Aft2p, a Novel Iron-regulated Transcription Activator That Modulates, with Aft1p, Intracellular Iron Use and Resistance to Oxidative Stress in Yeast*

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The yeast, Saccharomyces cerevisiae, contains a transcription activator, Aft1p, that regulates the transcription of the high affinity iron transport system genes. This report describes the properties of Aft2p, a protein 39% homologous to Aft1p. Aft2p was found to activate transcription. Overproduction of Aft2p activates the transcription of the AFT1 target gene FET3. The double aft1aft2 mutant was unable to grow in iron-deprived conditions. Because a fet3 mutant does not show this deficiency, the defect is not solely caused by mis-regulation of iron transport but also involves defective iron use by the cells. The aft1 cells were unable to grow in aerobic conditions on plates containing raffinose as the sole carbon source. The inability to grow on raffinose is not caused by the cell iron content being too low to sustain respiratory metabolism, because the oxygen consumption of aft1 mutants showed that their respiratory activity is 2-fold higher than that of controls. The double aft1aft2 mutant also has many phenotypes related to oxidative stress such as H2O2 hypersensitivity, oxygen-dependent copper toxicity, and oxygen-dependent methionine auxotrophy, which are suppressed in anaerobiosis. These results suggest that Aft2p and Aft1p have overlapping roles in the control of iron-regulated pathway(s) connected to oxidative stress resistance in yeast.

Iron is required by all organisms; it is used as a cofactor in key redox enzymes involved in such diverse biological processes as cell respiration and the synthesis of metabolic intermediates. However, iron can damage cells by reacting with hydrogen peroxide to form the hydroxyl radical. This highly toxic compound damages DNA, proteins, and lipids (1). Prokaryote and eukaryote cells therefore have evolved various systems for obtaining and using iron, depending on several homologous transporters of the major super-facilitator (MSF) family encoded by ARN1, SIT1, TAF1, and ENB1 (9–12). All the genes involved in the high affinity iron transport systems are under the control of the iron-dependent transcription activator Aft1p (12–14). However, Aft1p does not affect the regulation of FET4 (15). In addition to the high affinity transport genes, Aft1p regulates the transcription of other iron-responsive genes such as those encoding Ccc2p (the intracellular copper transporter responsible for delivering copper to Fet3p) (16), Fth1p (which forms an iron transport complex on the vacuole with Fet5p) (17), Fes3p/Fre4p (which are potential siderophore-iron reductases) (18), and Fre5p/Fre6p of unknown function (19). Finally, Aft1p seems to be involved in, but is not required for, the transcription of genes such as AFT1, which encodes the copper chaperone that delivers copper to Ccc2p, and ISU1/ISU2, the products of which are involved in the mitochondrial assembly of iron-sulfur clusters (20, 21). In conditions of iron deprivation, Aft1p activates the transcription of target genes by binding directly to the consensus sequence T/C/G/ACACC, a motif in its 5′-upstream regions. Although the functional DNA binding and activation domains of Aft1p have not yet been characterized, its similarities to other transcription factors suggest that the N-terminal region rich in basic residues is involved in recognizing DNA, and the C-terminal glutamine-rich domains may be required for the transcriptional activation function. The dominant allele AFT1up, which leads to Phe-291 in place of Cys-291 in the N-terminal region of the protein, constitutively binds to DNA (13, 14). Just how Aft1p senses iron and activates gene expression is not understood, and recent data indicate that the Aft1p-mediated response is not restricted to iron deprivation conditions but can be induced by the concentrations of molecular oxygen or copper in the culture medium (22, 23). We have now attempted to obtain further information about the regulation of iron homeostasis by inspecting the yeast genome by blast analysis. We found a 416-amino acid protein encoded by the YPL202c gene that presents 39% overall identity to Aft1p, which we designated Aft2p (Fig. 1). This paper describes some of the characteristics of Aft2p. It is a second iron-regulated transcriptional activator in yeast. Aft2p is required for iron homeostasis and resistance to oxidative stress when Aft1p is absent. Molecular data and phenotypic analysis suggest that Aft1p and Aft2p have overlapping functions.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—The strains used in this study were CM3260 (MATa, trp1-63, leu2-3, 112 gcn4-101, his3-609, Y18 (MATa, trp1-63, leu2-3, 112 gcn4-101, his3-609, aft1::TRP1), and Y19 (MATa, trp1-63, leu2-3, 112 gcn4-101, his3-609, fet3::URA3). The CM3260aft2Δ

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zymes. The plasmid pSH18 plasmid pEG202 (25) digested with the appropriate restriction enzyme deleted and replaced by four lexA operators (26). Plasmid pFC-W, and ligated to the DNA binding domain of LexA derived from the following primers were used to amplify the open reading frame replacement:

\[
\text{TATAATTATTTAGTTTTCAACTC-3} \quad \text{and} \quad \text{TATATTATTTATGCACAGGC-3}
\]

The sequences underlined are homologous to the kanMX4 marker sequence of a drug-resistant clone were selected on YPD plates containing G418 (200 \(\mu\)g/ml). Deletions were confirmed by polymerase chain reaction using primers flanking the insertion region.

