A second iron-regulatory system in yeast independent of Aft1p

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Iron homeostasis in the yeast *Saccharomyces cerevisiae* is regulated at the transcriptional level by Aft1p, which activates the expression of its target genes in response to low-iron conditions. The yeast genome contains a paralog of *AFT1*, which has been designated *AFT2*. To establish whether *AFT1* and *AFT2* have overlapping functions, a mutant containing a double *aft1*Δ*aft2*Δ deletion was generated. Growth assays established that the single *aft2*Δ strain exhibited no iron-dependent phenotype. However, the double-mutant *aft1*Δ*aft2*Δ strain was more sensitive to low-iron growth conditions than the single-mutant *aft1*Δ strain. A mutant allele of *AFT2* (*AFT2-1^{up}*), or overexpression of the wild-type *AFT2* gene, led to partial complementation of the respiratory-deficient phenotype of the *aft1*Δ strain. The *AFT2-1^{up}* allele also increased the uptake of ^{59}Fe in an *aft1*Δ strain. DNA microarrays were used to identify genes regulated by *AFT2*. Some of the *AFT2*-regulated genes are known to be regulated by Aft1p; however, *AFT2-1^{up}*-dependent activation was independent of Aft1p. The kinetics of induction of two genes activated by the *AFT2-1^{up}* allele are consistent with Aft2p acting as a direct transcriptional factor. Truncated forms of Aft1p and Aft2p bound to a DNA duplex containing the Aft1p binding site *in vitro*. The wild-type allele of *AFT2* activated transcription in response to growth under low-iron conditions. Together, these data suggest that yeast has a second regulatory pathway for the iron regulon, with *AFT1* and *AFT2* playing partially redundant roles.

metalloregulation | iron homeostasis

Iron is an essential nutrient but it is toxic in the absence of effective homeostasis. Mechanisms have therefore evolved to ensure adequate but not excess levels of reactive intracellular iron. In the yeast *Saccharomyces cerevisiae*, transcriptional regulation of the iron regulon is mediated by the transcriptional activator Aft1p (1). The Aft1p regulon consists of many genes that are involved in the acquisition, compartmentalization, and utilization of iron. These include genes involved in iron uptake (*FET3*, *FTR1*, and *FRE1,2*), siderophore uptake (*ARN1-4* and *FIT1-3*), iron transport across the vacuole membrane (*FTH1*), and iron-sulfur cluster formation (*ISU1*,*2*; refs. 1–6). Aft1p binds to a conserved promoter sequence in an iron-dependent manner and activates transcription under low-iron conditions (2). Mutants lacking a functional Aft1p grow poorly in iron-limiting conditions (1, 7). A Cys^{291}Phe substitution within Aft1p results in derepressed transcriptional activation in iron-replete cells (1). Because Cys^{291} is part of a Cys-X-Cys motif, an attractive hypothesis is that Aft1p is capable of binding iron; however, the mechanism by which Aft1p senses iron levels is not known.

The *S. cerevisiae* genome has many duplicated chromosomal regions that account for up to 16% of the yeast proteome, and it has been proposed that these regions have arisen from an ancient duplication of the entire genome (8). *AFT1* lies within such a region of chromosome VII, with its duplicate ORF YPL202c (designated *AFT2*), located on chromosome XVI (9). Characterization of paralogs that have arisen from the genome duplication has revealed differences in the extent to which genes within a pair have functionally diverged. The transcription factors Swi5p and Ace2p have nearly identical DNA-binding domains, yet they activate the transcription of different genes (10). Alternatively, there may be redundancy of function so that a clear phenotype is apparent only in a mutant lacking both paralogs, as in the case of the Pcl8p and Pcl10p cyclins (11).

Previous work relating to the function of *AFT1* has suggested that there is an *AFT1*-independent pathway for iron regulation in *S. cerevisiae* (1, 7). We were interested to learn whether such a pathway involves *AFT2*. We have used a combination of phenotypic analysis, functional complementation, and analysis of global gene expression to identify the relationship between *AFT1* and *AFT2*. We present evidence that *AFT2* codes for a transcription factor that activates gene expression in response to low-iron conditions. Although Aft1p and Aft2p are functionally similar, and have overlapping functions, comparison of gene activation by both transcriptional factors suggests that they also have distinct functions.

Materials and Methods

Yeast Strains and Culture Conditions. The following *S. cerevisiae* strains were purchased from Research Genetics (Huntsville, AL) and used in this study: BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) as wild type; BY4741*aft1*Δ (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 aft1*::*kanMX4*); BY4742*aft1*Δ (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 aft1*::*kanMX4*); and BY4741*aft2*Δ (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 aft2*::*kanMX4*). A haploid *aft1*Δ*aft2*Δ strain (*MATα his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 aft1*::*kanMX4 aft2*::*kanMX4*) was isolated after the mating of BY4741*aft2*Δ and BY4742*aft1*Δ, and its allele status was verified by using PCR and DNA sequencing. Cells were grown in either 1% yeast extract, 2% peptone medium (YP), complete-synthetic medium (CM), or, when appropriate, complete-synthetic medium lacking uracil [CM(−Ura)]. These media were supplemented with either 2% glucose, 2% raffinose, or 3% glycerol as indicated. For plate phenotypes, 10-fold serial dilutions of cells were spotted onto agar plates and grown at 30°C. For anaerobic growth, agar plates were incubated in a GasPak (Becton Dickinson) anaerobic chamber.

