulated YFH1 gene) and an integrated FET3-LacZ reporter construct were incubated with iron ascorbate for 30 min to allow the accumulation of intracellular iron. The cultures were then supplemented with methionine to repress the expression of YFH1. As expected, the inhibition of YFH1 expression resulted in activation of the FET3-LacZ reporter construct (Fig. 4). Despite the increase in cytosolic iron, similar kinetics were observed for activation of FET3-LacZ in the strain carrying the integrated FET3/FTR1 construct. This is in contrast to the delay observed when iron levels are decreased by either low media iron or by CCC1 overexpression.

Induction of FET3-LacZ independent of cellular iron content was also seen when expression of the FET3-LacZ reporter construct was assayed after cells had been incubated for 6 h with a range of media iron. In the absence of Yfh1p, there was a significant activation of β-galactosidase, even in the presence of 250 μM iron (data not shown). These results suggest that activation of the iron regulon in the absence of Yfh1p is independent of cytosolic iron levels and appears to be dependent on a mitochondrial product. This result was confirmed by measuring the sterol levels in cells carrying the erg25-2 allele and a regulated YFH1 construct, erg25-2 METYFH1. The absence of Yfh1p in iron-replete media results in the induction of the FET3-LacZ reporter construct comparable with that seen in severely iron-starved cells. Compared with wild-type cells, however, there was no change in sterol profile (ergosterol was high and 4,4-DMZ was low), suggesting that cytosolic iron levels are sufficient to iron load Erg25-2p (Fig. 5).

Although studies suggest that the primary function of Yfh1p/frataxin is involvement in Fe-S biosynthesis (3, 13, 14), other functions for Yfh1p have been suggested, including effects on mitochondrial iron storage (15) and heme synthesis (16). The deletion of other genes that affect Fe-S biosynthesis also leads to increased mitochondrial iron accumulation and increased
expression of the iron regulon. NFS1 encodes a cysteine desulferase, which generates sulfide for Fe-S biosynthesis (17, 18). We examined the effect of reduced expression of NFS1 on both FET3-LacZ induction and the sterol profile in an erg25-2 METYFH1 strain. Similar to the loss of Yfh1p, the inhibition of NFS1 expression resulted in high levels of β-galactosidase activity with a wild-type sterol profile (Fig. 5). This result confirms the conclusion that defects in Fe-S cluster synthesis can induce the iron regulon even when cytosolic iron is high.

Growth of erg25-2 cells can also be used to monitor cytosolic iron levels in cells depleted of Yfh1p. In the absence of Yfh1p, if iron accumulates in mitochondria at the expense of cytosolic iron, then depletion of cytosolic iron should exacerbate the low iron growth defect of the erg25-2 allele. In iron-replete media, erg25-2 METYFH1 grew as well as wild-type or erg25-2 cells independent of YFH1 expression (Fig. 6). On iron-restricted plates, erg25-2 cells showed the expected growth defect as did erg25-2 METYFH1 cells that expressed YFH1. In the absence of YFH1 expression, erg25-2 METYFH1 cells grew better than erg25-2, suggesting that cytosolic iron was sufficient to iron-load Erg25p and maintain ergosterol biosynthesis. Although overexpression of CCC1 also leads to induction of FET3, the erg25-2 METCCC1 strain failed to grow on iron-restricted media. These results indicate that although deletion of YFH1 increases mitochondrial iron, it does not deplete the pool of cytosolic iron.

Our data suggest that cellular iron acquisition is regulated by the availability of iron for the metabolic needs of Fe-S biosynthesis. We hypothesized that decreases in cytosolic iron, resulting from media iron deprivation or overexpression of the vacuolar iron transporter, should lead to a decrease in the activity of the Fe-S cluster containing the enzyme aconitase and also to a subsequent induction of FET3 expression. We measured aconitase activity and FET3-LacZ induction in cells incubated under varying media iron concentrations (Fig. 7A). We performed a linear regression analysis on the FET3-LacZ and aconitase activity data from Fig. 7A (Fig. 7B, closed circles). There was a strong inverse correlation between FET3 induction and aconitase activity \( r^2 = 0.90 \). We next examined FET3-LacZ induction and aconitase activity in METCC1, METYFH1, and METNFS1 strains under conditions in which the MET3-regulated gene was either turned on or off (Fig. 7B, open squares). Again, there was a strong inverse correlation between aconitase activity and FET3 induction. Including these points in the linear regression analysis increased the \( r^2 \) value to 0.94. Together, these data suggest that expression of FET3 is largely regulated by mitochondrial Fe-S cluster synthesis and not directly through the cytosolic iron pool.