The plasmid pEG202 and the derivative plasmids pEG202-AFT1 and pEG202-AFT2 contained the DNA binding domain of LexA. Plasmid pEG202-AFT1 expressing LexA-Aft2p and plasmid pEG202-AFT2 encoding LexA-Aft2p were constructed by integrating the open reading frames of AFT1 or AFT2 in frame with the DNA binding domain of LexA. Open reading frames were amplified with the following oligonucleotides:

- AFT1, 5'-CAGAAAGATTCCTACGAGAAGAGCCCTC-3' and 5'-TCTCTGCAGAATCTCTTCTGGACATC-3' (cognate to the AFT1 promoter and terminator)
- AFT2, 5'-AGGGCACCAGGAAGAAGGAGAGCTAC-3' and 5'-GATGAAAATCATTTGCTTTGGTC-3' (sequences 3'-end are homologous to the lexA operator)

The amplification products were digested by HindIII and Xhol for AFT1 and by BamHI and Xhol for AFT2 and ligated to the DNA binding domain of LexA derived from the plasmid pEG202 (25) digested with the appropriate restriction enzymes. The plasmid pSH18–34 contained the GAL1-lacZ reporter gene from which the upstream activation sequence GAL1 had been deleted and replaced by four lexA operators (26). Plasmid pFC-W, generously supplied by Yanaguchi-Iwai, contained a 30-base pair cassette of the FET3 upstream activation sequence (–283 to –234) fused to LacZ (14).

Media, Growth Conditions, and Plate Assays— Yeast strains were grown in rich medium (1% yeast extract/2% peptone) containing either 2% glucose (YPD) or 2% raffinose, or in copper and iron limiting yeast medium with or without 100 \(\mu\)M iron for 18 h, collected by centrifugation, washed twice in 10 mM EDTA and once in metal-free water. The total iron was measured by inductively coupled plasma atomic emission spectroscopy at the Microanalysis Facility of the Centre National de la Recherche Scientifique (Vernaison, France) on cells suspended in 0.1 N nitric acid.

Oxygen Consumption— The designated isogenic yeast strains were grown in the YPD medium without \((-\text{Fe})\) or with 100 \(\mu\)M iron \((+\text{Fe})\). The respiratory activity of whole cells was evaluated by an oxygen consumption assay. The rate of oxygen consumption was measured using a 2.2-m1 thermostated oxygraph equipped with a Clark-type electrode. The respiratory medium was a 0.1 M potassium phosphate buffer, pH 7.2 (30 °C), saturated with air (236 \(\mu\)M dissolved O2) containing 2 mM glucose. The cells were prepared as 50% (w/v) suspensions in 0.1 M potassium phosphate buffer, pH 7.2.
FIG. 1. Sequence alignment of Aft1p and Aft2p. Identical amino acids are boxed, and similar residues are shaded. Conserved cysteines are marked by a triangle except for Cys-291 corresponding to the substitution of Cys for Phe in the dominant allele AFT1<sup>dom</sup>, which is marked by an asterisk. The highly charged N-terminal sequence of Aft1p, which is absent from Aft2p, is also marked by a triangle.

TABLE I

LexA-Aft2p as a transcription activator

| LexA fusion present | LexAop-GAL1-lacZ expression |
|---------------------|-----------------------------|
|                     | –Fe | +Fe |
| LexA                | <10 | <10 |
| LexA-Aft1p          | 2850 ± 140 | 2510 ± 35 |
| LexA-Aft2p          | 1460 ± 110 | 770 ± 120 |

The double a<sup>ft1</sup>a<sup>ft2</sup> mutant was completely abolished under iron-deprived conditions. It was only restored by adding 100 μM iron to the ferrozone-containing plates. Thus, the a<sup>ft1</sup> strain was sensitive to iron deprivation, and this sensitivity was exacerbated by the absence of Aft2p. The inability of the a<sup>ft1</sup> and the a<sup>ft1</sup>a<sup>ft2</sup> strains to grow on iron-poor medium was not caused by a deficiency in the high affinity iron transport system, because the growth of the isogenic fet3 mutant was not affected under these conditions.