Vectors. Yeast-genomic DNA from strain BY4741 was isolated and used as template for PCR to amplify a 2.6-kb fragment containing *AFT2*, with both upstream and downstream sequences, which was ligated into the *Bam*HI/*Xba*I site of the YCp vector pRS416 (12) to create p*AFT2*. QuikChange (Stratagene) mutagenesis was used to generate an allele of *AFT2* that codes for a Cys^{187}Phe mutation by using p*AFT2* as template to create p*AFT2-1^{up}*. To place the *AFT2-1^{up}* allele under the control of the *GAL10* promoter, p*AFT2-1^{up}* was used as template for PCR to

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Abbreviations: BPS, bathophenanthroline disulfonate; EMSA, electrophoretic mobility-shift assay; YPD, yeast extract/peptone/dextrose; CM, complete medium.

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amplify a 1.3-kb fragment containing *AFT2-1^{up}*, which was ligated into the *Bam*HI/*Cla*I site of GFP-pYeF2, a derivative of pYeF2 (13), to generate p*GAL-AFT2-1^{up}*. Plasmid p*AFT1-1^{up}* contains a 3-kb fragment, consisting of *AFT1* with both upstream and downstream sequences, that originated from pCM16 (ref. 14; a kind gift from Enrique Herrero, Universitat de Lleida, Lleida, Spain), which was ligated into the *Xho*I/*Sac*I site of pRS316 (12). Site-directed mutagenesis by using the pALTER mutagenesis system (Promega) was used to generate an allele of *AFT1* that codes for a Cys^{291}Phe mutation. To generate plasmids that express truncated forms of Aft1p (pAft1-313) and Aft2p (pAft2-214), PCR was used to amplify the 5′ ends of *AFT1* and *AFT2* containing the first 313 codons of Aft1p and the first 214 codons of Aft2p, which were then ligated into pET20 and pET3 (Novagen), respectively. All of the *AFT1*, *AFT2*, *AFT1-1^{up}*, and *AFT2-1^{up}* sequences were confirmed by DNA sequencing. All yeast transformations were performed by using the lithium acetate procedure. In the case of transformations using the *aft1*Δ*aft2*Δ strain, cells were pregrown in yeast extract/peptone/dextrose (YPD) under nitrogen, and the agar plates were supplemented with $FeCl_2$ (100 μM).

Electrophoretic Mobility-Shift Assay (EMSA). The identification of specific protein-DNA complexes was analyzed as described with the following modifications (15). The DNA probe consisted of oligonucleotide 5′-ATCTTCAAAAGTGCACCCATTTG-CAGGTGC-3′ and its reverse complement, which contains the Aft1p-binding site within the *FET3* promoter.

Crude protein extracts were isolated from *Escherichia coli* cells (BL21-CodonPlus(DE3)-RIL from Stratagene) that had been transformed separately with pET3, pAft1-313, and pAft2-214. Cells were grown to mid-log phase at 37°C with ampicillin and chloramphenicol, protein expression was induced, and the cells were incubated for an additional 2 h before harvesting. The cells were pelleted and resuspended in 1/50th vol of lysis buffer (20 mM Tris·HCl, pH 7.5/1 mM DTT) and sonicated, and the cell debris was pelleted. The standard EMSA binding reaction consisted of equal volumes of an *E. coli* cell extract and the hybridization mix with the addition of poly(dI-dC)·poly(dI-dC) to a final concentration of 4 ng·μl^{-1}. The binding reaction was incubated for 15 min and applied to a 6% polyacrylamide nondenaturing gel and electrophoresed by using Tris-borate-EDTA buffer.

mRNA Quantification by S1 Nuclease Analysis. Total RNA was isolated from mid-log cells and was hybridized with ^{32}P-labeled, single-stranded DNA oligonucleotides that were complementary to the candidate gene (between 50 and 69 nt) and the control gene *CMD1* (40 nt). After digestion with S1 nuclease, the samples were electrophoresed through an 8% polyacrylamide/5 M urea gel.

Microarray Analysis. Cells were harvested from 300 ml of culture at an *A* at 600 nm of 0.4. Total RNA was isolated by the hot phenol method. mRNA was isolated from total RNA by using the PolyATtract mRNA Isolation System IV kit from Promega following the manufacturer's instructions. Cy3-dUTP or Cy5-dUTP (Amersham Pharmacia) was incorporated during reverse transcription of the polyadenylated RNA. The fluorescently labeled product was recovered and hybridized to microarrays, which were washed and scanned as described (16).