**DISCUSSION**

The control of cellular iron acquisition by mitochondrial Fe-S cluster synthesis is a mechanism that would sense both cytosolic iron levels and mitochondrial iron metabolism. Our data strongly suggest that Aft1p may not directly sense elemental iron but rather senses Fe-S clusters or a signal arising from an Fe-S-containing enzyme. This conclusion is based on the observation that induction of the iron regulon by the inhibition of Fe-S cluster synthesis occurs when cytosolic iron levels are high. Measurement of cytosolic iron has been problematic for organisms as diverse as yeast and man. The major problem is that cell homogenization destroys organelles, thus preventing accurate assessment of cytosolic iron. The cytosolic metal-binding chelator calcein has been used to estimate cytosolic iron in cultured vertebrate cells (19). Unfortunately, calcein has not been of use in yeast. We took advantage of a mutant enzyme in cultured vertebrate cells (19). Unfortunately, calcein has not been of use in yeast. We took advantage of a mutant enzyme in the sterol biosynthetic pathway to provide a measure of cytosolic iron. Erg25p is an enzyme localized to the endoplasmic reticulum. Based on sequence, Erg25p is an oxo-diiron-containing enzyme, and iron is essential for enzymatic activity. The erg25-2 allele was identified because the activity of the enzyme, and consequently cell growth, was dependent on cytosolic iron. Reduction in cytosolic iron led to decreased growth as a consequence of decreased ergosterol biosynthesis (6). In this paper, we have shown that changes in sterol profile are responsive to media iron. Thus, although we do not know the location of the active site of the enzyme (facing the lumen or facing the cytosol), the activity of the mutant enzyme is responsive to cytosolic iron. We utilized the erg25-2 allele to show that reduction in Fe-S cluster synthesis, which induces transcription of the iron regulon, does not lead to a reduction in cytosolic iron; the sterol profile of the erg25-2 cell appeared normal. Further,
Fe-S biosynthesis, the cytosolic iron level is high, thus permitting mitochondrial iron accumulation.

The prediction from our data is that Aft1p, or a protein that associates with Aft1p, may be an Fe-S-containing protein and that the status of the Fe-S may regulate transcription of genes required for iron acquisition. Aft1p contains a CXC motif that could potentially participate in binding Fe-S. Further, mutation in one of the cytochromes (C293P), which might prevent Aft1p from binding an Fe-S, results in a constitutively active allele (21). Expression of Aft1p in bacteria under conditions in which the Fe-S cluster operon was overexpressed did not provide evidence that Aft1p contains Fe-S clusters. It is worth noting that although Aft1p transitions between cytosol and the nucleus, the metal-sensing transcription factors for the copper regulon (Mac1p) and zinc regulon (Zap1p) are always nuclear (1). We speculate that the difference in the localization of metal-sensing transcription factors is because of the binding of elemental metals by Mac1p and Zap1p in contrast to Aft1p, which senses a mitochondrial product rather than elemental iron. A precedent for Fe-S regulation of gene expression is demonstrated by mammalian IRP1, in which the status of an Fe-S cluster regulates the turnover and expression of mRNA involved in iron transport and storage (22). In bacteria, the Fe-S state of FNR and IscR regulates the transcription of genes required for anaerobiosis and the lsc operon, respectively (23, 24). It may well be that Aft1p binds to other proteins that contain Fe-S clusters or that Aft1p senses the product of an Fe-S cluster-containing protein.

Cells respond to defective Fe-S cluster synthesis by increasing the amount of iron in the mitochondria and in the cell. By increasing iron in the mitochondria, the concentration of one of the essential substrates for Fe-S cluster synthesis is also increased. Recently, we demonstrated that transcription of the cell-surface iron transport system requires heme (8). In the absence of heme, transcription is inhibited even though Aft1p occupies the promoters of genes such as FET3. This observation suggests that transactivation of Aft1p is regulated by other gene products. In yeast, however, heme synthesis is dispensable, as yeast can live anaerobically without heme. Fe-S cluster synthesis, however, is essential. Coupling transcription of the iron transport system to the presence of heme or the synthesis of Fe-S clusters provides mechanisms for integrating mitochondrial iron usage with cellular iron acquisition.

Fig. 7. Induction of FET3 expression is correlated with decreasing aconitase activity. A, wild-type cells containing a FET3-lacZ reporter construct were grown in media with different amounts of bioavailable iron. The media were made iron-limited by adding 40 μM BPS with different concentrations of FeSO4 supplemented to the media. The cells were harvested after 12 h of growth, and aconitase and β-galactosidase activities were measured. B, the data from A were plotted as the relationship between aconitase and β-galactosidase activities (closed circles). The following strain cells were grown in the presence or absence of methionine for 12 h: METYPH1 (open squares; A, YFH1 on; B, YFH1 off), METNFS1 (open squares; C, NFS1 on; D, NFS1 off), and METCCC1 (wild-type cells with high copy plasmid of CCC1 under the MET3 promoter) (open squares; E, CCC1 off; F, CCC1 on). Each data point (aconitase activity versus β-galactosidase activity) was then plotted (open squares).

using iron-limited plates, we have shown that induction of the iron regulon by decreased Fe-S cluster synthesis can permit cells to acquire iron, which can allow erg25-2 cells to grow.

Although cytosolic iron is the substrate for mitochondrial Fe-S biosynthesis, conditions that lead to cytosolic iron deprivation (e.g. reduced media iron level, defective iron transport, or increased vacuolar iron storage) result in decreased Fe-S biosynthesis and increased expression of the iron regulon (3). Activation of the iron regulon by impaired mitochondrial Fe-S biosynthesis can explain the apparent conundrum in which loss of Fe-S leads to mitochondrial iron accumulation but only when the cytosolic iron level is high (12, 20). Conditions that result in cytosolic iron starvation will prevent mitochondrial iron accumulation in strains defective in Fe-S biosynthesis. Our results suggest that in the absence of

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