We next determined whether the requirement of more external iron by the a<sup>ft1</sup> and a<sup>ft1</sup>a<sup>ft2</sup> mutants was because of a smaller intracellular iron pool or a defect in the iron availability in the mutant cells. We measured the intracellular iron content of the cells before growing them in iron-deficient medium. The a<sup>ft1</sup> mutants had iron pools similar to those of the wild-type strain before the shift (Fig. 4A). However, the maximal cellular density reached in the medium without iron by the a<sup>ft1</sup> strain was lower compared with the wild-type and a<sup>ft2</sup> strains (Fig. 4B). The growth defect was exacerbated for the a<sup>ft1</sup>a<sup>ft2</sup> mutant. The growth of the a<sup>ft1</sup>a<sup>ft2</sup> strain was restored completely by adding a limited concentration of iron (Fig. 4C).

Effects of AFT1 and AFT2 on Growth Under Respiratory Conditions—The rates of oxygen consumption of the two a<sup>ft1</sup> mutants were much higher than those of the wild-type and a<sup>ft2</sup> strains (Table II). The oxygen consumption was sensitive to cyanide and was nearly the same with or without high iron concentrations in the growth medium. We investigated this further by analyzing the phenotypes of cells grown in a medium containing raffinose as the sole carbon source. Unlike glycerol, raffinose is not a strictly respirable carbon source. We thus could analyze the growth of the cells in the presence or absence of oxygen. The growth of the fet3 mutant on raffinose under aerobic conditions was slightly affected compared with the a<sup>ft2</sup> and wild-type strains (Fig. 5). In contrast, a<sup>ft1</sup> mutant strains were unable to use this carbon source under aerobic conditions. The a<sup>ft1</sup>-related growth deficiency was suppressed by overproducing Aft2p. The a<sup>ft1</sup> mutant phenotype could also be rescued by adding iron and also copper to the medium. The a<sup>ft1</sup>a<sup>ft2</sup> mutant phenotype was not rescued by the addition of iron or copper. Similar results were obtained when glycerol was used instead of raffinose (data not shown). Finally, the growth of both the a<sup>ft1</sup> and the a<sup>ft1</sup>a<sup>ft2</sup> strains was restored completely under anaerobic conditions without adding iron or copper.

Hypersensitivity of a<sup>ft1</sup> and a<sup>ft1</sup>a<sup>ft2</sup> Mutants to Oxygen Stress—We investigated the sensitivity of the a<sup>ft1</sup> mutants to...
iron-regulated control of transcription in yeast. The most similar domains of Aft1p and Aft2p are in the basic N-terminal regions of the proteins. This region has four conserved cysteine residues including the CDC sequence supposed to ligate iron in Aft1p (13). Aft2p was found to possess a transactivation activity that is lower relative to that of Aft1p (Table I). The transactivation activity of Aft2p was reduced by 50% when there was iron in the medium, whereas the transactivation activity of Aft1p was not affected. This confirms a previous report that indicates that the transcriptional activation function of Aft1p is independent of iron (31). In contrast, the transcriptional activation function of Aft2p may be iron-dependent.

Our data confirm previous studies that indicate that a residual iron-regulated transcription of FET3 is present in an AFT1 deleted strain (31). We show that Aft2p may be responsible for the remaining transcription of FET3. We have also demonstrated that overproduction of Aft2p activates, in an iron-regulated manner, the transcription of both the FET3 gene and the LacZ reporter gene placed under the control of the AFT1-responsive element. The modulation by iron of the AFT2-dependent regulation in aft1 strains seems to be greater than 2-fold, suggesting that iron could regulate both the transcription and DNA binding functions of Aft2p. These results suggest that Aft2p is recruited to a sequence identical or similar to the TGG/ACACC AFT1-responsive element and may take part in the transcription of genes that overlap AFT1 target genes. Our present findings show that the amount of ATX1 mRNA is regulated by iron. This regulation appears to be lost in the double aft1aft2 mutant. Moreover, the amount of the ATX1 mRNA is increased with the overproduction of Aft2p. These results support a previous report that indicates that ATX1 mRNA is unaffected in strains containing an aft1Δ null mutation but is increased in the hyperactive AFT1-up allele (20). It indicates that the Aft proteins in addition to other transcription factors may be involved in the iron-regulated transcription of ATX1.