^{59}Fe Uptake Analysis. Iron uptake was analyzed as described (17). Briefly, mid-log phase cells were grown in complete synthetic medium and harvested, and the cells were washed and resuspended in low-iron medium plus 1 mM ascorbate. ^{59}Fe (0.5 μM) was added to the cells, which were then incubated at 30°C for 10 min. The cell cultures were then filtered through Whatman GF/C filters, which were then washed, and the level of ^{59}Fe was measured. In some cases, the Fe(II) chelator bathophenanthroline disulfonate (BPS) was added to the cell cultures 4 h before the cells being harvested.

Results

***S. cerevisiae* Contains a Paralog of *AFT1*.** Analysis of the *S. cerevisiae* genome has revealed two similar regions of chromosomes VII and XVI that contain the paralogs *AFT1* and *AFT2* (8). The predicted product of *AFT2* exhibits strong identity to Aft1p from residues 38 to 285 (39% identity; Fig. 1). The homologous region includes the N-terminal basic region of Aft1p, which is predicted to represent the Aft1p DNA-binding domain, and the region that contains the Cys-X-Cys motif that confers iron sensitivity. The two histidine-rich regions of the extreme N terminus and C terminus of Aft1p are not conserved in Aft2p. It has been proposed that these regions may be involved in iron binding (1). The high degree of identity within the N terminus of both proteins encourages the prediction that *AFT2* codes for a DNA-binding protein.

The Effect of Iron on the *aft* Mutants. To determine whether *AFT2* plays a role in iron homeostasis, the growth of strains with either a single deletion of *AFT1* or *AFT2*, or the double deletion of both genes, was compared. The growth of a strain lacking *AFT1* is impaired under low-iron conditions, and this defect is exacerbated when the medium contains a nonfermentable carbon source (7). As expected, the *aft1*Δ strain failed to grow on medium containing glycerol, whereas the *aft2*Δ strain grew equivalently to the wild-type strain (Fig. 2). The growth defect of the *aft1*Δ strain was partially reversed in an *aft1*Δ strain harboring low-copy plasmids containing either the wild-type *AFT2* allele (p*AFT2*) or the *AFT2-1^{up}* allele (p*AFT2-1^{up}*). The *AFT2-1^{up}* allele contains a mutation that codes for a Cys^{187}Phe substitution, which corresponds to the mutation within the *AFT1-1^{up}* allele that codes for a Cys^{291}Phe substitution.

The growth defect of the *aft1*Δ strain is less pronounced on medium containing a fermentable carbon source, and it can be suppressed in three ways (Fig. 3*A*). The *aft1*Δ strain grows normally on complex YPD medium containing glucose, in the absence of oxygen. Alternatively, full aerobic growth can be attained by pregrowth of the *aft1*Δ strain in liquid medium supplemented with Fe(II) or by supplementing the agar with Fe(II) (Fig. 3*A*). The *aft1*Δ*aft2*Δ strain exhibits a more exaggerated iron-deficient phenotype under aerobic conditions (Fig. 3*A*). This growth defect is reversed only by supplementing both the preculture and the agar with exogenous Fe(II). As in the case of the *aft1*Δ strain, the *aft1*Δ*aft2*Δ strain grows fully under anaerobic conditions.

The exaggerated iron-deficient growth defect of the *aft1*Δ*aft2*Δ strain relative to the *aft1*Δ strain is also apparent on minimal medium (Fig. 3*B*). The addition of the Fe(II) chelator BPS to the agar markedly attenuates the aerobic growth of the *aft1*Δ*aft2*Δ strain even when it is pregrown in liquid medium supplemented with Fe(II). The addition of BPS to cultures is known to lower iron availability, resulting in Aft1p activation (1, 18). In contrast to growth on YPD, supplementation of the minimal medium agar with Fe(II) fully restores growth of the *aft1*Δ*aft2*Δ strain. The growth defect of the *aft1*Δ*aft2*Δ strain is also reversed when it is transformed with either p*AFT2* or p*AFT2-1^{up}* (data not shown).

The Effect of the *AFT2-1^{up}* Allele on Gene Expression Within *S. cerevisiae*. The exacerbated growth phenotype of the *aft1*Δ*aft2*Δ strain and the observed sequence similarity between Aft1p and Aft2p are consistent with both proteins having partial overlapping functions. We hypothesized that Aft2p may be a transcriptional activator and that the *AFT2-1^{up}* allele may result in the

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Aft1p   MEGFNPADIEHASPINSSDSHSSSFVYALPKSASEYVVNHNEGRASASGNPAAVPSPIMTLNLKSTHSLNIDQHVHTSTSP
Aft2p   ----------------MKAKSMKSIISVPISVSK------TGKMKLTASPD---------NLASMMSK------------
                          ..*   . ..* * *        *.   .  *          ** *  *

Aft1p   TETIGHIHHVEKLNQNNLIHLDPVPNFEDKSDIKPWLQKIFYPQGIELVIERSDAFKVVFKCKAAKRGRNARRKRKDKPKG
Aft2p   ------------D-QNKLIHLDPVPSFEDRHEIKPWLQKIFYPQGIDIVIERSDSSKVTFKCRSVR---------------
                     ** ******** ***. .*************..******. ** ***.. .

Aft1p   QDHEDEKSKINDDELEYASPSNATVTNGPQTSPDQTSSIKPKKKRCVSRFNNCPFRVRATYSLKRKRWSIVVMDNNHSHQL
Aft2p   -------SKVGLN---------------------------PKSKGSSSRSHACPFRIRAAYSVRLQKWNVVVMNNIHSHEL
              **..                              ** * . ** . ****.**.**.. ..* .*** * ***.*

Aft1p   KFN--PDSEEYKKFKEKLRKDNDVDAIKKFDELEYRTLANLPIPTATIPCDCGLTNEIQSFNVVLPTNSNVTSSASSSTVS
Aft2p   RFDLITKTDDYKKFKENLRQKNDEKAIKTFDELEYKASLNLPLVTPIISCDCGLTKEIEAFNNIFLPLSN-----------
        .*     ...****** **. **  *** ******..  ***. *  * ****** **..** .    **

Aft1p   SISLDSSNASKRPCLPSVNNTGSINTNNVRKPKS-QCKNKDTLLKRTTMQNFLTTKSRLRKTGTPTSSQHSSTAFSGYIDD
Aft2p   ------------PPLTSKKNLLKTNKNSVSKIKSRQMDN---------------SKPRPRLKTKLDADLHD----TGFLDN
                  * * *  *    * * * * ** *  *                .* * *       .  *     .*..*

Aft1p   PFNLNEILPLPASDFKLNTVTNLNEIDFTNIFTKSPHPHSGSTHPRQVFDQLDDCSSILFSPLTTNTNNEFEGESDDFVHS
Aft2p   FKTRNSCVKIEKED----SLTNLNEIDFTNMFCN-----------------DNFIQNYNQGLMELLTEPTPGPS-----S
          . *  . .   *    ..**********.*.                 *        *    .    * *     *

Aft1p   PYLNSEADFSQILSSAPPVHHDPNETHQENQDIIDRFANSSQEHNEYILQYLTHSDAANHNNIGVPNNNSHSLNTQHNVSD
Aft2p   --S------SCILPSTPTRPLS-----QSKMDIALSESTTSS----------------------PN--------------
              * ** *.*          *   **    ...*                          **

Aft1p   LGNSLLRQEALVGSSSTKIFDELKFVQNGPHGSQHPIDFQHVDHRHLSSNEPQVRSHQYGPQQQPPQQLQYHQNQPHDGHN
Aft2p   ----FMETDAPYGDEIIKVSKDTKSN----------´--APTADTDIATNLGKERNENFG-------MLNYN---------
              .  .*  *    *.  . *                       ...*  . *  ..*        *.*.

Aft1p    HEQHQTVQKDMQTHESLEIMGNTLLEEFKDIKMVNGELKYVKPED          690aa
Aft2p    --YEALLHFNDEHFNELNSIDPALISKY-----------------          416aa
               ..   .   *  .  .*.  .
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Fig. 1. Sequence comparison of Aft1p and Aft2p. The amino acid sequences of Aft1p and Aft2p of *S. cerevisiae* were aligned by using the CLUSTALW program. The conserved CXC motif is underlined and in bold (see text).

derepressed transcriptional activation of genes regulated by Aft2p. DNA microarray experiments were conducted to identify candidate target genes regulated by Aft2p. We compared the differential gene expression of the *aft2*Δ strain harboring either p*AFT2-1*^{up} or a control vector. These microarray experiments were conducted at both the Stanford Microarray Facility and the Huntsman Cancer Institute Microarray Facility at the University of Utah. A number of genes were reproducibly elevated in their expression in the strain containing the *AFT2-1*^{up} allele (Table 1). These include genes that are known to be regulated by Aft1p (*FIT1*, *FIT3*, *FTR1*, *FTH1*, *FRE1*, and *TIS11*) and by the zinc-responsive transcription factor Zap1p (*ZRT1* and YOL154w; refs. 1, 4, and 19). Genes that are not known to be metalloregulated by either Aft1p or Zap1p were also identified (*MRS4*, *UBC8*, *PRB1*, and *ECM4*) (Caroline Philpott and Dave Eide, personal communications).

To confirm the microarray data, S1 analysis was performed to quantify the expression of selected *AFT2-1*^{up} induced genes. The expression of *FIT3*, *FIT1*, and *MRS4* was clearly activated in the *aft2*Δ strain containing p*AFT2-1*^{up} but not in the control strain or the strain containing p*AFT2* (Fig. 4*A*). The *AFT2-1*^{up} allele also activated the expression of *FIT3*, *FIT1*, and *MRS4* in an *aft1*Δ strain, demonstrating that the *AFT2-1*^{up} allele activates transcription in an Aft1p-independent manner (Fig. 4*B*). The *ZRT1* and YOL154w genes were induced in only a subset of the S1 experiments (data not shown). Activation of these two genes was not observed in a *zap1*Δ strain harboring p*AFT2-1*^{up}, suggesting that their induction was the indirect result of fluctuating concentrations of intracellular zinc.