We also compared the growth of the aft strains with those of the fet3 and wild-type strains under iron-deprived conditions to identify the role of Aft2p in iron metabolism. Previous studies
have indicated that the \( \text{aft1} \) mutant is more sensitive to iron deprivation than is the \( \text{fet3} \) mutant despite their comparable measured ferrous iron uptakes. It has been suggested that Aft1p mediates some intracellular iron use in addition to its role in the regulation of iron transport (13). Our phenotypic analysis of growth on agar plates containing an iron-depleted medium indicates that the growth of the \( \text{aft1} \) mutant is affected by iron starvation, whereas that of the isogenic \( \text{aft1} \) mutant is not. The double \( \text{aft1aft2} \) mutant is totally unable to grow under these conditions. We have also shown that \( \text{aft1} \) and \( \text{aft1aft2} \) cells both have normal iron contents similar to those of wild-type cells. Thus, the inability of \( \text{aft2} \) mutants to grow in an iron-poor medium is not solely caused by defective iron uptake. It confirms the predicted role for \( \text{AFT1} \) in the control of intracellular iron use and suggests that Aft2p is critical, with Aft1p, for the modulation of iron homeostasis in yeast.

A previous report indicated that the \( \text{aft1} \) mutant is unable to grow in medium with glycerol as a respiratory carbon source (31). The authors attributed this to a defective high affinity iron uptake. This defect makes the intracellular iron content too low to sustain respiratory growth. Our data do not support this. First, the \( \text{aft1} \) mutants all have functional mitochondrial respiration that is 2-fold higher than in the wild-type and \( \text{aft2} \) strains whatever the iron concentration in the medium (Table II). This result is consistent with the measurements of cytochrome absorption spectra and cytochrome c oxidase activities (data not shown) that indicate that the \( \text{aft1} \) and \( \text{aft1aft2} \) mutants have a normal capacity to synthesize cytochromes and a normal respiratory cytochrome c oxidase activity. Second, the \( \text{aft1} \) mutant is unable to grow under aerobic conditions on agar plates containing raffinose as carbon source, whereas the isogenic \( \text{fet3} \) mutant does grow. Third, this oxygen-dependent phenotype is not specific to an iron deficiency, because adding copper restores the growth of the \( \text{aft1} \) mutant to the same extent as does iron (Fig. 5). But, adding iron or copper does not suppress the \( \text{aft1} \)-mediated mutant phenotypes in the absence of Aft2p. This suggests that elevated iron or copper concentration in the medium could induce some other pathway(s) to compensate for the deficiency of \( \text{aft1} \) and that this putative metal-mediated compensatory pathway requires the presence of Aft2p. Thus, these results do not support the notion that the inability of \( \text{aft1} \) mutants to grow on respiratory medium is due entirely to a defective high affinity iron uptake.

The \( \text{aft1} \), and particularly the \( \text{aft1aft2} \), mutants are hypersensitive to \( \text{H}_{2}\text{O}_{2} \), and the \( \text{aft1aft2} \) mutant has several oxygen-sensitive and iron-dependent phenotypes such as a hypersensitivity to copper and a methionine auxotrophy. Mutants, the copper detoxification of which is affected such as the \( \text{ace1} \) and \( \text{cup1} \) mutants, are hypersensitive to copper under aerobic con-
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many stress response or detoxification genes, also resulted in a 3.4-fold increase in the amount of AFT2 transcripts (38).

The phenotypic analysis presented herein indicates that the af1-mediated mutant phenotypes are exacerbated by the deletion of AFT2 and are suppressed by the overproduction of Af2p. Moreover, some phenotypes related to oxidative stress do not occur in the simple af1 and af2 mutants but are clearly present in the double af1af2 mutant. These results suggest that Af2p has functions that overlap those of Aft1p in the regulation of iron metabolism pathways. We are presently developing a global analysis to identify the putative AFT target genes that are involved in these pathways.

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FIG. 6. Sensitivity to H2O2 of the af1 and af1af2 mutants. Copper sensitivity and methionine auxotrophy of the af1af2 mutant under aerobic conditions. A, strains CM260 (WT), Y18 (af1), CM2960af2a (af2), Y18af2Δ (af1af2), Y18af2Δ harboring the plasmid pEG202-AFT2 (af1af2a AFT2), and Y19 (af2) were plated on YPD-rich medium containing 1 mM H2O2, with or without added 100 μM copper (+Cu). B, the indicated yeast strains were plated onto YPD-rich medium containing 100 μM copper. The Y18af2Δ (af1af2) strain was also grown in the same copper-containing medium with a 100 μM iron supplement (+Cu+/Fe2+) or placed in anaerobic conditions (+Cu2–/O2–). C, the indicated yeast strains were plated onto minimal iron and copper limiting medium lacking methionine. The Y18af2Δ (af1af2) strain was also grown in the same medium with a 100 μM iron supplement (+met+/Fe2+) or placed in anaerobic conditions (+met–/O2–).