The *AFT2-1*^{up} allele was placed under the control of a galactose-inducible promoter to test whether it directly regulates two of its target genes. The *aft2*Δ strain containing the *GAL10/AFT2-1*^{up} construct was precultured in raffinose. Galactose was then added to induce the expression of *AFT2-1*^{up}, which was maximal within 15 min (data not shown). Both *FET5* and *UBC8* were significantly induced within 15 min of the addition of galactose (Fig. 5*A*). No induction was observed in the *aft2*Δ strain lacking the *GAL10/AFT2-1*^{up} construct. The coinduction of *AFT2-1*^{up}, *FET5*, and *UBC8* is consistent with Aft2p being a direct transcriptional regulator.

Aft1p is activated in iron-deficient cells and inhibited in iron-replete cells (1). To determine whether wild-type Aft2p is similarly iron regulated, the *aft1*Δ*aft2*Δ strain containing either p*AFT2* or p*AFT2-1*^{up} was cultured under iron-deficient and iron-replete conditions (Fig. 5*B*). The *AFT2-1*^{up} allele induced the expression of *FIT3* in cells cultured in either BPS or Fe(II), demonstrating that the mutation in this allele results in derepressed activity (Fig. 5*B*). In contrast, the wild-type *AFT2* allele induced the expression of *FIT3* only in iron-deficient conditions. Therefore, Aft2p induces gene expression in iron-deficient cells.

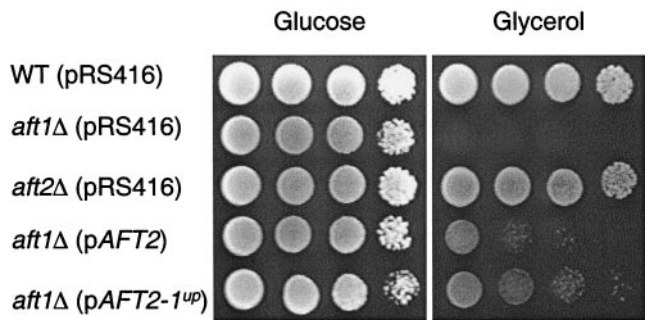

Fig. 2. *AFT2* partially complements the *aft1*Δ mutant strain. Overnight cultures of each strain were grown in CM(−Ura) medium containing glucose. The cultures were washed, and 10-fold serial dilutions were spotted onto CM(−Ura) agar plates containing either glucose or glycerol, which were then incubated at 30°C.

Aft1p and Aft2p Bind to the Same Region of the *FET3* Promoter. To determine whether the level of transcriptional activation by

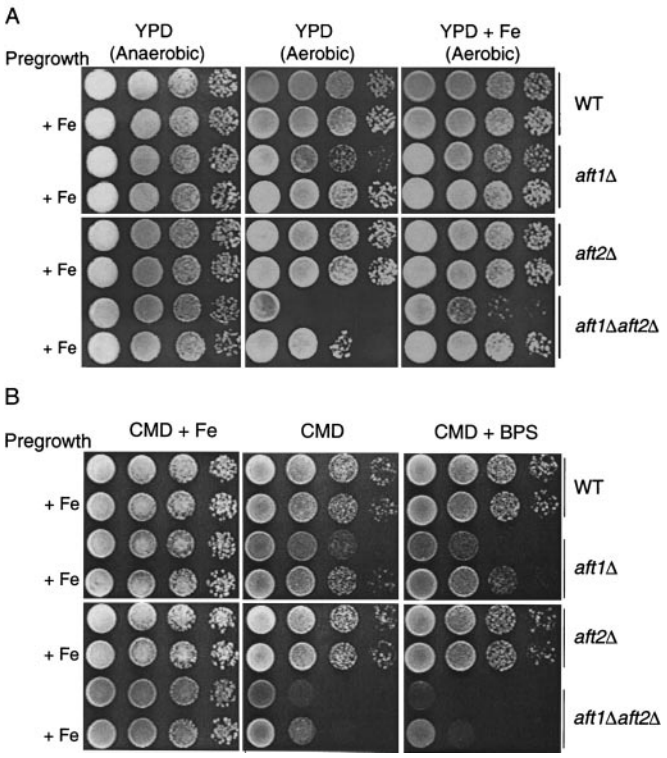

Fig. 3. The effect of iron on the growth of the *aft* mutant strains. Overnight cultures of the wild-type strain and each of the *aft*-mutant strains were grown in liquid CM(−Ura) with glucose medium. Each strain was grown in duplicate, with one of each pair being supplemented with $FeCl_2$ (100 μM). Iron supplementation in the pregrowth medium is indicated by "+ Fe." The cells were washed, and 10-fold serial dilutions were spotted onto (*A*) complex medium (YPD) agar plates with or without supplemented $FeCl_2$ (100 μM) or (*B*) synthetic medium (CMD) agar plates with or without supplemental $FeCl_2$ (100 μM) or BPS (10 μM). Plates were incubated at 30°C and, in the case of one YPD plate, under anaerobic conditions.

Aft1p and Aft2p is the same, the expression of *FET3*, *FET5*, and *MRS4* was analyzed in an *aft1*Δ*aft2*Δ strain containing either the *AFT1-1*^{up} allele or the *AFT2-1*^{up} allele (Fig. 6*A*). In the strain carrying the *AFT1-1*^{up} allele, *FET3* is more highly expressed than in the strain carrying the *AFT2-1*^{up} allele. This pattern of expression is reversed with respect to *MRS4*, which is more highly expressed in the strain carrying the *AFT2-1*^{up} allele. The level of activation of *FET5* is the same in the strains containing either the *AFT1-1*^{up} allele or the *AFT2-1*^{up} allele. Therefore, the *AFT1-1*^{up} and the *AFT2-1*^{up} alleles differentially activate gene expression.

To address whether Aft2p can bind to the same DNA site as Aft1p, EMSAs were performed using a 30-bp fragment that included the Aft1p-binding site within the *FET3* promoter. The Aft1p consensus binding site is PyPuCACCCPu and was identified by DNase1 footprinting of the *FET3* promoter and sequence comparison of Aft1p-regulated genes (2). Truncated forms of Aft1p (Aft1p313) and of Aft2p (Aft2p214) containing the conserved putative DNA-binding domains of each protein and the conserved Cys-X-Cys motif were used. A specific single complex was formed between the *FET3* promoter fragment and lysate from *E. coli* cells that contained plasmids that separately expressed the truncated forms of Aft1p and Aft2p (Fig. 6*B*). No complex was formed by using lysate from control *E. coli* cells that did not express Aft1p313 or Aft2p214. Aft1p and Aft2p are therefore capable of binding to the same DNA fragment *in vitro*.

Iron Uptake by the *aft* Mutants. Iron uptake is markedly attenuated in a strain lacking a functional *AFT1* (1). The observed *AFT2-1*^{up} induction of genes involved in iron uptake (*FET3*, *FTR1* and *FRE1*) suggested that iron uptake would be enhanced in cells containing *AFT2-1*^{up}. To determine whether *AFT2* stimulates

Table 1. Genes activated by the *AFT2-1*^{up} allele

Target gene	Cy5/Cy3 ratio	Function
FIT3	8.8	Cell wall protein
YOL154w	8.8	Zn-metalloprotease-like protein
ZRT1	6.2	High affinity zinc transporter
FIT1	5.7	Cell wall protein
ECM4	5.0	Cell wall structure/biosynthesis
YOL083w	4.2	Unknown
UBC8	4.1	Ubiquitin-conjugating enzyme
YJR078w	3.6	Similar to indoleamine 2,3-dioxygenase
MRS4	3.5	Member of mitochondrial carrier family
TIS11	3.3	Similar to mammalian *TIS11* family
FTR1	3.3	High affinity iron transporter
FTH1	3.2	Vacuolar iron transporter
LAP4	3.0	Vacuolar aminopeptidase I
PRB1	2.9	Vacuolar protease B
GRX4	2.8	Glutaredoxin
AHP1	2.7	Alkyl hydroperoxide reductase
HCR1	2.6	Putative component of eIF3
FET5	2.6	Vacuolar multicopper oxidase
PEP4	2.6	Vacuolar proteinase A
YDL124w	2.5	Unknown
FRE1	2.2	Cell surface iron reductase
SMF3	2.0	Vacuolar iron transporter

Transcripts of these genes were at least 2-fold more abundant in the strain containing the *AFT2-1*^{up} allele compared with the control strain. The value shown is the Cy5/Cy3 ratio (which reflects the ratio of the transcripts' abundance in the two strains) measured in one experiment at the Stanford Microarray Facility. The same genes were identified in two similar experiments conducted at the University of Utah Microarray Facility.

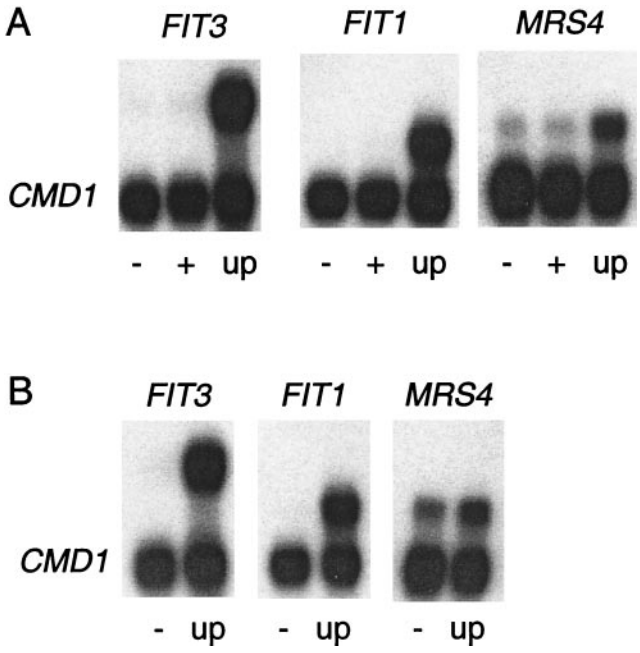

Fig. 4. S1 nuclease protection assays to quantify mRNA levels of genes activated by the *AFT2-1*^{up} allele. RNA was isolated from (*A*) the *aft2*Δ strain and (*B*) the *aft1*Δ strain transformed with pRS416 (−), p*AFT2* (+), and p*AFT2-1*^{up} (up) and grown in CM(−Ura) with glucose medium to mid-log phase. The upper band for each sample is the specified gene, and the lower band the calmodulin loading control (*CMD1*).

BIOCHEMISTRY

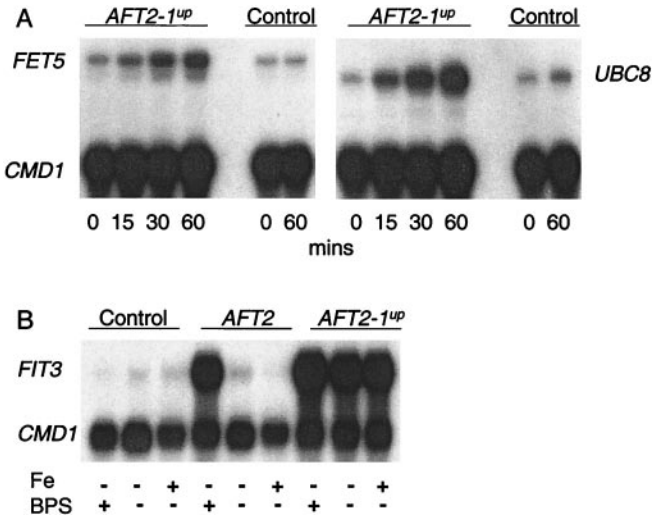

Fig. 5. Aft2p is a direct transcriptional activator that responds to low iron. S1 nuclease protection assays were carried out by using RNA isolated from (*A*) the *aft2*Δ strain transformed with either p*GAL-AFT2-1*^{up} or pYeF2 (control), which were grown in CM(−Ura) with raffinose to mid-log phase, at which time 2% galactose was added to induce the transcription of the *AFT2-1*^{up} allele. Cells were harvested at the specified times (*B*), the *aft1*Δ*aft2*Δ strain was transformed with pRS416 (control), p*AFT2*, and p*AFT2-1*^{up}, which were pregrown overnight with 10 μM $FeCl_2$, harvested, washed, and resuspended in CMD(−Ura) medium with or without supplemental $FeCl_2$ (100 μM) or BPS (100 μM) and then grown exponentially for 8 h. The *Upper* band for each sample is the specified gene, and the *Lower* band the calmodulin loading control (*CMD1*).

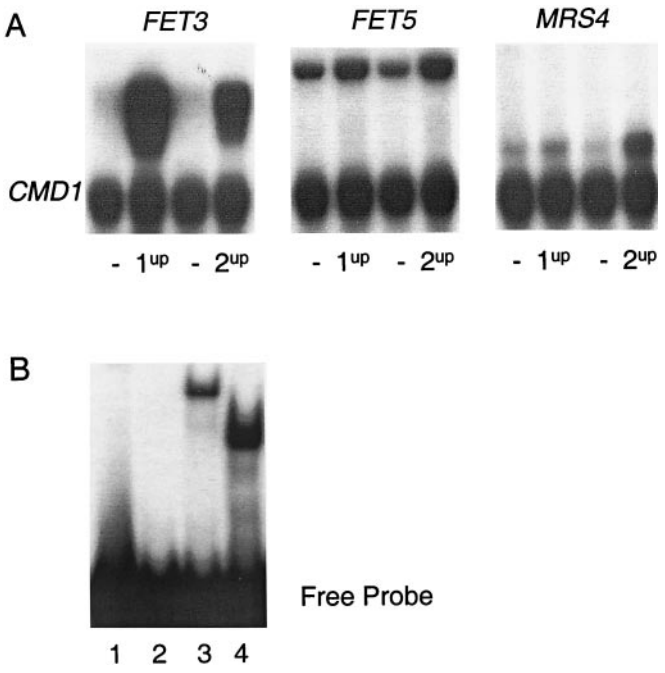

Fig. 6. Aft1p and Aft2p bind to the same promoter fragment *in vitro* but differentially regulate gene expression. (*A*) S1 nuclease protection assays to quantify mRNA levels of genes activated by the *AFT1-1*^{up} and *AFT2-1*^{up} alleles. RNA was isolated from the *aft1*Δ*aft2*Δ strain transformed with a control plasmid (−) (pRS316 in the case of p*AFT1-1*^{up} and pRS416 in the case of p*AFT2-1*^{up}), p*AFT1-1*^{up} (1^{up}), and p*AFT2-1*^{up} (2^{up}) and grown in CMD(−Ura) medium to mid-log phase. The *Upper* band for each sample is the specified gene, and the *Lower* band the calmodulin loading control (*CMD1*). (*B*) Gel retardation assays were performed by using a ^{32}P-labeled 30-bp duplex of the *FET3* promoter as probe with no protein (lane 1), and lysates from *E. coli* containing pET3 (lane 2), pAft1-313 (lane 3), and pAft2-214 (lane 4).

iron uptake, the level of ^{59}Fe uptake was quantified in all of the *aft* mutant strains. The level of ^{59}Fe uptake was similar between the wild-type and the *aft2*Δ strain but was significantly reduced in the *aft1*Δ and the *aft1*Δ*aft2*Δ strains (Fig. 7*A*). This result suggests that the chromosomally encoded Aft2p is not a significant factor in regulating iron uptake in exponential cells grown in synthetic glucose medium. However, expression of the *AFT2-1*^{up} allele in the *aft1*Δ strain significantly increased ^{59}Fe uptake (Fig. 7*B*). The ^{59}Fe uptake data are therefore consistent with the growth phenotypes of each strain.

Discussion

We found that Aft2p, like its paralog, Aft1p, is a functional transcriptional activator that responds to iron. A strain that lacks both Aft1p and Aft2p is more sensitive to growth in low-iron conditions than a strain lacking only Aft1p. An allele of *AFT2* that contains a mutation analogous to the *AFT1-1*^{up} mutation activates the transcription of a subset of the Aft1p regulon in an *AFT1*-independent manner. The expression profiles of two Aft2p target genes after the induction of the *AFT2-1*^{up} allele is consistent with Aft2p directly regulating these genes. The wild-type *AFT2* allele activates transcription of its target genes in response to treatment with the iron-chelator BPS. In addition, truncated forms of both Aft1p and Aft2p bind to the same DNA fragment *in vitro*, consistent with Aft2p being a direct transcriptional regulator. There are, however, differences in the extent to which Aft1p and Aft2p regulate the iron regulon. The *aft1*Δ strain and the *aft2*Δ strain exhibit different iron-deficient and respiratory-deficient phenotypes. The *AFT2-1*^{up} allele only partially suppresses the respiratory-deficient phenotype of the *aft1*Δ strain and partially stimulates iron uptake in the *aft1*Δ strain. Furthermore, the *AFT1-1*^{up} allele and the *AFT2-1*^{up} allele differentially regulate gene expression.

Microarray experiments identified a subset of the Aft1 regulon as being activated by the *AFT2-1*^{up} allele. Those genes that were not identified include *FET3* and *ARN1-4*. However, S1 analysis clearly shows that the *AFT2-1*^{up} allele is able to activate *FET3* expression in the absence of Aft1p. The microarray experiments were carried out in an *AFT1* wild-type strain, and S1 analysis showed that *FET3* is expressed in the control strain to such a level that no differential expression was observed in the strain carrying the *AFT2-1*^{up} allele (data not shown). This result implies that there are different thresholds of activation of genes within the iron regulon by Aft1p. Therefore, *AFT2* may be capable of

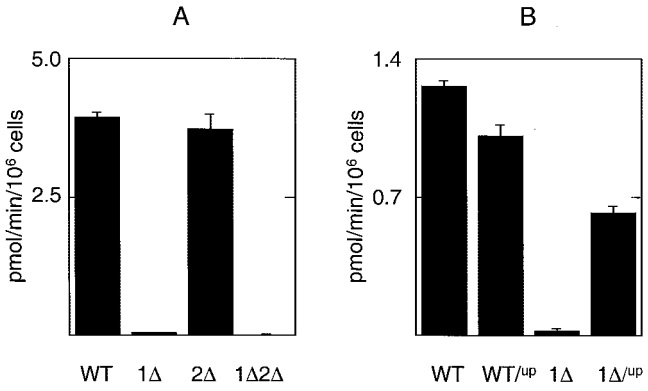

Fig. 7. Iron uptake in the *aft* mutants. The level of ^{59}Fe uptake was analyzed in the following cultures. (*A*) The wild-type strain and the *aft*-deletion strains grown to mid-log phase in synthetic medium (CMD) with BPS (100 μM). (*B*) The wild-type strain and the *aft1*Δ strain containing either pRS416 (WT, 1Δ) or p*AFT2-1*^{up} (WT/^{up}, 1Δ/^{up}) grown to mid-log phase in CMD(−Ura) medium.

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